Evaluation of the scientific quality of studies concerning genotoxic properties of glyphosate

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Preface

This document contains the authorized and final evaluation of industrial studies that concern the mutagenic properties of glyphosate and its formulations. These studies were used for the evaluation of mutagenic properties of the herbicide by health authorities (BfR and EFSA).

Prof. S. Knasmueller is currently employed at the Institute of Cancer Research, Medical University of Vienna. His opinion about the mutagenic properties of glyphosate does not necessarily reflect the position of the Medical University of Vienna.

Vienna, 25.03.21

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1. Introduction

This expertise contains a comprehensive evaluation of documents provided by the EFSA which concern unpublished investigations on mutagenic/genotoxic properties of glyphosate.

We analyzed if the individual reports are in agreement with the current international guidelines for the testing of chemicals and with recommendations of expert panels.

Each chapter describes findings which were obtained in a specific test system. In the introduction sections, the principles of the assays are briefly explained, at the end of each chapter a brief summary can be found.

We evaluated if the studies were conducted in agreement with the requirements which are defined in the current OECD and other guidelines, and focused on the most relevant criteria. On the basis of outcome of these evaluation, we provide in the conclusions section and in the "remarks", statements concerning the relevance of the findings and the reliability of the studies.

The last section of each study describes the results of the evaluation of glyphosate in the last version of the Renewal Assessment Report (RAR) of the BfR which came out in 2015. It contains statements concerning the acceptance of the studies by the authority. We mention in this section also the guidelines which were followed by the authors of the different studies (in some cases no guidelines are mentioned). This information was provided by Dr. Helmut Burtscher (Global 2000).

It is important to note that some studies followed guidelines which were outdated and were replaced by new ones that are currently used. We evaluated the studies in regard to these new guidelines which were developed by the experts in this field and are based on sound scientific evidence. Almost all NEWER guidelines were available already in 2015 when the last RAR was published. Old and new guidelines concerning the same tests differ in some cases

substantially. For example, the number of metaphase cells which was recommended for the evaluation of chromosomal aberrations in *in vitro* tests in older guidelines is lower as that recommended in newer versions.

In some *in vivo* micronucleus studies with bone marrow cells only one dose was tested. In OECD and other guidelines generally 3 doses are suggested. However, the guidelines mention that in some cases it is acceptable to test only one dose. This is justified (according to older guidelines) when a justification is provided which is lacking in all these studies. In a newer guideline (OECD, 2016) it is specified that it must be confirmed that the compound (or its metabolites) reaches the target cells in the bone marrow. This information is lacking in many studies.

The Salmonella/microsome ("Ames" test) was initially performed in routine tests with four strains [1]; however, in all newer guidelines (OECD, UK EMS, US EPA) five strains are mandatory. These strains detect different classes of mutagens and are complementary in this regard; therefore, it is not acceptable from the current status of knowledge to use a lower number of strains.

Also the number of bone marrow cells which should be evaluated in chromosomal aberration and micronucleus assays was increased (for more information wee chapter 2.3).

We interpret in our evaluation the results of different studies on the basis of the assumption that they should fulfill the criteria which were valid in 2015 when the RAR evaluation was published and not on the basis of criteria which were established several decades earlier.

Note that the correctness of statistical calculations was evaluated by a further expert, it is not included in this document.

2. Salmonella/microsome assays

2.1. Description

The test was developed by B. Ames and it is based on the detection of reverse mutations (back mutations) of specific gene of Salmonella typhimurium strains which lead to histidine auxotrophy [1]. Different strains were developed to detect different classes of compounds. They differ in regard to the target genes and also in regard to the repair capacity and membrane permeability.

We evaluated the results on the basis of the OECD Guideline # 471 (1997) [2], OECD update of guidelines 2014-2015 [3], the UK EMS guideline [4] and several other reports including the methodological description of Maron and Ames [5], Levy et al. [6] and Mortelmans and Zeiger [7].

More than 15,000 chemicals have been tested in the Salmonella/microsome assay. Sensitivity/specificity of the assay for the detection of genotoxic carcinogens is well known. Within the regulatory battery, the Ames test exhibits reasonable specificity, but poor sensitivity [8].

Most relevant criteria are described in OECD guideline # 471 [2]. A very important parameter (not explicitly described in this document) is the numbers of the revertant colonies in untreated control plates which should be in a defined range. When the rates are higher or lower as expected, results of experiments are inconclusive and the genetic identity of the strain has to be verified. One of the main reasons of such alterations can be loss of plasmids which encode for repair functions.

The most important criteria include, apart from the background levels, the adequate numbers of strains, the number of doses tested and the number of plates per dose. Only at the highest dose acute toxic effects should occur which leaf to reduction of his^+ revertants. Note that no statistical analysis is mandatory according to present OECD guideline. When negative results are obtained, the Agency suggests [2] to perform an additional experiment or provide an explanation why this not required. It is also notable that from 5 doses which should be tested from each compound, cytotoxicity leading to reduced number of his+ revertants should occur only at the highest dose.

2.2. Evaluation of individual studies

Study number 1

Performing laboratory: The Institute of Environmental Toxicology, Tokyo, Japan

Date: April 3, 1995

Title: Reverse mutation test

Report No.: IET-94-0142

Guidelines: U.S. EPA FIFRA Guidelines, Subdivision F [9].

Sponsor: Sankyo Co., Ltd, Japan

Test material: HR-001 (purity 95.69%); the test material is not described.

Test description: plate preincubation procedure with repetition

Dose finding test: included

Number of strains: 5

Number of doses tested: 6, the highest tested dose was 5,000 µg/plate

Number of plates/dose: 3

Negative controls: background levels of revertants are in all strains OK

Positive controls: results are provided, positive as expected. In all experiments with S9 2-AA was used for all strains without further information.

Statistical analysis: none; means ±SD are provided. No test for dose-response was applied. Only graphical evaluation was included.

Historical controls: not provided

Results: the test substance was not mutagenic in any strain

Remarks: negative results were obtained in a preincubation experiment but no follow-up experiment was performed; no justification is provided as requested in the OECD guideline [2]. 2-AA was used in all experiments with S9, no additional information is provided as requested in the OECD guideline [2].

RAR: Evaluation and Comments

Study identification in the report: *1st new Ames test (Akanuma, 1995)*

Evaluations (page 311): *The study is considered acceptable. No evidence of genotoxicity was obtained. However, it must be clarified that, according to the study report, only the pre-incubation method was used whereas the plate-incorporation assay is not described and was apparently not performed. At least, the results given in Table B.6.4-3 and Table B.6.4-4 were*

obviously obtained by means of the pre-incubation method. When the study description in the dossier was compared to the original study report, it was noted that the study director was Mie Akanuma. Erroneously, the first name had been mentioned in the dossier instead of the authors surname.

Comments (SK and AN): the preincubation method does not represent the standard procedure, and is only performed when negative results are obtained in the plate incorporation test. Furthermore, the study has additional minor shortcomings. Therefore, the study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 2

Performing laboratory: Central Toxicology Laboratory, Cheshire, UK

Date: February 16, 1996

Title: Glyphosate acid: An evaluation of mutagenic potential using *S. thyphimurium* and *E. coli*

Report No.: CTL/4874

Guidelines: OECD 471 [2], US EPA OPPTS 870.5100 [10] and 2000/32/EEC B.13/B.14 (2000) [11].

Test material: Glyphosate acid (purity 95.6%)

Sponsor: Zeneca Agrochemicals

Test description: preincubation test + plate incorporation assay

Dose finding test: not included

Number of doses tested: 6, the highest tested dose was 5,000 µg/plate

Number of strains: 6 (4 Salmonella and 2 *E. coli*)

Number of plates/dose: 3

Negative controls: background levels of revertants in all strains are OK

Positive controls: results are provided, positive as expected. However, in all experiments with S9 2-AA was used for all strains without further information.

Statistical analysis: Student's test; means ±SD are provided, no test for a dose-response was applied, only a graphical evaluation was included.

Historical controls: not provided

Results: the test substance was not mutagenic in any strain

Remarks: historical control data are not provided. 2-AA was used in all experiments with S9 without additional information as suggested as requested in the OECD guideline [2].

RAR: Evaluation and Comments

Study identification: *12th new Ames test (Callander, 1996)*

Evaluation (page 333)*: The study is considered acceptable. No evidence of mutagenicity was revealed. It should be clarified that the first experiment was performed by means of the plate incorporation method with and without metabolic activation. In the second trial, the same method was used in the absence of S9 mix. A pre-incubation assay was run with S9 mix.*

Comments (SK and AN): the study has minor deviations from OECD guidelines. The results are partly reliable.

Study number 3

Performing laboratory: LPT Laboratory of Pharmacology, Hamburg, Germany

Date: April 30, 2009

Title: Mutagenicity study of glyphosate TC in the *Salmonella. thyphimurium* reverse mutation assay (*in vitro*)

Report No.: LPT No. 23916

Guidelines: OECD 471 [2], Council Regulation (EC) No. 440/2008 B13/B14 [11], EPA OPPTS Guideline 870/5100 [10] and ICH Guideline S2A [12].

Test material: Glyphosate acid (purity; analyzed 97.52% w/w, authenticated 98.8% w/w)

Sponsor: Helm AG, Hamburg, Germany

Test description: preincubation test + plate incorporation assay

Dose finding test: included

Number of doses tested: 6, the highest tested dose was 3,100 µg/plate

Number of strains: 5

Number of plates/dose: 3

Negative controls: the background rates of revertants are OK.

Positive controls: results are provided, positive as expected. 2-AA and cyclophosphamide were used in experiments with S9.

Statistical analysis: Mann-Whitney U-test and Spearman R test were used, individual plates counts are not indicated as requested by the OECD guideline [2].

Historical controls: provided

Results: the test substance was not mutagenic in any strain

Remarks: background frequencies of all strains appear to be valid.

RAR: Evaluation and Comments

Study identification*: 7th new Ames test (Flügge 2009)*

Evaluation (page 322): *The study is considered acceptable. The highest concentration in the mutagenicity assays was chosen because there was evidence of cytotoxicity at this and above dose levels demonstrated at least for the strain TA100. This approach is reasonable and dose selection is supported.*

Comments (SK and AN): the results of the study are reliable.

Study number 4

Performing laboratory: Laboratory of Pharmacology and Toxicology (LPT), Hamburg, Germany

Date: January 25, 2010

Title: Mutagenicity study of Glyphosate TC in the *S. thyphimurium* reverse mutation assay (*in vitro*)

Report No.: LPT No. 24880

Guidelines: OECD #471 [2].

Test material: Glyphosate TC (purity 95.23%)

Sponsor: Helm AG, Hamburg, Germany

Test description: plate incorporation assay + preincubation test

Dose finding test: included, highest dose (5,000 µg/plate, precipitation)

Number of doses tested: 5, the highest tested dose was 3,160 µg/plate

Number of strains: 5

Number of plates/dose: 3

Negative controls: background levels of revertants in all strains are OK

Positive controls: results are provided, positive as expected, 2-AA and cyclophosphamide were used in experiments with metabolic activation.

Statistical analysis: Mann-Whitney U-test and Spearman R test were used.

Historical controls: provided

Results: the test substance was not mutagenic in any strain

Remarks: results of the study are reliable

RAR: Evaluation and Comments

Study identification: *8th new Ames test (Flügge, 2010)*

Evaluation (page 322): *The study is considered acceptable. The test substance proved non-gentotoxic. The choice of the highest concentration is sufficiently explained. In addition, some precipitation was observed in the pre-test with TA100 at 5000 µg/plate.*

Comments (SK and AN): the study fulfills the OECD criteria. The results are reliable.

Study number 5

Performing laboratory: SCANTOX A/S, Skensved, Denmark

Date: September 10, 1991

Title: Mutagenicity test: Ames Salmonella assay with glyphosate, batch 206-JaK25-1

Report No.: 12323

Guidelines: OECD #471 [2].

Test material: Glyphosate TC (purity 98.6%)

Sponsor: Cheminova Agro A/5, Lemvog, Denmark

Test description: plate incorporation assay + preincubation test

Dose finding test: included

Number of doses tested: 5, the highest tested dose was 5,000 µg/plate in experiments with S9, and 2,500 µg/plate in experiments without S9

Number of strains: only 4 strains!

Number of plates/dose: 3

Negative controls: all strains are OK

Positive controls: results are provided, positive as expected. 2-AA was used in all experiments with metabolic activation.

Statistical analysis: provided, ANOVA and Student's t-test were used.

Historical controls: not provided

Results: the test substance was not mutagenic in any strain

Remarks: only 4 strains were included, not five as requested by the OECD [2] and other guidelines [3, 4]. No historical controls were shown.

RAR: Evaluation and Comments

Study identification: *Jensen, 1991 (p.305) (Evaluation of 2001)*

Evaluation: *"Valid in vitro genotoxicity test" (page 305)*

Comments (SK and AN): only 4 strains were tested instead of 5. The study deviates substantially from the OECD guideline and the results are not reliable.

Study number 6

Performing laboratory: Monsanto Company

Date: February 7, 1992

Title: Ames/Salmonella mutagenicity assay of MON 2139 (ROUNDUP® herbicide formulation)

Report No.: MSL-11729 (91183)

Guidelines: not specified.

Test material: MON 2139 (ROUNDUP® herbicide formulation) (glyphosate 31%); the individual compounds are not specified, only the amount of glyphosate is specified

Test description: plate incorporation test + one repetition

Dose finding test: conducted but results are not clearly described (i.e. no toxic response, toxicity observed with no colonies, toxicity with colonies observed; only one strain was tested, TA100)

Number of doses tested: 5, highest dose 1,500 µg/plate in experiments with S9 and 500 µg/plate in experiments without S9

Number of strains: 4

Number of plates/dose: 3

Negative controls: background levels of revertants in all strains with and without S9 are OK

Positive controls: different compounds were used for different strains and positive results were obtained as expected.

Statistical analysis: provided. Results were log 10 transformed then analyzed with Student's t-test and Grubbs' test.

Historical controls: not provided

Results: in TA98+S9 a positive effect was seen in one series but not in the second one. The authors stated that the test compound is not mutagenic.

Remarks: only 4 strains were tested instead of 5 strains as requested by the current OECD guidelines [2, 3] and the UKEMS recommendations [4]. Historical controls are not presented as requested by the OECD guideline [2]. Invalid description of the test compound.

RAR: Evaluation and Comments

Study identification: *S.381 (Roundup) Kier, L.D.; Stegeman, S.D.; Costello, J.G. and Schermes, S. (1992, TOX1999-239)*

Evaluation: *"The study is considered acceptable."*

Comments (SK and AN): only 4 strains were tested. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 7

Performing laboratory: Monsanto Company

Date: February 18, 1992

Title: Ames/Salmonella mutagenicity assay of MON 14445 (DIRECT® herbicide formulation)

Report No.: MSL-11731 (EHL 91185/ML-91-442)

Guidelines: not described (in the original document it is stated that all procedures were realized according to Ames et al. (1975) [1].

Test material: MON 14445 (DIRECT® herbicide formulation) (glyphosate 72% acid equivalent); the content of glyphosate is specified, other compounds not!

Test description: plate incorporation test + one repetition

Dose finding test: conducted but results are not clearly described, no quantitative results are shown; only one strain was tested, TA100.

Number of doses tested: 5 (highest dose 1,500 µg/plate in experiments with S9, 500 µg/plate in experiments without S9)

Number of strains: 4

Number of plates/dose: 3

Negative controls: background frequencies of revertants are OK (with and without S9)

Positive controls: different compounds were used for different strains and positive results were obtained as expected. Three doses were tested and only one plate was used. Unusual but acceptable.

Statistical analysis: provided. Results were log 10 transformed then analyzed with Student's t-test and Grubbs' test.

Historical controls: not provided

Results: no evidence for positive effects but in some cases evidence for induction of toxicity was seen.

Remarks:

4 strains were tested instead of 5 strains as requested by the OECD guideline [2] and the UK EMS [4]. Historical controls are not shown as requested by the OECD guideline [2].

RAR: Evaluation and Comments

Study identification*: Kier, L.D.; Stegeman, S.D.; Costello, J.G. and Schermes, S. (1992, TOX1999-320): Ames/Salmonella mutagenicity assay of MON 14445 (DIRECT herbicide formulation)*

Evaluation*: "The study is considered acceptable."*

Comments (SK and AN): only 4 strains were tested instead of 5. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 8

Performing laboratory: Monsanto Company

Date: April 4, 1992

Title: Ames/Salmonella mutagenicity assay of RODEO®

Report No.: ML-91-441 (91184) Final

Guidelines: not specified (in original document it is stated that all procedure were according to Ames et al. (1975) [1].

Test material: RODEO® (glyphosate 40% acid equivalent); the content of glyphosate is specified, other compounds not

Test description: plate incorporation test + one repetition

Dose finding test: conducted, results are not clearly described, no quantitative results are shown (only Yes-NO); only one strain was tested, TA100.

Number of doses tested: 5 (highest dose 5,000 µg/plate in experiments with and without S9)

Number of strains: 4

Number of plates/dose: 3

Negative controls: background frequencies of revertants for all strains are OK (with and without S9)

Positive controls: different compounds were used for different strains and positive results were obtained as expected. Three doses were tested and only one plate was used. Unusual but acceptable.

Statistical analysis: provided. Results were log 10 transformed and analyzed with Student's t-test and Grubbs' test.

Historical controls: not provided

Results: no evidence for positive effects but in some cases evidence of acute toxicity was seen.

Remarks: only 4 strains were tested instead of 5 strains as requested by the OECD guideline [2] and the UK EMS [4]. The test substance is insufficiently described.

RAR: Evaluation and Comments

Study identification*: Kier, L.D.; Stegeman, S.D.; Costello, J.G. and Schermes, S. (1992, TOX9552373): Ames/Salmonella mutagenicity assay of Rodeo). Ames/Salmonella mutagenicity assay of MON 2139S.380*

Evaluation: *"The study is considered acceptable."*

Comments (SK and AN): only 4 strains were tested. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 12

Performing laboratory: TECAM Tecnologia Ambiental Ltda., Sao Paulu, Brazil

Date: December 13, 2007

Title: Bacterial reverse mutation test (Ames test) for GLIFOSATO TECNICO Helm

Report No.: 3393/2007 – 2.0AM

Guidelines: in the original study was mentioned that the procedures were according to Maron and Ames (1983) [13]. OECD 471 guideline was mentioned in the list of references, but was not mentioned in the text.

Test material: glyphosate (purity 98.01%)

Sponsor: Helm do Brazil Mercantil Ltda, Sao Paulo, Brazil

Test description: plate incorporation test without repetition

Dose finding test: conducted with TA100 ±S9, toxicity ca. 25% with the highest dose 5,000 µg/plate

Number of doses tested: 5 (highest dose 5,000 µg/plate in experiments with and without S9)

Number of strains: 5

Number of plates/dose: 3

Negative controls: background levels of revertants in all strains are OK.

Positive controls: different compounds were used for different strains without S9. 2-AA was used with all strains in experiments with S9. Positive results were obtained as expected.

Statistical analysis: not done, the results are presented as means±SD.

Historical controls: not provided

Results: no evidence for mutagenic effect was found.

Remarks: according to the OECD guideline [2], additional information should be provided when 2-AA is used as a positive control for all strains. This is lacking. Only one experiment was conducted. According to the OECD guideline [2], an explanation should be provided when no additional experiments are performed.

RAR: Evaluation and Comments

Study identification: *5th new Ames test (Ribeiro do Val, 2007)*

Evaluation: *The study is considered ~~supplementary because~~ acceptable although only the plate-incorporation assay was performed but not the pre-incubation method and ~~Furthermore~~, E. coli strains were not included. No evidence of mutagenicity was obtained. According to the study report, some cytotoxicity occurred that became obvious by a lower number of revertants when the*

strains TA1537 (with metabolic activiation) and TA102 (without) were treated at the highest concentration level of 5000 µg/plate.

Comments (SK and AN): the study has minor deviations from the current OECD guidelines and the results are partly reliable.

Study number 13

Performing laboratory: Bioservice Scientific Laboratories GmbH, Munich, Germany

Date: December 17, 2012

Title: Reverse mutation assay using bacteria (*Salmonella thyphimurium*) with glyphosyte tech.

Report No.: 126159 (Final)

Guidelines: the study followed EC Directive B.13-B.14 (2000) [11], EPA OPPTS 870.5100 (1998) [10] and OECD (1997) [2] guidelines.

Test material: glyphosate tech (purity 97%)

Sponsor: Industrias Afrasa S.A., Paterna, Spain

Test description: plate incorporation test + preincubation test

Dose finding test: results are described for TA100 and TA98, but the number of revertants is provided, only a "mutation factor" is presented which is not sufficient.

Number of doses tested: 5

Number of strains: 5

Number of plates/dose: 3

Negative controls: background frequencies of revertants in all strains are OK

Positive controls: 2-AA (2-aminoanthracene) was used in all experiments with S9 (with all strains). According to the OECD guideline [2], additional information is required.

Statistical analysis: not provided

Historical controls: provided

Results: No evidence for a positive effect; at the highest dose a substantial decrease of the number revertants was observed, possibly due to acute toxicity. In experiment I toxicity was observed at doses 2,500 and 5,000 µg/plate (±S9). In experiment II toxicity was found at concentrations 2,500 and 5,000 µg/plate (-S9) and at 5,000 µg/plate (+S9).

Remarks: additional information is lacking according to the OECD guideline [2] since 2-AA was used with all strains.

RAR: Evaluation and Comments

Study Identification (page 337)*: Schreib 2012*

Evaluation (page 337)*: "acceptable"*

Comments (SK and AN): the study has minor deviations from the OECD guidelines. The results are partly reliable.

Study number 14

Performing laboratory: Institute of Environmental Toxicology, Toxicology Division, Tokyo, Japan

Date: July 20, 1978

Title: The report of mutagenic study with bacteria for CP67573

Report No.: ET-78-241

Guidelines: the procedures were performed according to B. Ames et al. [1, 14].

Test material: CP67573 (Glyphosate purity 98.4%)

Description of the test: plate incorporation test

Dose finding test: not performed

Number of strains: 6 (5 Salmonella and 1 *E. coli* WP2 hcr)

Number of doses: 6 (highest dose 5,000 µg/plate)

Number of plates/dose: only 2 (therefore not possible to calculate means ± SD)

Negative controls: background levels of revertants in all strains are OK

Positive controls: different positive controls were used. In some experiments extremely high levels of revertants were obtained > 10,000/plate.

Statistical analyses: not provided

Historical control: not provided

Results: the authors stated that negative results were obtained.

Remarks: the test fails to a large extent to be in agreement with the OECD guidelines [2]. Only two plates/dose were used. No justification for the lack of a repeated experiment.

RAR – Evaluations and Comments:

Study identification*: Shirasu et al (page 305)*

Evaluation*: supplementary study, acceptable.*

Comments (SK and AN): the lack of a repeat experiment which is required and the use of only two plates/dose are not acceptable. Three plates are the minimal number to obtain reliable results according to all guidelines (OECD, ES EPA, UK EMS). The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 15

Performing laboratory: RCC Ltd, Itingen, Switzerland

Date: March 16, 2007

Title: *Salmonella thyphimurium* and *Escherichia coli* reverse mutation assay with Glyphosate technical (NUP-05068)

Report No.: Final report RCC – CCR 1061401

Guidelines: the study followed OECD (1997) [2], EC Directives B13-B14 (2000) [11] and Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), Guidelines for Study Results, Reverse mutation studies. Guideline NO.2-1-19-1. >Notification 12NohSan No. 8147, as partly revised in 16-Shouan-9260, on March 16, 2005. (English translation by ACIS on October 17, 2005.)

Test material: Glyphosate technical (purity 95.1%)

Sponsor: Nufarm Asia Sdn Bhd, Selangor, Malaysia

Test description: plate preincubation test + incorporation assay

Dose finding test: included

Number of doses tested: 8 in the first experiment, 6 in the second; the highest tested dose was 5,000 µg/plate

Number of strains: 5

Number of plates/dose: 3

Negative controls: background levels in all strains are OK.

Positive control: results are provided, positive as expected. However, in all experiments with S9 with all strains 2-AA was used without further information (as requested by OECD [2]).

Statistical analysis: means ±SD are provided, no test for a dose-response effect was provided.

Historical controls: provided

Results: the test substance was not mutagenic in any strain, a significant decrease of revertants number at the highest dose due to acute cytotoxicity.

Remarks: 2-AA was used in all experiments with S9 without additional information which is requested in the OECD guideline [2].

RAR – Evaluations and Comments:

Study identification*: 2nd new Ames test (Sokolowski, 2007)*

Evaluation*: The study is considered acceptable. No evidence of a mutagenic response was obtained. The lower number of revertants in one experiment with TA 1537 might point to a weak cytotoxic effect of the test substance to this strain at a high concentration. In the past, similar*

observations were occasionally made with glyphosate from different sources at high concentrations (see DAR, 1998, ASB2010-10302).

Comments (SK and AN): the study has minor deviations from the OECD guidelines. The results are partly reliable.

Study number 16

Performing laboratory: RCC Ltd, Itingen, Switzerland

Date: March 16, 2007

Title: *Salmonella thyphimurium* and *Escherichia coli* reverse mutation assay with Glyphosate technical (NUP-05070)

Report No.: Final report RCC – CCR 1061402

Guidelines: OECD [2], EC Directive [11], Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), Guidelines for Study Results, Reverse mutation studies. Guideline NO.2-1-19-1. >Notification 12NohSan No. 8147, as partly revised in 16-Shouan-9260, on March 16, 2005. (English translation by ACIS on October 17, 2005.)

Test material: Glyphosate technical (purity 97.7%)

Sponsor: Nufarm Asia Sdn Bhd, Selangor, Malasia

Test description: plate preincubation test + incorporation assay

Dose finding test: included

Number of doses tested: 8 in the first experiment, 6 in the second one. The highest tested dose was 5,000 µg/plate

Number of strains: 5

Number of plates/dose: 3

Negative controls: background levels of revertants in all strains are OK

Positive controls: results are provided, positive as expected. However, in all experiments with S9 2-AA was used with all strains without further information (as requested by OECD [2]).

Statistical analysis: means ±SD are provided. No test for a dose-response effect was applied.

Historical controls: provided

Results: the test substance was not mutagenic in any strain, a significant decrease of revertants number was seen at the highest dose due to acute cytotoxicity.

Remarks: 2-AA was used in all experiments with S9, no additional information was provided as suggested in the OECD guideline [2].

RAR: Evaluation and Comments

Study identification*: 3rd new Ames test (Sokolowski, 2007)*

Evaluation*: The study is considered acceptable. No evidence of a mutagenic response was obtained. Cytotoxic effects, if occurring, were confined to high concentrations and certain bacterial strains.*

Comments (SK and AN): the study has minor deviations from the OECD guidelines. The results are partly reliable.

Study number 17

Performing laboratory: RCC Ltd, Itingen, Switzerland

Date: March 16, 2007

Title: *Salmonella thyphimurium* and *Escherichia coli* reverse mutation assay with Glyphosate technical (NUP-05067)

Report No.: Final report RCC – CCR 1061402

Guidelines: OECD #471 (1997) [2], EC Directive B13./B.14 [11] and Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), Guidelines for Study Results, Reverse mutation studies. Guideline NO.2-1-19-1. >Notification 12NohSan No. 8147, as partly revised in 16-Shouan-9260, on March 16, 2005. (English translation by ACIS on October 17, 2005.) guidelines.

Test material: Glyphosate technical (purity 95.0%)

Sponsor: Nufarm Asia Sdn Bhd, Selangor, Malaysia

Test description: plate preincubation test + incorporation assay

Dose finding test: included

Number of doses tested: 8 in the first experiment, 6 in the second one. The highest tested dose was 5,000 µg/plate

Number of strains: 5

Number of plates/dose: 3

Negative controls: background levels of revertants are OK in all strains

Positive controls: results are provided, positive as expected. However, in all experiments with S9 with all strains 2-AA was used without further information (as requested by OECD [2]).

Statistical analysis: means ±SD are provided, o test for a dose-response effect was performed.

Historical controls: provided

Results: the test substance was not mutagenic in any strain, a significant decrease of the revertant numbers at the highest dose was observed due to acute cytotoxicity.

Remarks: 2-AA was used in all experiments with S9, no additional information was provided as suggested in the OECD guideline [2].

RAR: Evaluation and Comments

Study identification*: 3rd new Ames test (Sokolowski, 2007), pages 313-314*

Evaluation (page 314)*: The study is considered acceptable. No evidence of a mutagenic response was obtained. Cytotoxic effects, if occurring, were confined to high concentrations and certain bacterial strains.*

Comments (SK and AN): the study has minor deviations from the OECD guidelines. The results are partly reliable.

Study number 18

Performing laboratory: Harlan Cytotest Research GmbH (Harlan CCR), Rossdorf, Germany

Date: December 18, 2009

Title: Glyphosate technical – *Salmonella thyphimurium* and *Escherichia coli* reverse mutation assay

Report No.: 1264500 (Final)

Guidelines: OECD #471 (1997) [2], EPA, Health Effects Test Guidelines OPPTS 870.5100 (1998) [10], EC Directives B.13/14 (2000) [11] guidelines.

Deviations: None

Test material: Glyphosate technical (96.3% of glyphosate acid)

Sponsor: Syngenta Ltd, Berkshire, UK

Test design: plate incorporation test + preincubation test

Dose finding assay: included

Number of strains: 6 (4 Salmonella and 2 Escherichia)

Number of plates/dose: 3

Number of concentrations tested: 8

Negative controls: background levels of revertants in all Salmonella strains are OK.

Background levels of revertants in WP2 uvrA pKM101 (-S9 and +S9) and WP2 pKM101 (-S9 and +S9) are too high according to Levy et al. [6] and UK EMS recommendations [4].

Positive controls: yielded positive results as expected. 2-AA (2-aminoanthracene) was used in all experiments with S9 (for all strains)

Statistical analysis: not provided

Historical controls: provided

Results: No evidence for a positive effect for a tested compound.

Remarks: The main problem is that the spontaneous levels of revertants seen in *Escherichia coli* strains WP2 pKM101 and WP2 uvrA pKM101 with and without metabolic activation are substantially higher as guidelines (Levy et al. [6] and UK EMS [4]). 2-AA was used without providing further information as requested by OECD. "2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. If 2-aminoanthracene is used, each batch of S9 should also be characterized with a mutagen that requires metabolic activation by microsomal enzymes, e.g., benzo(a)pyrene, dimethylbenz(a)anthracene. [2]"

RAR: Evaluation and Comments

Study identification (pages 332 - 337): *13th new Ames test (Sokolowski, 2009)*

***Evaluation**: The study is considered acceptable. It could be shown that also technical glyphosate was not mutagenic when manufactured via the Nantong Jiangshan (glycine) route.*

Comments (SK and AN): abnormal background rates are not acceptable as they indicate that the genetic identity of a specific strain is questionable. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 19

Performing laboratory: Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany

Date: April 07, 2010

Title: *Salmonella thyphimurium* and *Escherichia coli* reverse mutation assay with Glyphosate TC spiked with Glyphosine

Report No.: 1332300

Guidelines: OECD #471 (1997) [2]

Test material: Glyphosate TC (purity 95.23%) and Glyphosine (purity 99.0%)

Sponsor: Helm AG, Hamburg, Germany

Test description: plate preincubation test + incorporation assay

Dose finding test: included for all 5 strains

Number of doses tested: 8 in the first experiment, the highest tested dose was 5,000 µg/plate

Number of strains: 5

Number of plates/dose: 3

Negative controls: background levels of revertants in all Salmonella strains are OK. That of WP2 uvrA with and without metabolic activation are substantially higher than recommended by Levy et al. [6].

Positive control: results are provided, positive as expected. However, in all experiments with S9 with all strains 2-AA was used without providing further information (as requested by OECD [2]).

Statistical analysis: means ±SD are provided.

Historical controls: provided

Results: the test substance was not mutagenic in any strain.

Remarks: 2-AA was used in all experiments with S9 without additional information is provided as suggested by the OECD guideline [2]. The background levels of revertants in *E. coli* strain WP2 uvrA were higher than recommended [6].

RAR – Evaluations and Comments:

Study identification*: 9th new Ames test (Sokolowski, 2010) (pages 324-326)*

Evaluation*: The study is considered acceptable. No evidence of mutagenicity was obtained. The reason for glyphosine spiking of the test material is not clear but it is assumed that this substance*

(similar to glyphosate) may occur as an impurity in the technical active ingredient. Thus, this test might become particularly important if a certain specification needs to be assessed from a toxicological point of view.

Comments (SK and AN): abnormal background rates are not acceptable as they indicate that the genetic identity of a specific strain is questionable. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 20

Performing laboratory: SafePharm Laboratories, Derby, UK

Date: January 2, 1996

Title: Technical glyphosate: reverse mutation assay "Ames test" using *Salmonella thyphimurium* and *Escherichia coli*

Report No.: 434/014 (Final)

Guidelines: OECD 471 (1983), EEC [11], US EPA (TSCA) guidelines

Deviations: None

Test material: Glyphosate technical (95.3%)

Sponsor: Mastra Industries SDN. BHD

Co-sponsor: Maruzen Kako Co., Ltd

Study description: plate incorporation test + one repetition

Dose finding assay: included, results for 2 strains are described (five doses).

Number of stains: 5 (4 Salmonella and 1 *E. coli*)

Number of plates/dose: 3

Number of doses tested: 5

Negative controls: the background number of revertants were in normal range in all strains

Positive controls: 3 compounds were used as positive controls in different strains. Positive results were obtained as expected.

Statistical analysis: no analysis was performed.

Historical controls: not provided

Results: No evidence for a positive effect was found. With 5,000 µg/plate and 1,500 µg/plate toxicity was observed, nevertheless, both concentrations were included in the main experiment. According to OECD guideline, toxicity should occur only at the highest dose [2].

Remarks: At two higher doses acute toxicity was observed which is not in agreement with the OECD guideline [2].

RAR: Evaluation and Comments

Study identification*: 11th new Ames test (Thompson, 1996)*

Evaluation: *There was no evidence of mutagenicity obtained although there was some cytotoxicity. The study is considered ~~supplementary since acceptable~~ although only the plate incorporation method was applied for testing.*

Comments (SK and AN): the study has minor deviations from the OECD guidelines. The results are partly reliable.

Study number 21

Performing laboratory: Harlan Laboratories Ltd., Derbyshire, UK

Date: September 2, 2014

Title: Glyphosate: reverse mutation assay "Ames test" using *Salmonella thyphimurium* and *Escherichia coli*

Report No.: 41401854

Guidelines: the procedures were performed according to Ames and coauthors [1, 13, 14].

Test material: Glyphosate (purity 85.79%)

Sponsor: Albaugh Europe Sarl, Lausanne, Switzerland

Study description: plate incorporation test + preincubation test

Dose finding assay: not included

Number of stains: 5 (4 Salmonella and one *E. coli*), highest dose tested was 5,000 µg/plate

Number of plates/dose: 3

Number of doses tested: 5

Negative controls: the background levels of revertants was in the normal range

Positive controls: all positive controls yielded positive results as expected. 2-AA was used in all experiments with metabolic activation (S9) with strains TA100, TA1535, TA1537 and WP2uvrA (without further information) and benzo(a)pyrene with strain TA98.

Statistical analysis: was performed but not described.

Historical controls: provided

Results: No evidence for positive effects for a tested compound. In some strains acute toxicity was seen at highest doses.

Remarks: 2-AA was used in all experiments with metabolic activation (S9) with all strains without further information.

RAR: Evaluation and Comments

Study identification: *Thompson 2014 (page 337)*

Evaluation: *(page 306) supplementary study; (page 337) the study is considered acceptable*

Comments (SK and AN): the study has minor deviations from the OECD guidelines. The results are partly reliable.

Study number 22

Performing laboratory: BioAgri, Sao Paulo, Brazil

Date: December 23, 1996

Title: The *Salmonella thyphimurium* reverse mutation by Glifos

Report No.: G. 1. 1 – 050/96

Guidelines: not specified. In the original document it is stated that tests with metabolic activation were performed according to Maron and Ames [13]

Test material: Glifos (glyphosate; 96%)

Sponsor: Chemonova Agro S. A., Lemvig, Denmark

Test description: plate incorporation test without repetition (no justification for lack of a follow-up study provided)

Dose finding test: not included

Number of doses tested: 4

Number of strains: 4

Number of plates/dose: 3

Negative controls: the background levels of revertants are OK with and without S9

Positive controls: severe mistakes were made. It is stated that sodium azide was used for experiments with and without metabolic activation for strains TA100 and TA1535, and 2-aminofluorene (2-AA) for strains TA98 and TA97a with S9. Sodium azide cannot be used in experiments with S9 as it is directly active.

Statistical analysis: provided (ANOVA)

Historical controls: not provided

Results: at the highest dose and in some cases also with the second highest dose acute toxic effects were found. According to the OECD guideline [2], cytotoxicity should occur only at the highest dose. The authors stated that "Glifos did not exhibit genetic activity on the strains of *Salmonella thyphimurium* tested." Results obtained with positive controls are not clearly presented, it is only indicated > 300 revertants/plate.

Remarks: This study has severe shortcomings. i) only 4 strains were used; ii) the positive control (sodium azide) can be only used in experiments without S9 but not as a proof for the activity of the S9 mix. Another positive control has to be used for experiments with S9 mix in strains TA100 and TA1535. No historical controls were presented.

A very strong difference in the dose range by a factor of 10 was used. This is not with agreement with the OECD guideline which states that "the test substance should be used with approximately half log (i.e. √10) intervals between test points for an initial experiment [2]"

RAR – Evaluations and Comments:

Study identification: *Vargas, A.A.T. (1996, TOX1999-884) (page 381)*

Evaluation: *"The study is considered of limited value for risk assessment only since a legal statement on GLP compliance is lacking and since there were some minor reporting deficiencies in particular regarding the negative (absolute and solvent) and positive control values."*

Comments (SK and AN): the study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 23

Performing laboratory: BioService, Scientific Laboratories GmbH, Planegg, Germany

Date: August 4, 2010

Title: Reverse mutation assay using bacteria (*Salmonella thyphimurium*) with Glyphosate TC

Report No.: 101268 (Final)

Guidelines: OECD #471 (1997) [2], EC Commission Regulation B.13/B.14 (2000) [10] and US EPA OPPTS 870.5100 (1998) [9] guidelines

Test material: Glyphosate TC (purity 95.8%)

Sponsor: Helm AG, Hamburg, Germany

Test description: plate incorporation test + preincubation assay

Dose finding test: not included

Number of doses tested: 6 (5,000 µg/plate was the highest)

Number of strains: 5

Number of plates/dose: 3

Negative controls: the background levels of revertants with and without S9 are OK for all strains

Positive controls: included, positive results were obtained as expected. In all experiments with S9 2-AA was applied with all strains without further information as required by the OECD guideline [2].

Statistical analysis: not provided.

Historical controls: provided

Results: in the first experiment toxicity was observed in some strains at concentrations of 2,500 µg/plate and 5,000 µg/plate (±S9); in the second experiment toxicity was observed in some strains at concentration of 5,000 µg/plate (±S9). The authors stated that the compound is considered to be non-mutagenic in this assay.

Remarks: apart from use of 2-AA in all strains with S9, no other shortcomings are noted in the study.

RAR: Evaluation and Comments

Study identification: *10th new Ames test (Wallner, 2010), pages 326-328*

Evaluation: "*The study is considered acceptable. Glyphosate proved non-genotoxic. Cytotoxicity was confined to very high concentrations."*

Comments (SK and AN): the study has minor deviations from the OECD guidelines. The results are partly reliable.

Study number 54

Performing laboratory: Monsanto Company

Date: March 3, 1981

Title: Ames/Salmonella mutagenicity assay of MON 8080

Report No.: Final ML-80-294/800281; MSL 1538

Guidelines: in the original document is mentioned that the metabolic activation part of the study was performed according to Ames et al. (1995) [1].

Test material: MON 8080 (purity 87.6%); the product is not specified

Test type: Salmonella/microsome mutagenicity test

Test description: spot test and plate incorporation assay (no repetition, i.e. no preincubation assay was performed)

Dose finding test: a toxicity test is described (Table 3; only with TA100).

Number of doses tested: 6

Number of strains: 4

Number of plates/dose: 3

Negative controls: included, background levels of revertants are OK.

Positive controls: included; 2-AA was used for experiments with S9 in TA98 and TA1537 strains, benzo(a)pyrene for TA100 and tris(2,3-dibromopropyl)phosphate for TA1535. In experiments without metabolic activation 4-nitroquinoline (TA98 and TA100), $NaNO_2$ (TA1535) and 9-aminoacrodone (TA1537) were used. No means ± SD were calculated.

Statistical analysis: t-test and regression analysis were used for dose-response calculations.

Historical controls: not provided

Results: it is stated that tested compound was not mutagenic. The results cannot be assessed since no means ± SD were presented. The results of the spot-test are irrelevant. The amount of test substance is given in µl (which is not adequate) and no quantitative results are provided (only P for positive and N for negative). In this form presentation of results is not useful.

Remarks: the results are completely irrelevant. The study has major severe shortcomings. Only four strains were used instead of 5; no quantitative results are provided and no means ± SD were calculated. The test substance is a surfactant used in glyphosate formulations

RAR: Evaluation and Comments

Study identification: *Flowers, L.J. (1981) (page 395)*

Evaluation: *"The study is considered acceptable"*

Comments (SK and AN): the study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 97

Performing laboratory: Hoechst, Pharma Development Central Toxicology, Frankfurt, Germany

Date: July 10, 1992

Title: Study of the mutagenic potential in strains of Salmonella thyphimurium (Ames test) and Escherichia coli

Report No.: 92.0467

Guidelines: in original document it is indicated that the metabolic activation part of the study was done according to Ames et al. [1].

Test material: Dodigen 4022 (purity 82%); surfactant used for herbicide formulations; does not contain glyphosyte

Study type: Salmonella/microsome mutagenicity test

Test description: two independent experiments, plate incorporation assay

Dose finding test: provided

Number of doses tested: 6, the highest tested dose was 10,000 µg/plate (5,000 mg/plate is the highest dose for a non-toxic compound as suggested by the OECD guideline [2].

Number of strains: 6 (TA 98, TA 100, TA 1535, TA 1537, TA 1538, WP2uvrA)

Number of plates/dose: 3

Negative controls: background levels of revertants in all strains are OK.

Positive controls: results are provided, positive as expected. In all experiments with S9 with all strains 2-AA was used without further information (as requested in the OECD guideline [2]).

Statistical analysis: not provided

Historical controls: not provided

Results: the authors stated that in all experiments test compound did not induce mutations. No SD are provided (as requested by OECD [2]).

Remarks: no historical control data are presented. 2-AA was used in all experiments with S9 without additional information requested by the OECD guideline [2]. The study was not conducted with glyphosate. No preincubation experiments were conducted.

RAR – Evaluations and Comments:

Study identification: *Stammberger, I. and Mayer, D. (1992, TOX1999-324): Dodigen 4022: Study of the mutagenic potential in strains of Salmonella typhimurium (Ames test) and Escherichia coli. (S.395)*

Evaluation: *"The study is considered acceptable."*

Comments (SK and AN): the study was not conducted with glyphosate; the study has minor deviations from OECD guidelines. The results are partly reliable.

Study number 99

Performing laboratory: Monsanto Company

Date: October 26, 1990

Title: Ames/salmonella mutagenicity assay of MON 0818

Report No.: MSL-10625

Guidelines: in original document is mentioned that metabolic activation part of the study was performed according to Ames et al. [1].

Test material: MON 0818 (purity: ethylene glycol 4.3%, polyethylene glycol 18.3%, polyoxyethylene tallowamine 71.9%, water 4.1%); the test compound is a mix and does not contain glyphosate

Study type: Salmonella/microsome mutagenicity assay

Test design: plate incorporation test + one repetition

Dose finding assay: results are presented in non-quantitative way.

Number of strains: 4

Doses tested: 5.

Number of plates/dose: 3.

Negative controls: indicated; Background levels of all strains are OK.

Positive controls: different compounds were used and positive results are presented as expected. In experiments with S9 benzo(a)pyrene was used with strain TA100, with all others 2-AA.

Statistical analysis: Student's t-test

Historical controls: not provided

Results: no evidence for positive effects for a tested compound. Acute toxicity was observed in some experiments not only with highest dose but also with the second highest dose. According to OECD guideline, acute toxicity should take place only the highest dose. The highest dose was too high and no revertants could be counted at all in any of experiments. Therefore, only 4 doses provided relevant information.

Remarks: the highest dose tested was inadequate (no colonies). Historical controls are missing. Only 4 strains were tested in contrast to requirements of the OECD guideline [2] and the UK EMS guidelines [4]. The test material is a surfactant used in glyphosate formulations, but does not contain glyphosate.

RAR – Evaluations and Comments:

Study identification: *Stegeman, S.D. and Li, A.P. (1990, TOX1999-241) (page 395)*

Evaluation: *"The study is considered acceptable"*

Comments (SK and AN): the study deviates substantially from the OECD guidelines and the results are not reliable.

Study number KIIA.5.4.1/06

Performing laboratory: BioAgri Laboratory, Piracicaba, Brazil

Date: September 15, 2008

Title: Evaluation of the mutagenic potential of the test substance Glyphosate Technical by reverse mutation assay in Salmonella thyphimurium (Ames test)

Report No.: Final RF 3996.401.392.07

Guidelines: OECD #471 (1997) [2].

Test material: Glyphosate Technical (purity 98.05%)

Sponsor: Jingma Chemicals Co., Ltd, Zheijang, China

Test description: plate incorporation assay, no repetition (no justification for lack of repetition)

Dose finding test: included only for one strain, TA100

Number of doses tested: 7, the highest tested dose was 5,000 µg/plate

Number of strains: 5

Number of plate/dose: 3

Negative controls: included, background levels of revertants are OK in all strains.

Positive controls: different compounds were used for experiments without S9. In all experiments with S9 with all strains 2-AA was used without further information (as requested by OECD [2]).

A significant induction of his+ revertants was detected in TA98+S9 at concentrations glyphosate 0.001 and 0.01 mg/plate). Results are provided, positive as expected. In some case extremely high numbers of revertants were found (> 6500/plate).

Statistical analysis: provided (ANOVA)

Historical controls: provided

Results: the authors stated that the test substance was not mutagenic in any strain.

Remarks: the study has a number of shortcomings. 2-AA was used in all experiments with S9 without additional information is provided as suggested in the OECD guideline [2]. No additional preincubation test was conducted, no rationale was provided why it was not performed as requested by the OECD guideline [2]. Significant induction of his+ revertants was detected in TA98+S9 with doses of 0.001 and 0.01 mg/plate. This observation merits a follow-up study. The positive control 2-AA induced too many revertants. Such a high dose of 2-AA should not be used as it does not reflect the sensitivity of the Salmonella strains adequately.

RAR – Evaluations and Comments:

Study identification: *6th new Ames test (Miyaji, 2008) (pages 318-320)*

Evaluation: ***"****No evidence of mutagenicity was obtained in the plate-incorporation assay. However, since the highest concentration of 1000 µg/plate was much lower than in most other studies and since no apparent reason for not using higher dose levels was given, the study is considered not acceptable."*

Comments (SK and AN): the study deviates moderately from the OECD guidelines and the results are not reliable.

2.3. Summary: individual tests

Salmonella/microsome assays

In total, 24 individual Salmonella/microsome studies were evaluated.

<u>In 6 studies repeat experiments are lacking (without justification)</u>:

Study 1

Study 12

Study 14

Study 22

Study 54

Study KIIA.5.4.1/06

<u>In 7 studies number of strains is not adequate</u>:

Study 5

Study 6

Study 7

Study 8

Study 22

Study 54

Study 99

In total 7 Salmonella/microsome assays (No. 5, 6, 7, 8, 22, 54 and 99) were found acceptable by the BFR, in which only **<u>four</u>** tester strains were used. Several strains were initially

developed by Bruce Ames, who invented this test system and it was suggested by him to use at least four of them in routine testing of chemicals in 1975 [1]. The strains have different target genes and other properties (repair capacity, membrane permeability etc.) and were complementary in regard to the detection of different classes of mutagens. One of the shortcomings of the initial version of this assay (with a set of four strains) was that it did not detect reactive oxygen species (ROS), therefore, further strains (TA102 and TA104) were developed to compensate for this disadvantage [15-19]. Since some laboratories experienced difficulties with these strains, it was decided on the basis of the evaluation of the sensitivity of different *E. coli* WP strains, that it is acceptable to use them as a replacement of TA102/TA104 (see UKEMS guidelines [4]). In the OECD guidelines (1997) [2], US EPA (1998) [10] and also in the UKEMS guideline (1990) [4] it is consistently mentioned that five strains (including either TA 102 or at least one *E. coli* strain) have to be used in routine testing of chemicals. This is of particular importance in the case of experiments with glyphosate. It is assumed that the herbicide may cause DNA-damage via generation of reactive oxygen radicals (ROS; see for details IARC Vol. 112 [20]). Therefore, it is absolutely not acceptable to conduct Salmonella/microsome assays without including strains that are sensitive towards ROS such as TA102 or TA104 or *E. coli* WP strains (that can replace these latter strains).

In 12 studies 2-AA was used in all experiments with S9, no additional information is provided:

Study 1

Study 2

Study 5

Study 13

Study 15

Study 16

Study 17

Study 18

Study 19

Study 23

Study 97

Study KIIA.5.4.1/06

Wrong positive control in experiments with S9:

Study 22

Inadequate number of plates per dose:

Study 14

In 2 studies inadequate results were found in negative controls:

Study 18

Study 19

In these studies the background levels of revertants are not in expected range. This indicate that the genetic background as altered and may affect the sensitivity. Studies with abnormal frequencies in Salmonella TA102/104 or *E.coli* WP strains were considered as not reliable as

they are in particularly sensitive to ROS which are considered to play a role in glyphosate toxicity [20].

Historical controls is not provided in 7 studies:

Study 1

Study 2

Study 6

Study 7

Study 8

Study 97

Study 99

Inadequate selection of doses:

Study 22

Study 99

Individual plate counts are not shown:

Study 3

Study 4

Study 14

A more detailed description of the test material is warranted in 7 studies:

Study 1 (HR-001)

Study 6 (Roundup)

Study 7 (Direct)

Study 8 (Rodeo)

Study 54 (MON 8080)

Study 97 (Dodigen)

Study 99 (MON 0818)

Test material:

- five studies tested formulations containing glyphosate (studies #6, #7, #8, #54, # 99)
- one investigation was conducted with surfactant Dodigen 4022 (study # 97)
- in one study the tested material is not characterized (#1)

2.4. References

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3. Rec-A assay

3.1. Description

The test system was developed in the early 1970's by T. Kada and his co-workers in Japan [1, 2]. It is based on comparisons of the viability of two isogenic strains of *Bacillus subtilis* which differ in regard to their repair capacity (repair proficient vs. repair deficient in regard to the "rec A" function). Genotoxic agents lead to different killing effects in the two strains, i.e. the repair deficient stains are more sensitive as the repair proficient strains. The test is based on the growth inhibition of the two strains. The procedure was used in 1970s and 1980s and was partly validated to regard of its sensitivity/specificity for the detection of genotoxic carcinogens and is not included in guidelines for routine testing of chemicals. Only a relatively small number of chemicals was included in a validation by Matthews et al. (2006) [3]. The specificity of the test for detection of carcinogens was quite low (61.9%). Notably, not all genotoxins cause activation of the repair function on which the test is based. Certain genotoxins do not induce the repair system in which recA is involved, these compounds give negative results.

A relatively precise description of the test can be found in the "Handbook of Mutagenicity Test Procedures" (Kilbey et al., 1984; [4]). Two isogenic strains are usually used for the experiments. The test can be conducted with bacterial cells but with also spores (this procedure was postulated to increase the sensitivity). The test compounds are placed on filter disc on Petri dishes on which cells or spores are seeded. The growth inhibition is determined after inoculation of the plates. Positive and negative controls should be included in the test. The number of parallel treatments is usually three. Experiments are conducted with and without metabolic activation.

3.2. Evaluation of individual studies

Study number 14

Performing laboratory: Institute of Environmental Toxicology, Toxicology Division, Tokyo, Japan

Date: July 20, 1978

Title: The report of mutagenic study with bacteria for CP67573

Report No.: ET-78-241

Test material: CP67573 (Glyphosate purity 98.4%)

Description of the test: Rec Assay with *B. subtilis*

Protocol: "streak-out" experiments with filter discs with the parental strain (H17, rec+) of *B. subtilis* and a recA- mutant (M45, rec-).

Number of plates: 3 per points.

Concentrations tested: 6 (20 – 2,000 µg/disc)

Negative control: included, results are as expected

Positive control: included (mitomycin C) but only for experiments with S9; positive control was effective as expected.

Statistical analyses: not provided

Results: negative results were obtained with all concentrations in both strains without S9. No test with S9 mix was included.

Remarks: inconclusive results as no experiments with S9 mix were included, the test is not contained in international guidelines.

RAR: Evaluation and Comments

The study is not included in the RAR document

Study number 31

Performing laboratory: Institute of Environmental Toxicology, Toxicology Division, Tokyo, Japan

Date: March 14, 1995

Title: HR-001: DNA repair test (Rec-Assay)

Report No.: IET-94-0141

Guidelines: "required under U.S. EPA FIFRA Guidelines, Subdivision F [5].

Test material: HR-001 (purity 95.68%)

Sponsor: Sankyo Co., Ltd, Tokyo, Japan

Description of the test: Rec Assay with *B. subtilis* strains H17 (*rec*+) and M45 (*rec*-)6

Protocol: spot test with spore agar plates (growth inhibition)

Concentrations tested: 5 (7.5 – 240 µg/disc)

Negative control: included, rates are OK

Positive controls: included, for experiments without S9 mitomycin C, and for experiments with S9 Trp-P-1 were used.

Results: a moderate positive result was obtained with the highest dose (1.0 mm larger growth inhibition in the repair deficient strain). Positive results were obtained with the positive controls.

Remarks: The authors explained that the result is negative but it is unclear why they come to this conclusion as a moderate positive result was seen with the highest tested concentration.

It is also unclear why higher concentrations were not tested (e.g., 2,000 mg/disc as in study number 14). The results are inconclusive in our opinion; furthermore, the test is not included in internationally accepted guidelines (e.g. OECD, US EMS, US EPA).

RAR: Evaluation and Comments

Study identification*: in RAR document the study is indicated as IIA, 5.4.3/02 (Akanuma, 1995)*

Evaluation*: The study is considered to provide supplementary information only because the Rec assay is not a standard method for this endpoint (DNA damage and repair). Furthermore, dose selection was not explained. However, the study results are valid and it is agreed that the test compound glyphosate proved negative in this experiment, both with and without metabolic activation. This conclusion can be drawn because the evaluation criteria for a positive response were not met. The difference of growth inhibiting zone between the two strains was 1 mm at the highest concentrations and differences were 0 at lower dose levels. Thus, they were below the value obtained for the negative control (kanamycin). When the study description in the dossier was compared to the original study report, it was noted that the study director was Mie Akanuma. Erroneously, the first name had been mentioned in the dossier instead of the authors surname.*

Comments (SK and AN): the test detects only compounds which cause activation of a specific DNA-repair pathway; its specificity is very low; the results of the study do not provide "supplementary information; the results of the studyare not reliable.

3.3. Summary

Rec-A test

The test is only partly validated and was never implemented in international guidelines for routine testing of chemicals. The test procedure is based on the detection of induction of an improved survival in presence of a specific repair gene. Since several classes of mutagens do not induce this specific repair pathway, they cannot be detected with this assay. If the experiments are conducted adequately, it can be used as supplementary information concerning mechanisms. At present it is outdated and not used in mutation research and for routine testing of chemicals.

Both reports have several shortcomings.

Lack of results from high doses:

Study 31 the compound can be tested up to doses of 2,000 µg/plate but only 240 µg/plate were tested which caused a marginal positive effect.

Study 14: no experiments with S9 mix were conducted which are mandatory according to the study protocol.

Characterization of the test material:

In study # 31 the material which was tested is not specified.

3.4. References

[1] T. Kada, Y. Sadaie, Y. Sakamoto, Bacillus subtilis repair test, in: B.J. Kilbey, M. Legator (Eds.) Handbook of Mutagenicity Test Procedures, Elsevier, London, 1984.

[2] T. Kada, K. Tutikawa, Y. Sadaie, In vitro and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected, Mutat Res, 16 (1972) 165-174.

[3] E.J. Matthews, N.L. Kruhlak, M.C. Cimino, R.D. Benz, J.F. Contrera, An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: I. Identification of carcinogens using surrogate endpoints, Regul Toxicol Pharmacol, 44 (2006) 83-96.

[4] Kilbey, B.J., *Bacillus Subtilis Repair Test*, in *Handbook of Mutagenicity Test Procedures* B.J. Kilbey, et al., Editors. 1984, Elsevier Science: Amsterdam.

[5] B. Jaeger, Pesticide assessment giudelines: Subdivision F. Hazard evaluatuin - humans and domenstic animals, in: H.E. Division (Ed.), EPA, Washington, DC, 1984.

4. Gene mutations in mammalian cells *in vitro* (*Hprt* test)

4.1. Description

The test system is based on the detection of (forward) gene mutations in the hypoxanthine-guanine phosphoribosyl transferase gene (*Hprt* in rodent cells, *HPRT* in human cells) and was developed in the 1980s [1, 2]. The experiments can be conducted with several suitable cell lines. Mutant cells which are deficient in *Hprt* enzyme activity are resistant to the cytostatic effects of the purine analogue (antimetabolite) 6-thioguanine (6-TG) while non-mutant cells cannot proliferate in presence of 6-TG [1, 2]. Important parameters of the test are the relative survival rate which is determined by plating indicator cells on non-selective medium plates and the mutant frequencies (MF) which indicate the number of mutants, that are found when the cells are seeded on plates which contain the selection agent (6-TG).

The procedure is described in detail in the OECD guideline # 476 (1997), in an update of the OECD guidelines 2014-2015) [3, 4] and the criteria and requirements for an adequate report are specified. The procedure is also explained in the UK EMS guideline [5]. The experiments are conducted with and without metabolic activation (S9 mix); at least 3-4 doses of test compound as well as positive and negative controls should be included.

4.2. Evaluation of individual studies

Study number 25

Performing laboratory: Scantox A/S, Denmark

Date: September 13, 1991

Title: Mutagenicity test: In vitro mammalian cell gene mutation test with glyphosate, batch 206-JaK-25-1.

Report No.: 12325

Guidelines: not specified

Test material: Glyphosate (purity 98.6%)

Sponsor: Cheminova Argo A/S, Denmark

Test type: Hypoxanthine-guanine phosphoribosyl transferase assay with mouse lymphoma cells (L5178Y) with and without metabolic activation.

Test protocol: a preliminary toxicity test and two main experiments are described. Two cultures were used per experiment. Four doses were tested, the exposure time was 3 h.

Negative controls: included; however, the results are presented in transformed form; it is not possible to assess background levels.

Positive controls: included in experiments with and without S9 (ethyl methanesulfonate (EMS) for experiments without metabolic activation) and dimethylbenzanthracene (DMBA) with S9. Positive results were obtained as expected.

Statistical analyses: ANOVA is mentioned but was obviously not applied; no p values are shown.

Historical controls: not shown.

Results: the authors stated that they did not find evidence for positive effects.

Remarks: the protocol is in general in agreement with the OECD guideline [3] with several deviations. However, it is not possible to assess the actual spontaneous mutations as only transformed data are shown. Historical controls data are not shown. Relative survival data are not indicated in % as required. The results of a preliminary toxicity study are not shown. No p values from statistical analyses are shown.

RAR: Evaluation and Comments

Study identification: *Jensen, 1991 (page 338)*

Evaluation: "*confirmed to be acceptable upon re-evaluation"*

Comments (SK and AN): the study has deviations from the OECD guidelines. The results are partly reliable.

Study number 27

Performing laboratory: Monsanto Company, US

Date: October 20, 1983

Title: CHO/HGPRT gene mutation assay with glyphosate

Report No.: ML-83-155; Project: 830079

Guidelines: not specified

Test material: Glyphosate (purity 98.7%)

Test type: Hypoxanthine-guanine phosphoribosyl transferase assay with Chinese hamster ovary (CHO) cells with and without metabolic activation

Test protocol: two experiments were performed namely 1) a study with different S9 mix concentrations and 2) dose-response experiments with a fixed concentration of S9 (5.0%) and different concentrations of the test compound. The treatment time was 3 hours.

Negative controls: included, the background level is OK

Positive controls: included, ethyl methanesulfonate (EMS) for experiments without metabolic activation and benzo(a)pyrene for experiments with S9. Both produced positive results as expected.

Historical controls: not provided.

Statistical analyses: mentioned but not described in detail. The authors state that they used an approach described by Snee et al. [6] (Student's t-test).

Results: the authors state that using method of Snee et al. [6] no statistically significant increases of the mutant rates were found. However, the experimental series with S9 showed a clear increase which was most pronounced in experiments with 1.0% of S9. Also with 2.0% and 5.0% S9 an indication for an increase was seen. An increase was also observed in the follow-up study (several doses of glyphosate were tested in presence of 5.0% S9).

Remarks: the protocol follows in general the OECD guideline [3] with some deviations.
Relative survival values were not indicated in % as suggested. A concentration response relationship was not evaluated. Historical control values are not provided.

RAR: Evaluation and Comments

Study identification: *TOX9552369 Monsanto (page 338)*

Evaluation: "*considered still acceptable although it is not entirely clear from the original study report which dose level was actually the highest under activation conditions.*

Comments (SK and AN): the study has some deviations from the OECD guidelines. The results are partly reliable.

Study number 32

Performing laboratory: Central Toxicology Laboratory, Cheshire, UK

Date: May 24, 1996

Title: Glyphosate acid: L5178Y $TK^{+/-}$ mouse lymphoma gene mutation assay

Report No.: CTL/P/4991

Guidelines: OECD #476 (1997) [3]; US EPA OPPTS 870.5300 (1998) [7]; EC Directive 2000/32/EEC B.17 (2000) [8].

Test material: Glyphosate (purity 95.6%)

Sponsor: Zeneca Argochemicals

Test type: Hypoxanthine-guanine phosphoribosyl transferase assay with mouse lymphoma cells (L5178Y $TK^{+/-}$) with and without metabolic activation.

Study protocol: a preliminary dose range study performed. Subsequently, two main experiments with dublicate cultures with 4 doses and exposure 4 h were realized (with and without S9 mix).

Negative controls: Included, in normal range.

Positive controls: ethyl methanesulfonate (EMS) for experiments without metabolic activation and nitrosodimethylamine (NDMA) for experiments with S9. Positive results were obtained as expected.

Historical controls: not shown.

Statistical analyses: provided

Results: the authors conclude that the compound did not induce a mutagenic effect. Strong acute toxicity was found in the first experiment with 1,500 µg/ml (highest dose); therefore, only three doses could be evaluated in this experiment. In the following (second experiment) lower concentrations were used, and all 4 concentrations could be evaluated. Notably, the mutation frequency (MF) increased with the dose in the first experiment (with S9; from 0.9 in negative control to 1.4 at concentration of 1,000 µg/ml) and also in the second one without S9 (1.3 in negative control and 2.3 at highest dose).

Remarks: the protocol is only partly in agreement with the OECD guideline [3]. In the first experiment only three doses could be evaluated due to acute toxicity at the highest dose. Historical controls are not presented. Results of statistical analyses (p values) are not provided. The lack of results of statistical analyses and the increase of the mutation frequencies (from 1.3 to 2.3) at the highest dose (without S9) may indicate that the results of this study are wrongly interpreted.

RAR: Evaluation and Comments

Study identification: *Clay, 1996; reference IIA, 5.4.3/01; TOX2000-1994 (page 338)*

Deviations: *The stability, homogeneity and achieved concentration of the test or control substances in the vehicle used were not determined by analysis and the certified purity and stability of the control substances are not available. These deviations from the current regulatory guideline are considered not to compromise the scientific validity of the study.*

Evaluation: *The study is considered acceptable and the conclusion is agreed with. The selection of the highest concentration of 1000 µg/mL because of pH reduction at dose levels above is reasonable. It is widely accepted that pH changes (as well as increases in osmolality) may alter the mutant frequency. However, it was noted that the resulting top dose level was much lower than in the study by Jensen (1991, TOX9552372) who did not report a decline in pH at concentrations above 1 mg/mL. This obvious difference might suggest some variability in the acidic properties of the test materials although it seems not entirely clear from the study report if Jensen (1991, TOX9552372) had in fact measured the pH after treatment.*

Comments (SK and AN): the study has major deviations from the OECD guidelines and the results are not reliable.

4.3. Summary

Gene mutation test with mammalian cells (HPRT- assay)

None of the studies is in full agreement with the current OECD guideline.

Results of statistical analyses not shown:

Study 25 an analysis is mentioned but no results (p values) are shown; requested by the OECD guideline)

Study 27 a concentration response was statistically not evaluated

Study 32 an analysis is mentioned but no results (p values) are shown; requested by the OECD guideline). This makes the study inconclusive as the mutation frequencies (MF) were increased with some doses

Historical control data not shown (required to confirm a clear negative results; requested in the OECD guideline):

Study 25

Study 27 in the main experiment an effect was seen with S9 with individual doses. Therefore, historical controls are mandatory

Study 32

4.4. References

[1] E.H. Chu, Mutation systems in cultured mammalian cells, Ann N Y Acad Sci, 407 (1983) 221-230.

[2] A.W. Hsie, D.A. Casciano, D.B. Couch, D.F. Krahn, J.P. O'Neill, B.L. Whitfield, The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. A report of the gene-tox program, Mutat Res, 86 (1981) 193-214.

[3] OECD, In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes No. 476, 1997.

[4] OECD, ENVIRONMENT DIRECTORATE. JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY. Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014-2015, OECD, Paris, 2017.

[5] J. Cole, M. Fox, R.C. Garner, D.B. McGregor, J. Thaker, Gene mutation assays in cultured mammlian cells, in: D.J. Kirkland (Ed.) Basic Mutagenicity Tests: UKEMS Recommended Procedures, Press Syndicate of University of Cambridge, New York, 1990, pp. 87-114.

[6] R.D. Snee, J.D. Irr, Design of a statistical method for the analysis of mutagenesis at the hypoxanthine-guanine phosphoribosyl transferase locus of cultured Chinese hamster ovary cells, Mutat Res, 85 (1981) 77-93.

[7] EPA, Health Effects Test Guidelines OPPTS 870.5300 In Vitro Mammalian Cell Gene Mutation Test, in: P.a.T.S. Prevention (Ed.), US EPA, Washongton, DC, 1998.

[8] EC, B.13/14. MUTAGENICITY REVERSE MUTATION TEST BACTERIA, Official J EC, 8.6.2000 (2000) L136/131-L 136/189.

5. DNA repair test with primary rat hepatocytes

5.1. Description

The procedure ("Bromodeoxyuridine Incorporation Assay") is based on a method which was developed in the early 1960s'and measures DNA synthesis as a consequence of chemically induced DNA repair. Cells are incubated in presence of the heavy thymidine analogue, 5-bromodeoxyuridine (BrdUrd), radioactive deoxycytidine (^{3}H-dCyd) and the test chemical. DNA strands synthesized during the incubation time by normal replicative DNA synthesis incorporate large amounts of heavy BrdUrd and, thus, have a higher density than unreplicated DNA strands. If a test chemical causes damage in unreplicated DNA which is subject to excision repair, short stretches of newly synthesized DNA are incorporated containing both BrdUrd and ^{3}H-dCyd. The incorporated BrdUrd is not sufficient however, to increase significantly the density of the DNA strands. Repaired and replicated DNA strands can subsequently be separated on alkaline cesium salt gradients and quantitatively determined by measuring the incorporated radioactivity.

The method was standardized in an international guideline (EPA OPPTS 870.5550 [1]). A similar procedure, the unscheduled DNA synthesis (UDS) test is described in OECD guideline #482, 1986 (DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells in vitro) which was removed on April 2nd, 2014 [2].

The evaluation of study No. 28 is based on that outdated guideline [2] and on recommendations from expert's panel [3-5].

5.2. Evaluation of individual studies

Study number 28

Performing laboratory: ANAWA, München AG, International Bioscience Park, Germany

Date: March 18, 1994

Title: DNA repair test wit primary rat hepatocytes.

Report: TEST 931564

Guidelines: OECD guideline #482, which was removed on April 2nd, 2014 [2].

Test material: Glyphosate technical grade (purity > 98.0%)

Sponsor: M/s Feinchemie Schwebda GmbH, Germany

Study type: DNA repair in primary mammalian cells (hepatocytes)

Test design: two independent experiments were performed with freshly isolated hepatocytes from male Sprague-Dawley rats.

Cultures per experimental point: one culture per point

Concentrations tested: 6 (0.2 – 11.7 mM)

Negative control: included (solvent)

Positive control: two compounds were used, dimethylnitrosamine for experiments with S9 and 2-acetamidofluorene for experiments without metabolic activation. Positive effects were obtained as expected.

Results: no increase DNA repair synthesis at any concentration tested.

Remarks: A clear guideline for the method has never been published. Therefore, we evaluated the quality of the study on the basis of the description for the unscheduled DNA synthesis (UDS) assay which is related and for which several guidelines (recommendations) were developed [4, 5].

Only one culture was treated per experimental point. According to UDS guideline by Madle et al. [3] at least 3 duplicate cultures should be analyzed per concentration.

Only an 18 h exposure period was used. According to recommendations for UDS experiments this is OK for the first experiment but a shorter period (2 – 4 h) should be used when negative results were obtained in a follow-up study.

A major shortcoming is the lack of statistical analyses.

RAR: Evaluation and Comments

Study identification*: Rossberger (1994, TOX9400697) (page 341)*

Evaluation: *"is still considered acceptable." and "valid in vitro test" (page 341)*

Comments (SK and AN): this test system is outdated and the method is not included anymore in the panel of tests which are recommended by the OECD. It is also not included in the UK EMS guidelines. The assay has methodological shortcomings and the results are not reliable.

5.3. Summary

DNA repair test with rat hepatocytes

The study # 28 has major shortcomings.

The method (DNA repair test with primary rat hepatocytes) is outdated.

5.4. References

[1] EPA, Health Effects Test Guidelines OPPTS 870.5550 Unscheduled DNA Synthesis in Mammalian Cells in Culture, in: P.a.T.S. Prevention (Ed.), EPA, Washington, DC, 1998.

[2] OECD, Genetic Toxicology: DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells in vitro No. 482, OECD1986.

[3] S. Madle, S.W. Dean, U. Andrae, G. Brambilla, B. Burlinson, D.J. Doolittle, C. Furihata, T. Hertner, C.A. McQueen, H. Mori, Recommendations for the performance of UDS tests in vitro and in vivo, Mutat Res, 312 (1994) 263-285.

[4] F. Nesslany, Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo, Methods Mol Biol, 1044 (2013) 373-387.

[5] B.E. Butterworth, J. Ashby, E. Bermudez, D. Casciano, J. Mirsalis, G. Probst, G. Williams, A protocol and guide for the in vitro rat hepatocyte DNA-repair assay, Mutat Res, 189 (1987) 113-121.

6. Chromosomal aberration test in mammalian cells *in vitro*

6.1. Description

This classical procedure was developed to detect structural and numerical chromosomal aberrations (chromosome and chromatid type) and is performed with mammalian cells, either with stable cell lines or with primary cells.

The sensitivity of the test system depends strongly on the cell type. When the experiments are performed with indicator cells which do not possess drug metabolizing enzymes, an exogenous activation mix has to be added to the incubation mixtures.

The selection of the concentrations, as well as positive and negative controls are described in detail in OECD guideline # 473 (1997, 2014, 2016, update of 2014-2015) [1-4]. The number of concentrations that has to be tested and the number of metaphases which are evaluated per concentration should be clearly specified. Chromosome and chromatid type aberrations should be recorded separately and classified by subtypes (breaks, exchanges, gaps). Data concerning CA numbers should be presented with and without gaps.

Apart from OECD [1-4] and the US EPA OPPTS 870.5375 [5] guidelines, the test procedure is also described in the UK EMS recommendations [6] and in the "Handbook of Mutagenicity Test Procedures" (two chapters [7] and [8]). A very important issue is the number of metaphases which should be evaluated. Relevant information can be found in Table 1.

In vitro CA analyses were largely replaced by the MN experiments which detect structural as well as numerical CA. However, the biological relevance of CA in regard to the detection of genotoxic carcinogens is relatively high, therefore, the results of earlier investigations are important.

Table 1. Number of metaphases which should be evaluated according to individual guidelines

Authority	Year	Number of cells which should be evaluated	Reference
OECD	1997	200	[4]
OECD	2014	300	[1]
OECD	Update 2014/2015	300	[3]
OECD	2016	300	[2]
FDA Redbook 2000: IV.C.1.b.	2003	200	https://www.fda.gov
US EPA	1998	200	[5]

6.2. Evaluation of individual studies

Study number 24

Performing laboratory: Central Toxicology Laboratory, Cheshire, UK

Date: October 29, 1998

Title: Glyphosate acid: *in vitro* cytogenetic assay in human lymphocytes

Report No.: CTL/P/6050

Guidelines: OECD #473 (1997), US EPA OPPTS 870.5375 (1998) [5] and EC Directive 2000/32/EC B10 (2000) guidelines [9].

Test material: Glyphosate acid (purity 95.6%)

Sponsor: Zeneca Agrochemicals, Surrey, UK

Study type: Cytogenetic study with human lymphocytes *in vitro* with and without metabolic activation

Study design: Cultures from 2 donors were prepared and 3 concentrations (100, 750 and 1,250 µg/ml) were tested. Sampling time: 68 and 96 h. In experiments with metabolic activation the cells were treated for 3 h. In a second independent experiment, lymphocytes were exposed to the test substance in presence and absence of S9-mix. Duplicate cultures were tested. The mitotic indexes were calculated based on the count of 1,000 lymphocytes.

Negative controls: included, background aberrations are OK

Positive controls: included [cyclophosphamide for experiments with S9 and mitomycin C for experiments without S9].

Selection of the concentrations: concentrations > 1250 µg/ml induced acute cytotoxicity.

Concentrations tested: 3 (100, 750 and 1250 µg/ml)

Scoring of slides: Cells were stained with 10% Giemsa. One hundred cells ("where possible" as stated by the authors), were analyzed. The exact total number of analyzed cell is unclear.

Statistical evaluation: Fisher's exact probability test was applied.

Historical controls: not provided.

Results: no increase of the number of chromosomal aberrations was observed at any concentration. With both chemicals used as positive control, positive results were obtained as expected (the results are OK).

Remarks: in contrast to international guidelines, 100 cells instead of 200 - 300 were analyzed. Different types of chromosomal aberrations are not specified as suggested in the OECD guidelines. Chromosomal aberrations are presented only excluding gaps which is in contrast to the guidelines.
In contrast to the OECD Guideline 473 (2014) [1] and US EPA OPPTS 870.5375 (1998) [5], the difference between the selected doses were not 2-3-fold but 1.66- and 7.5-fold.

RAR: Evaluation and Comments

Study identification*: 3rd new clastogenicity study in vitro, IIA, 5.4.2/03 (Fox, 1998), page 345*

***Deviations**: The stability and achieved concentration of the test substance and control substances in the vehicles used were not determined by analysis. This deviation from the current regulatory guideline is considered not to compromise the scientific validity of the study.*

***Evaluation: "**The study is considered acceptable and the conclusion is agreed with. The long duration of this study is surprising but was apparently due to the fact that the in-life phase was run early in 1996 but slides were evaluated not before 1998."*

Comments (SK and AN): it is stated by the authors of the study that it was performed according to several international guidelines. But in all international guidelines 200 – 300 cells are mentioned as mandatory (see Table 2) and only 100 cells were evaluated. The study deviates substantially from the guidelines and the results are not reliable.

Study number 26

Performing laboratory: The Institute of Environmental Toxicology, Tokyo, Japan

Date: May 29, 1995

Title: HR-001: In vitro cytogenetic test

Project No.: IET 94-0143

Guidelines: the U.S. EPA FIFRA Guideline, Subdivision F [10].

Deviations: None

Test material: HR-001 (purity 95.68%)

Sponsor: Sankyo Company, Ltd., Tokyo. Japan

Study type: cytogenetic study (metaphase analysis) with Chinese hamster lung (CHL) cells.

Study design: cells were treated with HR-001 at 4 dose levels, with and without metabolic activation (S9 from male Sprague-Dawley rats). Duplicate cultures were used for each dose.

Exposure time: 24 and 48 h without S9-mix. In experiments with metabolic activation cells were treated for 6 h.

Selection of the concentrations: provided, concentrations > 2,000 µg/ml were cytotoxic

Concentrations tested: 4 in 24 h exposure experiments (125, 250, 500 and 1,000 µg/ml); 4 in 48 h exposure experiments (without S9; 62.5, 125, 250 and 500 µg/ml); 4 in 48 h exposure experiments with S9 (250, 500. 1,000 and 2,000 µg/ml)

Negative controls: included, CA are in normal range

Positive controls: included; 40 µg/ml benzo(a)pyrene for experiments with S9 and 0.1 µg/ml mitomycin C with experiments without S9.

Staining: 2.0% Giemsa

Scoring of slides: 200 metaphases were analyzed for each concentration (100 metaphase cells/culture) and also from negative and positive controls.

Statistical evaluation: chi-square test.

Historical control: provided, the values are based on 39 independently conducted experiments.

Results: The data are presented adequately. Cell growth values are presented graphically. Types of all chromosomal aberrations are presented.
No increase of chromosomal aberrations was observed at any concentration. With both chemicals which were used as positive controls, positive results were obtained as expected.

Remarks: Only 200 cells as suggested in old guidelines (US EPA OPPTS 870.5235 [5] and OECD (1997) [4] were analyzed in the study instead of 300 recommended in the OECD guideline # 473 from 2014 and 2016 and in update of OECD guidelines published in 2014-2015 [3, 10].

RAR: Evaluation and Comments

Study identification*: 1st new clastogenicity study in vitro (Kyomu, 1995)*

Evaluation: "*The study is considered acceptable. No evidence of an increase in structural or numerical chromosome aberrations was obtained. With regard to the study description in the dossier, it must be clarified that metaphases were analysed up to a concentration of 500 µg/mL in the first series of experiments without metabolic activation (called above and in the study report "direct method") and 24- or 48-hour treatment periods. In the experiments with and without activation and an exposure period of 6 hours (thereafter, cells were fixed for 18 hours), metaphases could be analysed up to a concentration of 1000 µg/mL. At concentrations above, evaluation was avoided by severe cytotoxicity."*

Comments (SK and AN): According to the current OECD guidelines, 300 cells must be evaluated per dose. The study deviates moderately from the OECD guidelines and the results are partly reliable.

Study number 29

Performing laboratory: NOTOX B. V., The Netherlands

Date: July 30, 1996

Title: Evaluation of the ability of glyphosate to induce chromosome aberrations in cultured peripheral human lymphocytes (with independent repeat)

Report No.: TOX96-51525

Guidelines: OECD #473 (1983) and EEC Directive 92/69/EEC [9]

Test material: Glyphosate (purity 96%)

Study type: study of chromosomal aberrations induced by test-substance in human lymphocytes with and without metabolic activation (S9 from male Wistar rats).

Study design: cultures from 3 donors were prepared. Sampling time: 24 and 48 h. In experiments with metabolic activation the cells were treated 3 h. The second independent experiment was carried out in the presence of and absence of S9-mix with sampling time 69 and 92 h.

Negative controls: included (DMSO, vehicle of the test substance)

Positive controls: included [cyclophosphamide for experiments with S9 and mitomycin C for experiments without S9].

Selection of concentrations: included

Concentrations tested: 3 concentrations (33, 237 and 333 µg/ml) in experiments without S9, 3 concentrations (333, 422 and 562 µg/ml) in experiments with S9.

Staining: 5.0% Giemsa

Scoring of slides: the number of cells scored per each sampling point was in total 200.

Statistical evaluation: the chi-square test was applied.

Historical controls: provided

Results: the data are presented adequately. All types of chromosomal aberrations were evaluated. No increase of the chromosomal aberrations was observed at any concentration in lymphocytes obtained from both donors. With both chemicals which were used as positive controls, results were obtained as expected. The mitotic indices were calculated. Only 200 cells were evaluated per experimental point.

Remarks: In contrast to the actual OECD Guidelines [1, 2], number of analyzed cells is 200 (instead of 300). In contrast to the OECD Guidelines [1, 2], the differences between the selected doses were not 2-3-fold but 1.3- and 7-fold.

RAR: Evaluation and Comments

***Study identification**: Van de Waart (1995, TOX9651525) (page 345)*

***Evaluation**: "In contrast, a study by Van de Waart (1995, TOX9651525) is of sufficient quality and may still be used to address this endpoint. It is considered now supplementary since the dose levels were rather low if compared to the 3 further studies that were provided for this new evaluation of glyphosate."*

Comments (SK and AN): it is stated in the actual OECD guidelines (2014, 2016, update 2014-2015) that evaluation of 300 cells per dose is mandatory. The study deviates moderately from the OECD guidelines and the results are partly reliable.

Study number 30

Performing laboratory: SafePharm Laboratories Limited, Derby, UK

Date: March 13, 1996

Title: Technical glyphosate: chromosome aberration test in CHL cells in vitro

SPL Project No.: 434/015

Guidelines: not specified

Test material: Glyphosate technical (95.3%)

Sponsor: Mastra Industries Sdn. Bhd, Port Klang, Malaysia

Co-Sponsor: Mazuren Kako Co., Ltd, Tokyo, Japan

Study type: Cytogenetic study (metaphase analysis) with Chinese hamster lung (CHL) cells

Study design: CHL cells were treated with Glyphosate technical at 3 dose levels, in duplicate with and without metabolic activation (S9 was obtained from male Sprague-Dawley rats).

Exposure time: 24 and 48 h without S9-mix and 6 h with and without S9.

Negative controls: included

Positive controls: included (cyclophosphamide for experiments with S9 and mitomycin C for experiments without S9).

Selection of the concentrations: provided

Concentrations tested: 3 (312.5, 625 and 1,250 µg/ml both for experiments with and without S9).

Staining: 2.0% Giemsa

Scoring of slides: 200 metaphases were analyzed from each concentration and in the negative controls. 200 cells were analyzed in positive control without S9 and 150 with S9 mix.

Statistical evaluation: Fisher's exact test.

Historical controls: not provided; it is mentioned that many earlier experiments (not specified) established a range of aberration frequencies (0.0 to 3.0%) in negative control.

Results: The data are presented adequately. Cell growth indexes are presented. Types of all chromosomal aberrations are presented. No increase of chromosomal aberrations was observed at any concentration. With both chemicals used as positive control, adequate results were obtained as expected.

Remarks: The number of metaphases which was evaluated is not sufficient. Also the relative increase of cell counts (RICC) or relative population doubling (RPD) should be calculated [1, 2], but no such data are provided.

RAR – Evaluations and Comments:

Study identification*: 2nd new clastogenicity study in vitro (Wright, 1996) (page 351 - 353)*

***Evaluation**: The study is considered acceptable. There were no indications for more frequent occurrence of chromosome aberrations. Thus, the conclusion is agreed with.*

Comments (SK and AN): the study has deviations from OECD guidelines. The results are partly reliable.

Study number 96

Performing laboratory: Pharma Development Central Laboratory, Hoechst, Germany

Date: December 16, 1992

Title: Dodigen 4022: chromosome aberrations in vitro in V79 Chinese hamster cells

Project No.: 920337

Guidelines: not specified

Test material: Dodigen 4022 (purity 82%); used for herbicide formulations as a surfactant, does not contain glyphosate

Study type: Cytogenetic study (metaphase analysis) with Chinese hamster lung (V79) cells

Study design: cells were treated with Dodigen 4022 at 3 dose levels 18 h, with and without metabolic activation (S9 obtained from male Sprague-Dawley rats). In experiments with metabolic activation cells were treated 4 h. The compound was also tested with an exposure time of 7 h and 28 h with and without S9 mix (at the highest concentration, 6,000 µg/ml).

Concentrations tested: 3 (600, 3,000 and 6,000 µg/ml).

Negative control: provided, background chromosomal aberration levels are in the normal range

Positive controls: provided, 5.0 µg/ml cyclophosphamide for experiments with S9 and 1,750 µg/ml ethylmethanesulfonate with experiments without S9

Selection of concentrations: the compound was tested for cytotoxicity in the range between 3.9 – 5,000 µg/ml in preliminary toxicity test (13 concentrations were tested). Based on the results, 3 concentrations were chosen (600, 3,000 and 6,000 µg/ml).

Staining: 2.0% orcein

Scoring of slides: 100 metaphases were analyzed for each concentration (100 metaphase cells/culture), negative and positive controls; mitotic indexes were calculated.

Statistical evaluation: Fisher's exact test.

Historical controls: not presented.

Results: The data are presented adequately. Mitotic indexes are described. Types of all chromosomal aberrations are presented. No increase of chromosomal aberrations was observed at any concentration. With both chemicals used as positive control, positive results were obtained as expected.

Remarks: Instead of mitotic indexes presented by the authors, other parameters, i.e. Relative Increase in Cell Counts (RICC) or Relative Population Doubling (RPD) should be calculated. Instead of 200 - 300 cells per dose level which is recommended by the OECD and other guidelines, only 100 cells were analyzed.

Note: Dodigen is a surfactant which does not contain glyphosate.

RAR – Evaluations and Comments:

Study identification: *Stammberger, I. (1992, TOX1999-325) (page 395)*

Evaluation: "*The study is considered acceptable."*

Comments (SK and AN): the number of evaluated cells is not in agreement with the OECD guidelines (2014, 2016 and update 2014-2015) (i.e. 300 cells should be scored) and also with US EPA guideline (1998) which mentions 200 cells per experimental point. The study deviates substantially from the guidelines and the results are not reliable.

6.3. Summary

Chromosomal aberration (CA) test *in vitro*

All studies have shortcomings.

Inadequate presentations of the results:

Study 96: MI (mitotic indexes) are presented instead of RICC (Relative Increase in Cell Count) or RPD (Relative Population Doubling) (not a severe mistake)

Study 30: RPD or RICC are not shown

Study 24: CA are presented only with excluding gaps (not in agreement with OECD guideline).

Inadequate number of evaluated cells (less than 300 as required by actual OECD guidelines):

Study 24

Study 29

Study 96

Inadequate dose selection:

Study 24: differences between selected doses are not adequate

Study 29: differences between selected doses are not adequate

Inadequate description of the test compound:

In study 26 the product which was tested (HR-001) is not specified.

6.4. References

[1] OECD, IN VITRO MAMMALIAN CHROMOSOMAL ABERRATION TEST, OECD, Paris, 2014.

[2] OECD, In Vitro Mammalian Chromosomal Aberration Test No. 473, OECD2016.

[3] OECD, ENVIRONMENT DIRECTORATE. JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY. Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014-2015, OECD, Paris, 2017.

[4] OECD, In Vitro Mammalian Chromosome Aberration Test, OECD, Paris, 1997.

[5] EPA, Health Effects Test Guidelines OPPTS 870.5375 In Vitro Mammalian Chromosome Aberration Test, in: P.a.T.S. Prevention (Ed.), EPA, Washongton, DC, 1998.

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[9] EC, B.10. MUTAGENICITY IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST, Official J EC, 8.6 (2000) L 136/135.

[10] B. Jaeger, Pesticide assessment giudelines: Subdivision F. Hazard evaluatuin - humans and domenstic animals, in: H.E. Division (Ed.), EPA, Washington, DC, 1984.

7. Chromosomal aberration test in rodents *in vivo*

7.1. Description: Mammalian bone marrow chromosomal aberration (CA) test

This test is a classical approach which detects structural and numerical CA in mammals. Chromosomal and chromatid types of CA are scored, the experimental protocol is similar to that of bone marrow micronucleus (MN) assay.

The selection of animal species and of the doses are described in detail in OECD guideline # 475 (1997, and in the update from 2014-2015) [1-3]. Positive and negative controls have to be included, three doses should be tested. The collection time of cells is important and depends on the treatment schedule. The animals are treated at the end of the experiment with a metaphase arresting chemical before the cells are sampled.

CA experiments are at present only rarely performed; they were largely replaced by MN experiments with bone marrow cells of rodents which are less time consuming and provide essentially the same information. The main shortcoming of the procedure is the same as that encounted in MN experiments with bone marrow cells, i.e. it is often not clear if the test compound reaches the target cells. Furthermore, it is not known if and to which extent target cells metabolize the test chemicals. According to our knowledge, the predictive value of the procedure for the detection of genotoxic carcinogens has not been validated in the last two decades, presumably it is similar to that of the MN assays *in vivo* (relatively low).

As mentioned above, the procedure is described in detail in OECD guidelines [1] and in US EPA (OPPTS 870.5385 Guideline) [4] and also in a number of other publications (see for example the UK EMS recommendations [5], Adler [6] and Preston et al. [7]).

Table 2. Number of cells which should be evaluated according to different guidelines.

Authority	Year	Number of cells which should be evaluated	Reference
OECD	1997	100	[1]
OECD	2014	200	[3]
OECD	Update 2014/2015	200	[3]
OECD	2016	200	[2]
US EPA	1998	100	[4]

It is constantly mentioned in the guidelines that three doses should be tested. However, if the compound does not cause acute toxicity, it is acceptable that only one dose is investigated. If only one dose is tested, it is mentioned in the OECD guidelines that this should be discussed/justified. In the newest OECD guideline (2016) it is mentioned that testing of one dose is only justified when it is shown that the compound or its metabolites reach target cells in the bone marrow.

7.2. Evaluation of individual studies

Study number 72

Performing laboratory: Monsanto Company Environmental Health Laboratory, US

Date: October 20, 1983

Title: In vivo bone marrow cytogenetic study of Glyphosate in Sprague-Dawley rats

Study No. 830083; DMEH Project No. ML-83-236

Guidelines: not specified

Test material: Glyphosate (purity 98.7%)

Study type: chromosomal aberration test in bone marrow of rats

Study design: single i.p. administrations of test compound in HBSS. Rats were sacrificed 6, 12 and 24 h after treatment.

Doses tested: 1 (1,000 mg/kg)

Animals: n=36 in CA study; 10 - 12 weeks old, 6 ♂ and 6 ♀ Sprague-Dawley per group.

Negative control: included (Hank's balanced salt solution, HBSS

Positive control: included, cyclophosphamide (25 mg/kg, i.p.).

Acute toxicity (dose selection): not performed.

Staining: 2.0% Giemsa

Scoring of slides: 50 mitotic cells per animal (300 cells per treatment) were scored for chromosomal aberrations

Statistical evaluation: Student's "t"-test.

Historical control: not presented.

Results: the data concerning CA in the negative and positive controls are OK. In the positive group, however, cyclophosphamide was toxic in female animals, and only 21 metaphases were available for scoring. The authors stated that the results of the study are clearly negative in the glyphosate group.

Remarks: only 50 metaphases were analyzed per animal instead of 200 as suggested by several international guidelines (OECD; see Table 2). Only one dose was tested (1000 mg/kg) which is not justified. The route of administration is not justified. A dose selection experiment was not performed; mitotic indexes were not calculated.

RAR: Evaluation and Comments

Study identification: *Monsanto, TOX9552375 (page 358)*

Evaluation: *"valid in vivo study"*

Comments (SK and AN): the number of cells which was evaluated per animal is substantially lower than the recommended number. No justification/discussion is included why only one dose was tested. It was not shown that the compound (or its metabolite) reaches the target cells. the study is not acceptable. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 101

Performing laboratory: Rallis Agrochemical Research Station. India

Date: January 22, 1994

Title: Genetic toxicology – in vivo mammalian cytogenetic test – chromosomal aberrations

Report No. TOXI: 890-MUT-CH.AB.

Guidelines: not specified

Test material: Glyphosate technical (purity 96.8%)

Sponsor: M/s Feinchemie Schwebda GmbH, Germany

Study type: chromosomal aberration test in bone marrow cells of mice

Study design: two oral administrations of test compound in oil, 24 h after second treatment mice were injected with colchicine, and subsequently sacrificed 1.5 h later.

Doses tested: 3 (50, 500 and 5,000 mg/kg for 2 consecutive days).

Animals: n=50 in CA study, 10 - 12 weeks old, 5 ♂ and 5 ♀ Swiss albino mice per group.

Negative control: included (ground nut oil)

Positive control: included (cyclophosphamide, 50 mg/kg).

Acute toxicity test (dose selection): not included

Staining: 2.0% Giemsa

Scoring of slides: 50 metaphases per animal were analyzed

Statistical evaluation: CA frequencies changes were evaluated with a paired "t"-test and with Z-test.

Historical control: not presented.

Results: the mitotic indexes (MI) were calculated. The MI was significantly decreased at the highest dose in males and females. A significant increase of the incidence of gaps in females of highest dose group was observed. Negative and positive controls are OK. The authors stated that the results of the study are clearly negative.

Remarks: Only 50 metaphases were analyzed per animal instead of 200 suggested by the OECD guidelines.

RAR: Evaluation and Comments

Study identification: *ADAMA 1994; TOX9400323 (page 358)*

Evaluation: *"valid in vivo cytogenetic test"*

Comments (SK and AN): the decision of the authority is not justified as the number of evaluated cells is not adequate (only 50 cells were evaluated instead of 200). The study deviates substantially from the OECD guidelines and the results are not reliable.

7.3. Chromosomal aberration (CA) test *in vivo*

Both studies on chromosomal aberrations in bone marrow cells have severe shortcomings.

Inadequate number of scored cells:

Only 50 metaphases were evaluated per animal instead of 200 (requested by OECD) in both studies

Study 72

Study 101

Inadequate number of tested doses:

Study 72 (only one dose was tested)

Inadequate route of administration (i.p.):

Study 72

7.4. References

[1] OECD, Mammalian Bone Marrow Chromosome Aberration Test, OECD, Paris, 1997.

[2] OECD, Mammalian Bone Marrow Chromosomal Aberration Test No. 475, OECD2016.

[3] OECD, ENVIRONMENT DIRECTORATE. JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY. Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014-2015, OECD, Paris, 2017.

[4] EPA, Health Effects Test Guidelines OPPTS 870.5385 Mammalian Bone Marrow Chromosome Aberration Test, in: P.a.T.S. Prevention (Ed.), US EPA1998.

[5] M. Richold, J. Ashby, J. Bootman, A. Chandley, D.G. Gatehouse, L. Henderson, In Vivo Cytogenetics Assays, in: D.J. Kirkland (Ed.) Basic Mutagenicity Tests, UKEMS Recommended Procedures, Cambridge University Press, Cambridge, 1990.

[6] I.D. Adler, Cytogenetic tests in mammals, in: S. Venitt, J.M. Parry (Eds.) Mutagenicitzy Testing: a Practical Approach, IRL Press, Oxford, 1984, pp. 275-306.

[7] R.J. Preston, W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff, J.S. Wassom, Mammalian in vivo and in vitro cytogenetic assays: a report of the U.S. EPA's gene-tox program, Mutat Res, 87 (1981) 143-188.

8. Micronucleus assays with polychromatic erythrocytes of rodents

8.1. Description

Micronuclei (MN) are extranuclear DNA containing bodies which can be detected by microscopy in a variety of different cell types [1, 2]. These structures were initially designated as “Howell-Jolly bodies”. They are formed as a consequence of structural and numerical chromosomal aberrations; i.e. they reflect chromosomal breaks and aneuploidy [1]. MN experiments are much easier to perform as chromosomal analyses with metaphase cells, therefore, they replaced classical chromosomal aberration (CA). MN can be scored in a variety of tissues, for example in polychromatic erythrocytes (PCE) from bone marrow, lymphocytes and blood erythrocytes, in liver cells and also in exfoliated cells from different organs in humans (for review see [2-5]).

The MN assay with PCE is at present the most widely used *in vivo* test for routine screening of chemicals [5]. One of the problems is that some of the test chemicals do not necessarily reach the target cells and that the bone marrow is not an important metabolic organ. The sensitivity of the MN assay with PCE for the detection of genotoxic carcinogens is therefore limited, only between 50 and 70% of the carcinogenic chemicals are identified with this approach [6]. A recent analysis indicates that other methods which were developed may provide more reliable results (for example, the single cell gel electrophoresis assay [7].

To ensure that the data obtained in MN experiments with PCE are correct, the sampling time after administration of the compound is of high importance as MN are only formed after cell divisions [1]. Positive results are obtained when MN frequencies are higher than that seen in negative controls. However, also historical negative controls can be included in the evaluation. In

our opinion, they should preferentially come from the same laboratory. Results obtained in other laboratories with other rodent strains under other feeding conditions are of very limited value.

It is also relevant that the route of administration reflects human exposure. In the case of glyphosate most studies were performed with oral and i.p. administration, notably the latter route is largely irrelevant for humans. According to our knowledge, no inhalation studies which reflect exposure of workers in agriculture and production were submitted by the industries.

Guidelines for MN experiments were published by the OECD and other authorities. The number of cells which should be evaluated per animal is of crucial importance (see Table 3)

Table 3. Number of cells which should be evaluated per animal according to individual guidelines

Authority	Year	Number of cells which should be evaluated	Reference
UK EMS	1990	2000	[8]
OECD	1997	2000	[9]
OECD	2014-2015 (update)	**4000**	[10]
OECD	2016	**4000**	[11]
US EPA OPPTS 870.5395	1998	2000	[12]
US FDA (RedBook)	2000	2000	[13]

8.2. Evaluation of individual studies

Study number 49

Performing laboratory: BIOAGRI Laboratorios Ltds, Piracicaba, Brazil

Date: September 29, 2008

Title: Evaluation of the mutagenic potential of Glyphosate technical by micronucleus assay in mice

Report No.: 3996.402.395.07

Guidelines: OECD #474 (1997) [9]; Commission Directive 2000/32/EC B.12 (2000) [14], USA EPA [12] and JMAFF guidelines.

Deviations: none

Test material: Glyphosate technical (purity 98%)

Sponsor: JINGMA Chemicals Co. Ltd, China

Study type: MN assay with bone marrow erythrocytes of mice.

Study design: Two i.p. administrations with 24 h intervals. Mice were sacrificed 24 h after the last administration.

Animals: 50 mice (5 ♂ and 5 ♀ per group) were used for MN study, 7-12 weeks old Swiss albino mice

Doses rested: 3 (15.62, 31.25 and 62.5 mg/kg; $1/8^{th}$, $1/4^{th}$ and ½ of MTD).

Negative control: provided (corn oil, vehicle), MN rates are OK

Positive control: provided (cyclophosphamide, 25 mg/kg, i.p.), MN rates are OK.

Acute toxicity test: results provided, the dose of 125 mg/kg was determined as a MTD.

Staining: Giemsa

Scoring of slides: PCE/NCE ratio was determined; 2,000 PCE were scored for MN. MN also were scored in NCE which is not mandatory.

Statistical evaluation: U-test Mann-Whitney.

Historical control: presented.

Results: all data obtained from individual animals are presented.

Results are clearly negative.

Remarks: the route of administration is not justified as requested in the OECD guideline [9]. The absence of MN in 20 exposed animals which were treated with different doses of the test compound (5 males and 15 females; no MN in 40,000 PCE) is very unusual. In negative controls (males and females) no PCE with MN were found but in historical controls the frequencies were 0.87‰ and 0.76‰, respectively.

RAR: Evaluation and Comments

Study identification: *3rd new micronucleus test in mice (Costa, 2008) (pages 364 – 367)*

Evaluation: ***"****When this section of the RAR was reviewed, this table (Table B.6.4-16: Summary of results) was found to be wrong, due to a technical error. The (expected) in increase in the positiv control group had been erroneously allocated to the group receiving 62.5 mg glyphosate/kg bw in the GTF dossier and the RMS reviewers had not noticed this error when the original RAR was prepared. Furthermore, not all figures were precisely those that are given in the original report (2008, ASB2012-11481). Therefore, it was replaced now by a new one giving the appropriate allocation of test results to the individual test groups. The sampling time was always 24 hours after the second i.p. dose."*

"The study is considered not acceptable since the dose levels were much too low for any meaningful conclusion with regard to micronucleus formation. Some of the information given in the dossier was apparently wrong but, unfortunately, was corrected in the revised RAR only. In the original RAR, the study was considered not acceptable since the dose levels were much too low for any meaningful conclusion with regard to micronucleus formation even though it must be taken into consideration that the exposure was via the intraperitoneal route. (Thus, the study can be hardly compared to most other micronucleus tests with glyphosate in which the test substance was administered via the oral route.) In the original report, dose selection for the"definitive test" was justified with the outcome of a preliminary test. In this range finding experiment, 3 males and 3 females per dose level received i.p. glyphosate doses of 62.5, 125, 250, 500, or 1000 mg/kg bw. The top dose level resulted in 100 % mortality and at the next lower dose level of 500 mg/kg bw, one male and two female mice died. Based on a clear decrease in the PCE/NCE ratios in both

sexes, the intermediate dose of 250 mg/kg bw was found to be cytotoxic. It was recommended that 125 mg/kg bw was the most appropriate high dose to be employed in the definitive test but, without further justification, 62.5 mg/kg bw was actually the highest dose used. That was much

lower than in other studies in which the i.p. route had been also chosen, 1999, ASB2012-11482; 2006, ASB2012-11478). In 2014, an amendment to this study was submitted (2010, ASB2014-9284). In this document, some results of testing glyphosate at dose level of 125, 250, and 375 mg/kg bw are reported. Clinical signs but no mortality were seen at all dose levels. It is not clear in which way this data is linked to the preliminary test that was performed as part of the original study since the dose levels were not exactly the same and the number of animals was different (this time 5 per sex and dose). Furthermore, in the amendment, more data on micronucleus incidences and PCE/NCE ratios at the dose levels of 15.62, 31.25, and 62.5 mg/kg bw was given, apparently based on 10 animals per sex and dose. It was confirmed that there was no clastogenic potential of the test substance. However, treatment of these animals was simply not described in the original report and the amendment cannot be considered a full study report. Taking all these deficiencies and uncertainties in the amendment as well as the use of only very low dose levels into account, assessment of the study as "not acceptable" by the RMS is maintained."

Comments (SK and AN): the number of evaluated cells is not sufficient. The results of the study are not plausible because of several shortcomings. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 53

Performing laboratory: SafePharm Laboratories Limited, Derbyshire, UK

Date: February 08, 2006

Title: Glyphosate technical: Micronucleus test in the mouse

Report No.: SPL 2060/014

Guidelines: OECD #474 (1997) [14]; Commission Directive 2000/32/EC B.12 (2000) [14], USA EPA [12] and JMAFF.

Test material: Glyphosate technical (purity 95.7%)

Sponsor: Nufarm Asia Sdn Bhd, Malaysia

Study type: micronucleus test in mouse bone marrow cells

Study design: single i.p. administration of the test compound was used. Mice were sacrificed 24 h (all doses) and 48 h (only highest dose) after administration.

Doses tested for MN study: 3 (150, 300 and 600 mg/kg)

Animals: 5-8 weeks old Crl:CD-1TM(ICR)BR mice; n=49 for MN study.

Negative control: PBS (vehicle); MN rates are OK but % of PCE per 1000 erythrocytes is unusual (lower than should be, ca. 50%).

Positive control: cyclophosphamide (27 mg/kg, i.p.); rates are OK

Acute toxicity test: provided, a MTD was defined (600 mg/kg).

Staining: May-Gruenwald-Giemsa

Scoring of slides: in total 1000 total erythrocytes were scored for the determination of PCE/NCE ratio; 2,000 PCE were scored for MN.

Statistical evaluation: the statistical analysis of MN frequencies was carried out with square root transformation followed by Student's t-test and ANOVA.

Historical control: vehicle control data obtained in 60 independent experiments are presented (MN results but not %PCE).

Results: clinical signs of toxicity (lethargy, ataxia, ptosis) were observed in mice dosed with glyphosate at and above 150 mg/kg (at all doses). The highest dose (600 mg/kg) induced a significant decrease of PCE ($p < 0.01$) at 24 h exposure and a not significant decrease at 48 h exposure time (because of high SD values). All data from individual animals are presented. Results are clearly negative.

Remarks: the study is not in agreement with the actual OECD guideline [9]. All doses induced symptoms of toxicity (ataxia and lethargy) in mice. Possibly lower doses should have been tested. Normally the ratio of PCE to NCE is approximately 1 : 1 (50% each) [15]. In the vehicle (negative) control the content of PCE was 36-38% while it was higher in the group treated with

glyphosate at dose of 150 mg/kg and in the positive control. Furthermore, the route of administration is not justified (as requested by the OECD [10]).

RAR: Evaluation and Comments

Study identification: *1st new micronucleus test in mice (Durward, 2006) (page 359)*

Evaluation: ***"****The study is considered acceptable. The selection of the highest dose level of 600 mg/kg bw is appropriate since the application route was intraperitoneal (for oral dosing, it might be too low). In fact, in a range-finding test, deaths were observed after i.p. application of 800 and 1000 mg/kg bw. The use of only male mice is also justified because males are known to be the more sensitive sex for micronucleus formation. The conclusion is supported. The increase in micronucleated PCE in high dose males after 24 hours (3.9/2000 = 1.95/1000) is covered by the historical controls even though it is close to its upper edge. Furthermore, no evidence of an increase was seen after 48 hours. Thus, there is no concern about clastogenicity because of this study and the conclusion of the notifiers may be agreed with."*

Comments (SK and AN): the ratios of PCE to NCE in the negative control are not plausible. Cyclophosphamide which was used as positive control either should decrease the number of PCE or have no effects, but it should not increase the rates of PCE. The route of administration is irrelevant and not justified. The number of evaluated cells is not sufficient. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 55

Performing laboratory: Laboratory of Pharmacology and Toxicology (LPT) GmbH & Co. KG, Hamburg, Germany

Date: May 18, 2009

Title: Micronucleus test of glyphosate TC in bone marrow cells of the CD rat by oral administration

Report No.: 23917

Guidelines: OECD #474 (1997) [9], Commission Directive 2000/32/EC B.12 (2000) [14], USA EPA [12] and JMAFF guidelines.

Test material: Glyphosate TC (98.8%)

Sponsor: Helm AG, Hamburg, Germany

Study type: micronucleus test in bone marrow erythrocytes

Study design: Single oral administration of the test compound. Rats were sacrificed 24 h and 48 h after administration.

Doses tested: 3 (500, 1,000 and 2,000 mg/kg).

Animals: 32 – 34 days old CD rats [stock Crl:CD(SD)]; in total 70 rats were used (5 ♂ and 5 ♀ per group).

Negative control: included, 0.8% aqueous hydroxypropyl methyl cellulose

Positive control: included, cyclophosphamide (27 mg/kg, i.p.).

Acute toxicity test: included.

Scoring of slides: 2,000 total erythrocytes were scored for the determination of the PCE/NCE ratios; 2,000 PCE were scored for MN. Staining of cells was with Mayers Haemaleum.

Statistical evaluation: the MN frequencies were analyzed with the chi-square test.

Historical control: presented

Results: all data concerning each individual animal are presented. The PCE content in erythrocytes is OK at both time points in exposed mice; negative and positive controls are OK. Results are clearly negative at all time points (24 and 48 h) and all doses.

Remarks: the design of study is adequate but the number of evaluated cells is not in agreement with the actual guidelines (2,000 were scored instead of 4,000).

RAR: Evaluation and Comments

Study identification: *1st new micronucleus test in rats (2009) (IIA, 5.4.4/02; pages 376 – 378)*

Evaluation: ***"****The study is considered acceptable and the conclusion is agreed with. Thus, absence of clastogenicity in vivo was also confirmed in the rat."*

Comments (SK and AN): the study is partly in agreement with OECD guidelines. The results are partly reliable.

Study number 56

Performing laboratory: Central Toxicology Laboratory, Alderley Park Macclesfield, Cheshire, UK

Date: March 21, 1996

Title: Glyphosate acid: mouse bone marrow micronucleus test

Report No.: CTL/P/4954

Guidelines: OECD #474 (1997) [9] and Commission Directive 2000/32/EC B.12 (2000) [14].

Test material: Glyphosate acid (95.6% purity)

Sponsor: Zeneca Agrochemicals

Study type: mouse bone marrow micronucleus test

Study design: single oral administration of test compound. Mice were sacrificed 24 h and 48 h after administration.

Doses tested: 1 (5,000 mg/kg)

Animals: n=50 in MN assay were used; 6-7 weeks old ♂ and ♀ CD-1 mice, (♂/♀ ratio in the group 1:1).

Negative control: included (0.9% saline); rates are OK

Positive control: cyclophosphamide (65 mg/kg); rates are OK

Acute toxicity test: single oral application of glyphosate at dose of 5,000 mg/kg did not induce clinical signs or lethality. This dose was used in MN experiments.

Staining: polychrome methylene blue and eosin using an automatic staining machine

Scoring of slides: the PCE/NCE ratio was determined; 2,000 PCE were scored for MN.

Statistical evaluation: Student's t test was used for root square transformed data.

Historical control: not presented.

Results: all results obtained in individual animals are presented. %PCE is OK at both time points in exposed mice, negative and positive controls are OK.

Results are clearly negative

Remarks: In contrast to the actual OECD guideline (2016) [11] and US EPA OPPTS 870.5395 [12] only one dose was tested instead of mandatory 3 doses ("In order to obtain dose response information, a complete study should include a negative control group and a minimum of three dose levels"). Testing of only one dose is possible when it was shown that the test chemical reaches bone marrow and/or when justification is provided. This was not included in the present study. The number of evaluated cells is not in agreement with the actual guidelines (2000 were scored instead of 4000).

RAR: Evaluation and Comments

Study identification: *5th new micronucleus test in mice 1996 (IIA, 5.4.4/06; pages 370 – 372)*

Evaluation: *The study is considered acceptable and the conclusion is agreed with.*

Comments (SK and AN): it is notable, that it is mentioned in OECD guidelines (OECD, 1997, 2014) that it should be justified why only one dose is used. In the newer OECD guideline (2016) it is stated that it should be demonstrated that the compound reaches target cells in bone marrow. No such discussion/justification is included. It was not shown that the compound reaches the target cells. The number of evaluated cells is not sufficient. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 62

Performing laboratory: RCC (Cytotest Cell Research GmbH), Germany

Date: June 09, 2008

Title: Glyphosate technical – micronucleus assay in bone marrow cells of the mice. Final report

Project ID: No. 1158500, Study No. 1158500

Guidelines: OECD #474 (1997) [9]; Commission Directive 2000/32/EC B.12 (2000) [14] and EPA OPPTS 870.5395 [12].

Sponsor: Syngenta Ltd, UK

Test material: Glyphosate technical (purity 99.1%)

Study type: micronucleus assay in bone marrow cells of the mice

Study design: Single oral administration of "Glyphosate technical". Three doses were used: 2,000 mg/kg, 1,000 and 500 mg/kg. Mice were sacrificed 24 h (2,000 mg/kg, 1,000 and 500 mg/kg) and 48 h (2,000 mg/kg) after administration.

Doses tested: 3

Animals: n=35 in MN study, 7-8 weeks old ♂ NMRI mice.

Negative controls: included, 0.5% carboxymethylcellulose in water; MN rates are OK

Positive control: cyclophosphamide (40 mg/kg, orally); MN rates are OK.

Acute toxicity (dose selection): included.

Staining: May-Gruenwald-Giemsa

Scoring of slides: 2,000 erythrocytes in total were scored for determination of PCE/NCE ratios; 2,000 PCE were scored for MN.

Statistical evaluation: MN frequencies were transformed by Blom's method, then ANOVA was used.

Historical control: presented.

Results: data obtained with individual animals are presented. Results are clearly negative at all time points (24 and 48 h). No dose response was found.

Remarks: The design of the study is in partial agreement with the OECD guideline [11]. The number of evaluated cells is not in agreement with the actual guidelines (2000 were scored instead of 4000).

RAR: Evaluation and Comments

Study identification: *6th new micronucleus test in mice (2008) (IIA, 5.4.4/07; pages 372 – 372)*

Evaluation: "*The study is considered acceptable and the conclusion is agreed with. It seems that technical material from a different (Chinese) source and of rather high purity was tested."*

Comments (SK and AN): the study is only partly in agreement with OECD guidelines. The results are partly reliable.

Study number: 63

Performing laboratory: SCANTOX A/S

Date: September 12, 1991

Title: Mutagenicity test: Micronucleus test with Glyphosate, batch 206-JaK-25-1. Final report

Guidelines: OECD #474 (1997) [9] and FIFRA [16]

Client: Cheminova Agro A/S, Denmark

Test material: Glyphosate (purity 98.6%)

Study type: micronucleus test in bone marrow cells

Study design: Single oral administration of "Glyphosate". Mice were sacrificed 24 h, 48 h and 72 h after administration.

Doses tested: 1 (5,000 mg/kg).

Animals: n=50, 10 weeks old ♂ and ♀ NMRI SPF mice of the strain Bom:NMRI, 5/sex/per each time point.

Negative control: included (0.5% carboxymethyl-cellulose in water); rates of MN are OK

Positive control: cyclophosphamide (30 mg/kg, orally); rates of MN are OK

Acute toxicity (dose selection): included.

Staining: May-Gruenwald-Giemsa

Scoring of slides: 200 total erythrocytes were scored for determination of PCE/NCE ratio; 2,000 PCE were scored for MN.

Statistical evaluation: MN frequencies were transformed by Blom's method, then ANOVA was used.

Historical control: not presented.

Results: all results obtained with individual mouse are presented. Results are clearly negative at all time points (24, 48 and 72 h).

Remarks: according to the OECD guidelines the testing of only one dose is justified when it was proven that the test compound reaches the target tissue and/or when a discussion is provided which is not the case. The number of evaluated cells is not in agreement with the actual guidelines (2000 were scored instead of 4000).

RAR: Evaluation and Comments

Study identification: *Jensen, 1991 (TOX9552374; page 358)*

Evaluation: "*found by the RMS to be of acceptable quality" (page 357)*

Comments (SK and AN): it is notable, that it is mentioned in OECD guidelines (OECD, 1997, 2014) that it should be discussed why only one dose is used. In the newer OECD guideline (2016) it is stated that it should be demonstrated that the compound reaches target cells in bone marrow. No such justification is included, i.e. it was not shown that the compound reaches the target cells. The number of evaluated cells is not in agreement with the actual guidelines (2000 were scored instead of 4000). The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 66

Performing laboratory: Monsanto Company

Date: February 25, 1992

Title: Mouse micronucleus study of RODEO® herbicide formulation. Final report

Report No. MSL-11772; Project No. 91201/91205, ML-91-435/ML-91-438

Guidelines: not specified

Test material: RODEO® herbicide formulation (40% glyphosate; acid equivalent; other components are unknown)

Study type: micronucleus assay in bone marrow cells

Study design: Single i.p. injection of "RODEO® herbicide formulation". Three doses were used: 555 mg/kg, 280 and 140 mg/kg (80%, 50% and 25% of LD_{50}). Mice were sacrificed 24 h, 48 h and 72 h after administration.

Animals: n=130 in the MN study, 8-12 weeks old ♂ and ♀ CD-1 mice, 15/sex/ per each dose level.

Negative control: included, 0.9% saline, i.p.

Positive control: cyclophosphamide (40 mg/kg, i.p.).

Acute toxicity (dose selection): included, the LD_{50} value was determined (4239 mg/kg). Three doses were used: 3400 mg/kg, 1700 and 850 mg/kg (80%, 50% and 25% of LD_{50}).

Scoring of slides: the PCE/NCE ratios were determined; 1,000 PCE were scored for MN by 2 different scorers (500 cells each).

Staining: Wright-Giemsa stain.

Statistical evaluation: Dunnett's test

Historical control: not presented.

Results: all data concerning each individual mouse are presented. The numbers of PCE are OK at both time points, negative and positive controls are OK. Results are clearly negative at all time points (24, 48 and 72 h).

Remarks: only 1000 PCE were scored per animal by 2 scorers (500 cells each) instead of 4,000 PCE as suggested by the OECD and other guidelines (see Table 3). The route of administration (i.p.) is not justified as requested by the OECD [10]. The test compound is not clearly characterized.

RAR: Evaluation and Comments

Study identification: *Monsanto, 1992, TOX9552376 (page 381)*

Evaluation: "*The study is considered as acceptable."*

Comments (SK and AN): the conclusion is not justified as the study did not follow the current internationally accepted guidelines, i.e. the number of evaluated cells is substantially lower than recommended. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number: 67

Performing laboratory: Monsanto Company

Date: February 25, 1992

Title: Mouse micronucleus study of ROUNDUP® herbicide formulation. Final report

Report No. MSL-11771; Project No. 91202791206, ML-91-434/ML-91-437

Guidelines: not specified

Test material: ROUNDUP® herbicide formulation (31% glyphosate; acid equivalent; other components of the formulation are not specified)

Study type: micronucleus assay in bone marrow cells

Study design: Single i.p. injection of the "ROUNDUP® herbicide formulation". Mice were sacrificed 24 h, 48 h and 72 h after administration.

Doses tested: 3 (555 mg/kg, 280 and 140 mg/kg; 80%, 50% and 25% of LD_{50}).

Animals: n=136 in MN study, 8-12 weeks old ♂ and ♀ CD-1 mice, 5/sex/time point per group.

Negative control: included, 0.9% saline, i.p.

Positive control: included, cyclophosphamide (40 mg/kg, i.p.).

Acute toxicity: the LD_{50} was determined (643 mg/kg). Three doses were used for the MN assay: 555 mg/kg, 280 and 140 mg/kg (80%, 50% and 25% of LD_{50}).

Staining: May-Gruenwald-Giemsa

Scoring of slides: 1000 total erythrocytes were scored for determination of PCE/NCE ratio; 1,000 PCE were scored for MN by 2 different scorers (500 cells each).

Statistical evaluation: MN frequencies were transformed (root square), then Dunnett's test was applied.

Historical control: not presented.

Results: all data obtained from individual animal are presented.

Results are clearly negative.

Remarks: Only 1000 PCE were scored per animal by 2 scorers (500 cells each) instead of 4,000 as suggested in the OECD and other guidelines (see Table 3). The route of administration (i.p.) is not justified.

RAR: Evaluation and Comments

Study identification: *(Monsanto 1992, TOX1999-242) (page 381)*

Evaluation: "*The study is considered acceptable."*

Comments (SK and AN): the conclusion is not justified as the study did not follow the current internationally accepted guidelines, i.e. the number of evaluated cells is substantially lower as recommended. The choice of the rout of administration is not justified. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number: 68

Performing laboratory: Monsanto Company

Date: February 25, 1992

Title: Mouse micronucleus study of DIRECT® herbicide formulation. Final report

Report No. MSL-11773; Project No. 91202791206, ML-91—436/ML-91-439

Guidelines: not specified

Test material: DIRECT® herbicide formulation (72% glyphosate; acid equivalent; other components are not characterized)

Study type: micronucleus assay with bone marrow cells

Study design: Single i.p. injection of "DIRECT® herbicide preparation". Mice were sacrificed 24 h, 48 h and 72 h after administration.

Doses tested: 3 (365 mg/kg, 183 and 91 mg/kg; 80%, 50% and 25% of LD_{50}).

Animals: n=136 in MN study, 8-12 weeks old ♂ and ♀ CD-1 mice, 15 - 18 per group.

Negative control: included, 0.9% saline, i.p.

Positive control: cyclophosphamide (40 mg/kg, i.p.).

Acute toxicity: the LD_{50} value was determined (436 mg/kg). Three doses were used: 365 mg/kg, 183 and 91 mg/kg (80%, 50% and 25% of LD_{50}).

Staining: May-Gruenwald-Giemsa

Scoring of slides: 1000 total erythrocytes were scored for the determination of the PCE/NCE ratios; 1,000 PCE were scored for MN by 2 different scorers (500 cells each).

Statistical evaluation: MN frequencies were transformed (root square), then Dunnett's test was applied.

Historical control: not presented.

Results: all data obtained from individual animals are presented. Results are clearly negative.

Remarks: Only 1000 PCE were scored per animal by 2 scorers (500 cells each) instead of 4,000 PCE as suggested in the OECD guideline [10]. The route of administration (i.p.) is also not justified.

RAR: Evaluation and Comments

Study identification: *Monsanto, 1992, TOX1999-322 (page 381)*

Evaluation: "*The study is considered acceptable." (page 382)*

Comments (SK and AN): the conclusion is not justified as the study did not follow the current internationally accepted guidelines, i.e. the number of evaluated cells is substantially lower as recommended. No justification for the route of administration is provided. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number: 82

Performing laboratory: JAI Research Foundation, India

Date: September 13, 2012

Title: Micronucleus test of Glyphosate TGAI in mice. Final report

Laboratory Project study No. 485-106-4996

Guidelines: OECD #474 guideline (year not specified) and JMAFF (2000).

Test material: Glyphosate (purity 98.9%)

Sponsor: Dow AgroSciences Llc, USA

Study type: micronucleus test with bone marrow cells

Study design: Two oral gavage of Glyphosate TGAI at 0 h and 24 h. Mice were sacrificed 24 h and 48 h after administration.

Doses tested: only one (2,000 mg/kg)

Animals: n=18 in MN study, 8-9 weeks old ♂ Swiss albino mice, 5 - 7 per group.

Negative control: included, vegetable oil (not specified); rates are OK

Positive control: included, mitomycin C (1.0 mg/kg, i.p.).

Acute toxicity: six mice were treated orally with glyphosate at dose of 2,000 mg/kg. It was not toxic.

Staining: Giemsa (5.0%)

Scoring of slides: 200 erythrocytes were scored for the determination of PCE/NCE ratio; 2,000 PCE were scored for MN.

Statistical evaluation: Bartlett's test as well as ANOVA followed by the Dunnett's test were applied.

Historical control: not provided

Results: all data obtained from individual animals are presented. Results are clearly negative.

Remarks: only one dose was tested for MN induction. According to the current OECD guidelines (2016) the testing of only one dose is justified when it was proven that the test compound reaches the target tissue and/or a discussion is presented which is not the case. The number of cells which was evaluated is not in agreement with current guidelines.

RAR: Evaluation and Comments

Study identification: *7th new micronucleus test in mice Dow (2012) (ASB2014-9277, page 359)*

Evaluation: *"The study may be considered acceptable" (page 375)*

Comments (SK and AN): it is notable, that it is mentioned in all OECD guidelines that it should be discussed why only one dose is used. In the newer OECD guideline (2016) it is stated that it should be demonstrated that the compound reaches target cells in bone marrow. No such justification is included. The number of evaluated cells is not in agreement with the current guidelines. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number: 91

Performing laboratory: Harlan Cytotest Cell Research GmbH (Harlan CCR)

Date: September 28, 2012

Title: Glyphosate technical – micronucleus assay in bone marrow cells of the mice. Final report

Report No. 1479200; Task No. TK0112981

Guidelines: OECD #474 (1997) [9] and US EPA OPPTS 870.5395 (1998) [12].

Test material: Glyphosate technical (purity 96.3%)

Sponsor: Syngenta Ltd, UK

Study type: micronucleus assay in bone marrow cells of mice

Study design: Single oral gavage of glyphosate. Mice were sacrificed 24 h and 48 h after administration.

Doses tested: only one (2,000 mg/kg).

Animals: n=29, 8-9 weeks old ♂ NMRI mice, 5 - 7 per group.

Negative control: included, 1.0% carboxymethylcellulose; MN rates are OK

Positive control: included, cyclophosphamide (40 mg/kg).

Acute toxicity: included; the test chemical was not toxic, and a limit test was performed using a maximal dose of 2,000 mg/kg, lower doses were not tested.

Staining: May-Gruenwald-Giemsa

Scoring of slides: PCE/NCE ratios were determined; 2,000 PCE were scored for MN.

Statistical evaluation: Mann-Whitney test was applied.

Historical control: not presented.

Results: all data obtained from individual animals are presented. Results are clearly negative.

Remarks: only one dose was tested for MN test. According to the OECD guidelines the testing of only one dose is justified when it was proven that the test compound reaches the target tissue and/or when a discussion is provided which is not the case. **The number of evaluated cells is not in agreement with the current guidelines.**

RAR: Evaluation and Comments

Study identification: *8th new micronucleus test in mice Dow (2012) (ASB2014-9277, page 359)*

Evaluation: *"The study may be considered acceptable" (page 375)*

Comments (SK and AN): it is notable, that it is mentioned in all OECD guidelines that it should be discussed why only one dose is used. In the newer OECD guideline (2016) it is stated that it should be demonstrated that the compound reaches target cells in bone marrow. This was not shown in the study. The number of evaluated cells is not in agreement with the current guidelines. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number: 98

Performing laboratory: Monsanto

Final report; Project No. EHL 89182/ML-89-463

Date: March 26, 1998

Title: Mouse micronucleus screening assay of MON-0818

Guidelines: not specified

Test material: MON 0818; the test material is a mix and does not contain glyphosate

Study type: micronucleus assay with bone marrow cells

Study design: Single i.p. administration of MON 0818 in corn oil. Mice were sacrificed 24 h and 48 h after administration.

Doses tested: 1 (100 mg/kg)

Animals: n=25 mice were used in MN experiment; 10-12 weeks old ♂ and ♀ CD-1 mice, 5 of each sex per group.

Negative control: included, corn oil; rates of MN are OK

Positive control: included, cyclophosphamide (60 mg/kg, i.p.).

Acute toxicity test: included, the LD_{50} value was determined (165.2 mg/kg). A target dose of 100 mg/kg was chosen (61% of the LD_{50}).

Staining: Wright-Giemsa

Scoring of slides: 1000 erythrocytes for PCE and NCE ratio; 1000 PCE for MN.

Statistical evaluation: Dunnet's test

Historical control: Not presented

Results: all data obtained from individual animals are presented. Results are clearly negative.

Remarks: the number of evaluated PCE (1000) is substantially lower than that recommended by many international guidelines (2,000) (see Table 3). Since the compound produces acute toxicity, three doses should be investigated in genotoxicity experiments according to the OECD guidelines. The route of administration is not justified as requested in the OECD guidelines [9]. According to the OECD guidelines the testing of only one dose is justified when it was proven that the test compound reaches the target tissue and/or when a discussion is provided which is not the case. Therefore, the results obtained in this study are inconclusive. The test material does not contain glyphosate.

RAR: Evaluation and Comments

Study identification: *Monsanto, 1998 (TOX1999-240; page 395)*

Evaluation: *"The study is considered supplementary only since it was not in compliance with OECD recommendations for tests of this type. In particular, the only dose level used was too low for definitive assessment." (page 395)*

Comments (SK and AN): The number of evaluated cells is not in agreement with the current guidelines. Choice of route of administration is not justified. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 100

Performing laboratory: Rallis India Limited (Rallis Agrochemical Research Station)

Title: Mutagenicity micronucleus test in Swiss albino mice

Report TOXI: 889-MUT.MN (TOXI-889/1993 ES-GPT-MUT-MN)

Guidelines: OECD #474 guidelines (1984).

Date: May 6, 1993

Sponsor: M/s Feinchemie Schewebra GmbH, Germany

Test material: Glyphosate Technical (purity 96.8%)

Study type: micronucleus assay with bone marrow cells

Study design: Two gavage administrations of glyphosate with a 24 h interval in groundnut oil. Mice were sacrificed 24 h after the last treatment.

Doses tested: 3 (50, 500 and 5,000 mg/kg)

Animals: 60 mice/group were used in the MN experiment, 8-10 weeks old ♂ and ♀ Swiss albino mice, 5 of each sex per treatment group (in the negative control group 10 mice of each sex).

Negative control: included, groundnut oil; rates of MN are OK

Positive control: included, cyclophosphamide (100 mg/kg).

Acute toxicity: no data presented, a justification for selection of doses is not presented.

Staining: May-Gruenwald and Giemsa.

Scoring of slides: 1000 PCE and 1000 NCE were evaluated per mouse for MN. PCE/NCE ratio were scored.

Statistical evaluation: one-way ANOVA test and "t"-test for unequal number of observations were used.

Historical control: not presented

Results: all data obtained from individual animals are presented. In the high dose group (5,000 mg/kg) of males and in combined sexes, results do not differ from the control group. However, in females the incidence of PCE with MN was significantly higher than in the controls.

Remarks: this study has numerous serious shortcomings. The authors stated in the "Conclusions" that "Glyphosate Technical is not mutagenic by MN test in mice. However, at 5,000 mg/kg it may significantly increase the incidence of MN in female mice."

In contrast to actual OECD guidelines # 474 (2014, 2016) the number of evaluated PCE (1000) is substantially lower than that recommended (4,000) (see Table 3).

The dose levels are separated by a factor of 10 instead of 2-4 as recommended by OECD guidelines (the factor should not exceed 4!) [9]. It is stated that physiological saline was used to flush out the femura for the collection of the target cells. This is in contrast to all standardized procedures which use bovine or calf fetal serum [2, 17-19]. According to the personal experience of the authors of this document (A. Nersesyan and S. Knasmueller) the use of saline leads to clothing of the erythrocytes and it is not possible i) to distinguish between PCE and NCE and ii) to evaluate MN formation in PCE. Therefore, we anticipate that the results obtained in this study are faked!

RAR – Evaluations and Comments:

Study identification: *ADAMA,1993, page 358*

Evaluation: "*the study is still found by the RMS to be of acceptable quality" (page 357).*

Comments (SK and AN): the conclusion is not justified as the study did not follow the current internationally accepted guidelines (the dose selection is inadequate and the number of evaluated cells is substantially lower than recommended). The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 105

Performing laboratory: BIOAGRI

Title: A micronucleus study in mice for the product GLIFOS

Report: # G.1.2 – 60/96

Guidelines OECD #474 (1997) [9].

Date: November 18, 1996

Sponsor: Cheminova Agro A/S, Sao Paolo, Brazil

Test material: GLIFOS (glyphosate; purity not specified)

Study type: micronucleus test with bone marrow cells

Study design: two i.p. administrations of GLIFOS (with a 24 h interval) in water. Mice were sacrificed 24 h after the last injection.

Doses tested: 3 (68, 137 and 206 mg/kg; 25%, 50% and 75% of the LD_{50}).

Animals: n=50, 7-10 weeks old ♂ and ♀ Swiss albino mice, 5 of each sex per group.

Negative control: included, distilled water; MN rates are OK

Positive control: included, cyclophosphamide (25 mg/kg).

Acute toxicity test: included, the LD_{50} was determined (275 mg/kg).

Staining: Giemsa (3.3%)

Scoring of slides: PCE/NCE ratio were scored; 1000 PCE and 1000 NCE for MN were scored per animal.

Statistical evaluation: Mann-Whitney U test

Historical control: Mentioned, but not presented

Results: all data obtained with individual animals are presented. Results are clearly negative; no increase was observed in the exposed animals.

Remarks: in contrast to several international guidelines (see Table 3), the number of evaluated PCE (1000) is substantially lower as recommended (4,000). The route of administration (i.p.) is not justified. The number of PCE with MN was zero in males and females at the highest dose (206 mg/kg) which is strange since at all lower doses MN were found both in males and females.

It is also strange that no MN were found in 4 males in the negative control group, and in one 4.0‰. In three females (negative controls) no MN were observed. In 6 mice from 11 (5 males, 6 females; positive control) no MN were found. In the positive control (males) the range of MN was between 0 and 24‰, in females the range was between 0 and 16‰.

Although it is declared that 1000 cells (PCE) were scored for MN, in Table 3 is indicated that in males (negative control) 1000 cells were evaluated only in one animal, in all the other mice only 337, 445, 273 and 696 PCE were scored. At the lowest dose, 1000 PCE were evaluated only in three mice, the number of sored cells in two further animals were lower. At a dose of 137 mg/kg, only in one mouse 1000 PCE were evaluated, in four other mice the numbers of PCEs were between 653 and 870. At highest dose less than 1000 PCE were evaluated in two mice.

RAR – Evaluations and Comments:

Study identification: *TOX1999-253, 1996 (page 382)*

Evaluation: ***"****The study is considered of limited value for risk assessment only since a legal statement on GLP compliance is lacking and since there was no information regarding general health effects of treatment to the animals. Therefore, it is not clear whether the highest possible dose was actually reached."*

Comments (SK and AN): the number of evaluated cells is substantially lower than recommended) and the route of administration is not justified. The study deviates substantially from the OECD guidelines and the results are not reliable.

Study number 106

Performing laboratory: TECAM Laboratorios, Tecnologia Ambiental, Sao Rogue Ltda, Sao Rogue, Brazil

Title: Mammalian erythrocyte micronucleus test for GLIFOSATO TECHNICO HELM

Report: RL3393/2007 – 3.0MN-B (Final)

Guidelines: OECD #474 (1997) [9], Commission Directive 2000/32/EC B.12 (2000) [14], USA EPA OPPTS 870.5395 [12].

Date: December 13, 2007

Test material: GLIFOSATO TECHNICO HELM (glyphosate; 98.01% purity)

Sponsor: HELM Do Brasil Mercanti Ltda, Sao Paulo

Study type: micronucleus test in bone marrow cells

Study design: Oral gavage of "GLIFOSATO TECHNICO HELM**"** in deionized water. Mice were treated twice at 0 and 24 h and sacrificed 24 h after last administration.

Doses tested for MN study: 3 (8.0, 15 and 30 mg/kg)

Animals: n=30, 9-10 weeks old ♂ Swiss mice, 6 per group for MN study.

Negative control: included, deionized water; rates are OK

Positive control: included, cyclophosphamide (75 mg/kg); rates are OK

Acute toxicity test: included, the MTD value was determined (30 mg/kg). Two other dose were selected, 15 and 8.0 mg/kg (factor of 2).

Staining: Wright-Giemsa stain

Scoring of slides: 2,000 erythrocytes were scored for determination of PCE/NCE ratio; 3,000 PCE were scored for MN.

Statistical evaluation: the chi-square test was applied.

Historical control: mentioned (1.0 per 1,000 PCE for several experiments over 5 years).

Results: All data concerning each individual mouse are presented.

Results are clearly negative at doses 8.0 and 15 mg/kg; a significant increase was observed in 30 mg/kg exposed mice (1.39‰ vs 0.6‰ in negative control; p=0.020). The authors stated that the historical control for 5 years is 1.0/1,000 PCE. They also presented data of other publications

which indicate that the MN rates in negative control is between 1.88 and 1.95 per 1,000 PCE. On the basis of three doses they state that the significant increase is biologically not relevant because the number of MN is between historical control and published data.

Remarks: according to the OECD guideline [9], a result of MN study is clearly negative if it is inside the distribution of the historical negative control data. The authors stated that these results are within the historical control data from their laboratory (1.0‰) and other published data (results of 561 papers indicating that the range is between 1.88‰ and 1.95‰s) and hence they have no biological relevance. However, no results of individual studies are shown from their own laboratory which support this conclusion. Furthermore, no statistical calculations are provided in which the actual data were compared to historical controls.

RAR – Evaluations and Comments:

Study identification: *2nd new micronucleus test in mice (2007), (pages 362 – 364)*

Evaluation: "*The study is considered not acceptable since it was seriously flawed (see below) and because the dose levels were much too low for any meaningful conclusion with regard to micronucleus formation, in particular when application by the oral route is taken into consideration. In the original report, some justification for dose selection is given, based on a range-finding test suggesting effects at rather low dose levels. In fact, two animals that were administered 2000 mg/kg bw, died on day 3 after having shown ataxia and prostration before. The same observations were made in 3 animals which received an oral dose of 320 mg/kg bw. They all died on day 2. Even at a dose level of 50 mg/kg bw, one out of three treated animals died on day 1. No mortality was seen at 30, 20, and 12.5 mg/kg bw. Therefore, 30 mg/kg bw was considered the MTD and was selected as the highest dose for the micronucleus assay. These*

findings The occurrence of deaths and clinical signs at relatively low dose levels were was obviously in contradiction to more reliable the available acute toxicity tests with glyphosate in the mouse. In addition, in five other micronucleus assays or cytogenetic studies in mice with substance administration by the oral route described in this section much higher dose levels could be used. A single study cannot contravene or even outweigh all this data coming from a number of (independent) laboratories even though this was suggested by a comment that was provided in the public consultation. It is much more likely that the micronucleus assay by (2007) was seriously flawed by severe toxicity that was completely unexpected and cannot be explained if the whole toxicological profile of glyphosate is taken into consideration. Either, strong methodical mistakes have been made when the study was conducted or the test material was not glyphosate even though it was claimed as such. Both possibilities would turn the study completely unreliable and make it unsuitable for any regulatory use. Because of this general assessment, there is no need to discuss the weak "increase" in micronuclei at 30 mg/kg bw that is in complete contradiction to what was seen in the other, much more reliable studies" (page 364).

Comments (SK and AN): the number of evaluated cells is lower than recommended. The study deviates substantially from the OECD guidelines and the results are not reliable.

8.3. Summary

MN experiments *in vivo*

In total, we evaluated 15 studies which concern MN formation in bone marrow cells, the most widely used procedure for routine genotoxicity screening of chemicals with rodents. All experiments were conducted with mice. Almost all reports had shortcomings which were either moderate or severe and were not in agreement with the current OECD guideline.

Not plausible numbers of MN in control animals:

Study 49

Study 105

Lack of justification for i.p. administration in 7 studies (according to the current OECD guideline, it should be justified when the test compound is administered i.p.):

Oral and inhalative exposures are by far more relevant for glyphosate!

Study 49

Study 53

Study 66

Study 67

Study 68

Study 98

Study 105

Not plausible ratios of PCE/total erythrocytes in control animals (these unusual findings indicate that the results may be not reliable):

Study 53

Inadequate number of tested doses:

Study 56

Study 62

Study 63

Study 82

Study 91

Study 98

Insufficient number of scored cells per animal (according to the current protocol, 4,000 PCE should be scored):

All the studies; only in study #106 3,000 PCE were evaluated.

In the following studies only 1,000 PCE or less were scored:

Study 66

Study 67

Study 68

Study 98

Study 100

Study 105

In the following studies only 2,000 PCE were scored:

Study 49

Study 53

Study 55

Study 56

Study 62

Study 63

Study 82

Study 91

Inadequate choice of dose levels (according to the OECD guideline the dose should be separated by a factor of 2, but not greater than 4):

Study 106

In three studies formulations containing glyphosate (#66, #67 and #68) were investigated and in one study the material is not specified (#98; MON 0818).

8.4. References

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9. Dominant Lethal Assay

9.1. Description

The Dominant Lethal Assay was developed in 1960's and enables the detection of mutagenic effects in male germ cells of rodents [1, 2]. Dominant lethal mutations cause embryonic and fetal deaths and positive results indicate that the chemical affects germinal cells of the test animal. By definition, a dominant lethal mutation kills the fertilized egg when it has been inherited from only one parent. Theoretically, death may occur at any time from fertilization to sexual maturity. In practice, however, the lethals on which the assay is based kill the zygote at a very early stage, before or around the time of implantation of the egg. Thus, either they fail to induce a decidual response, or having induced that response, they fail to develop further. The former is detectable only as a discrepancy between the number of implantation sites. The latter are seen as dead implants (usually deciduomata) scattered amongst the live implants [1].

The procedure is described in detail in OECD guideline 478 (2016) [2] and in the US EPA guideline OPPTS 870.5450 (1998) [3]. It is based on mating of untreated virgin females with chemically treated males. Important parameters are the selection of doses, the administration period, the mating intervals and the number of animals, and also inclusion of negative and positive controls [2, 3]. Different germ cell types can be tested by use of sequential mating

intervals. Following mating the females are euthanized after appropriate time periods, and their uteri are examined to determine the numbers of implants and live and dead embryos. The dominant lethality of a test agent is determined by comparing the live implants per female in the treated group with the live implants per female in the vehicle/solvent control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the test-agent-induced post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants in the treated group compared to the ratio of dead to total implants in the control group. Pre-implantation loss can be estimated from *corpora lutea* counts or by comparing the total implants per female in treated and control groups [2].

9.2. Evaluation of individual studies

Study number: 107

Performing laboratory: Rallis Agrochemical Research Station, India

Date: November 04, 1992

Title: Dominant lethal test in Wistar rats

Report No.: TOXI-888-DLM/1992

Guidelines: OECD # 478 (1982)

Test material: Glyphosate technical (purity 96.8%)

Sponsor: M/s Feinchemie Schwebda GmbH, Germany

Guidelines: according to the authors the study followed the OECD (1982) guidelines

Study type: Dominant Lethal Assay with rats treated by gavage with glyphosate.

Study design: male rats were treated with single dose glyphosate and mated with virgin females (1:1). The paired females were separated after 6 days. On the 8^{th} day males were again paired with fresh batch of virgin females. One male was mated with 30 females. The female rats from each "week were sacrificed on 16^{th} day of pairing.

Animals: random bread Wistar rats (300 ♀ and 30 ♂ males)

Doses tested: 3 (200, 1,000 and 5,000 mg/kg).

Negative controls: included, refined groundnut oil.

Positive controls: included, ethyl methanesulfonate (100 mg/kg/day for 5 days and 500 mg/kg as a single dose).

Dose selection: not described, the authors noted that the doses were selected after the dose range study.

Statistical analysis: Bartlett's test for homogeneity, ANOVA and Dunnett' multiple pairwise comparison tests were used.

Historical control: not presented.

Results: Results obtained from all groups and individual animals are presented. Calculations of the results are clearly described. The authors conclude that that glyphosate does not induce dominant lethal mutations. It is evident that pre- and post-implantation losses were seen after different time points and with different doses. According to the authors (conclusions, page 12) "the compound did not induce dominant lethal effect in rats as most of the changes seen in fertility indices were inconsistent and not related to the dose of the test compound excepting at high dose during the first week when it may cause acute toxicity related effect on incidence of

pregnancy, early resorption, pre- and post-implantation losses and the duration after treatment. Under similar conditions the positive control, EMS, induced typical dominant lethal effects".

Remarks: According to the OECD guidelines [2], a clear negative result is obtained "if none of the test doses exhibits a statistically significant increase compared with the concurrent negative control, and there is no dose-related increase in any experimental condition". The latter criterion is not fulfilled as the effects increased with the dose, therefore, the assumption of negative results is possibly not justified. The acceptability criteria defined in the current OECD guideline also not fulfilled as no historical control data are shown. It is mandatory that "Concurrent negative control is consistent with published norms for historical negative control data, and the laboratory's historical control data." [2].

No experiments to define the maximally tolerated dose (MTD) were conducted which is suggested by the OECD [2].

No experiments were conducted to verify that the test chemicals reach the target tissue or the general circulation (this is mandatory when negative results are obtained) [2].

The statistical analysis is not in agreement with the OECD guideline [2]. It is suggested in the guidelines that for over- under-dispersion calculations tests such as Cochran's binomial variance test or Tarone's C(α) test for binomial overdispersion are recommended. If no departure from binomial dispersion is detected, trends in proportions across dose levels may be tested using the Cochran-Armitage trend test and pairwise comparisons with the control group may be tested using Fisher's exact test. However, the analysis used in the study is acceptable. It is notable that many chemicals which cause DNA damage in somatic cells do not cause damage in germ cells. Therefore, a negative result in the Dominant Lethal Assay does not prove that a compound is

devoid of mutagenic activity in inner organs. Hence, a negative result can be at best only used as supplementary information.

Evaluation by the European authorities:

Study identification: *ADM, TOX9551102 (1992)*

Evaluation: *"still considered acceptable" (page 378)*

Comments (SK and AN): the study deviates substantially from the OECD guidelines and the results are not reliable.

Study number: 108

Performing laboratory: International Research and Development Corporation, Michigan, US

Date: April 16, 1980

Title: Dominant lethal study in mice

Report No.: 401-064

Guidelines: not specified. The study was carried out according to method described in Epstein et al., Toxicol Appl Pharmacol, 1972.

Test material: Glyphosate technical (98.7%)

Sponsor: Monsanto Company, US

Guidelines: not specified

Study type: Dominant Lethal Assay with CD-1 mice.

Study design: Male CD-1 mice (n=10/group) were treated once with the test substance by gavage dissolved in "METHOCEL™" cellulose ether. Each male mouse was mated with 16 virgin females. Duration of the experiment was 10 weeks.

Doses tested: 3 (200, 800 and 2,000 mg/kg).

Total number of animals: 800 ♀ and 50 ♂ males per group.

Negative control: included, "METHOCEL™" cellulose ether (0.5%).

Positive control: included, cytoxan (cyclophosphamide; 240 mg/kg a single dose i.p.).

Toxicity assessment: MTD was not determined.

Statistical analysis: Bartlett's test for homogeneity, ANOVA and Dunnett' multiple pairwise comparison tests were used. Calculations of the results are clearly described.

Historical control: not presented.

Results: Results obtained from all the groups and individual animals are presented. No treatment –related effects were observed in the study. The authors found slight but significant decrease of the number of viable fetuses at dose of 800 mg/kg one week after mating and in 200 mg/kg group after week 3 of the mating. However, no increase in early fetal deaths accompanied this effects. Therefore, the authors conclude that the compound is negative in the DLM test. Clearly positive results were obtained in the positive control group.

Remarks: The dominant lethal factor which is suggested in the OECD guideline was not calculated [2]. The observation of reduced viability of the newborns which was seen is a serious

effect (even in the putative absence of positive results in DLM test and should be taken into consideration in the risk assessment of a compound.

The acceptability criteria defined in the OECD guideline also not fulfilled as no historical control data are shown. It is mandatory that "Concurrent negative control is consistent with published norms for historical negative control data, and the laboratory's historical control data."

No experiments to define the maximal tolerated dose (MTD) were conducted which is suggested by the OECD.

No experiments were conducted to verify that the test chemical reached the target tissue or the general circulation (this is mandatory when negative results are obtained) [2].

Statistical analyses are not in agreement with the current OECD guideline [2]. It is suggested in this guideline that for over- under-dispersion calculations tests such as Cochran's binomial variance test or Tarone's C(α) test for binomial overdispersion are recommended. If no departure from binomial dispersion is detected, trends in proportions across dose levels may be tested using the Cochran-Armitage trend test and pairwise comparisons with the control group may be tested using Fisher's exact test [2]. However, the analysis used in the study is acceptable. It is notable that many chemicals which cause DNA damage in somatic cells do not cause damage in germ cells. Therefore, a negative result in the Dominant Lethal Assay does not prove that a compound is devoid of mutagenic activity in inner organs. Hence, a negative result can be at best only used as supplementary information.

Evaluation by the European authorities:

Study identification: *Monsanto TOX9552377 (1980)*

Evaluation: *"still considered acceptable" (page 378)*

Comments (SK and AN): the study deviates substantially from the OECD guidelines and the results are not reliable.

9.3. Summary

Dominant Lethal Test

No historical data are shown (mandatory according to the OECD guideline):

Study 107 (highly relevant as an increase was found with some doses)

No experiments to define the maximally tolerated dose (MTD) were carried out:

Study 107

Study 108

The statistical evaluation of the results is possibly not in agreement with the OECD guideline but is acceptable (see evaluation of M. Kundi):

Study 107

Study 108

No study was conducted to verify that the compound reached the target tissue:

Study 107

Study 108

9.4. References

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10. Background information about the authors

Prof. Siegfried Knasmueller studied biology and chemistry at the University of Vienna; his thesis concerned bacterial mutagenicity tests. SK was visiting scientist at the Institute of Environmental Health (School of Medicine) of the University of Cleveland (Illinois) and at the US EPA (North Carolina). Since 1983 SK works at the Institute of Cancer Research, Medical University of Vienna. He is a head of the Environmental Toxicology Group. SK published 255 articles in peer-reviewed journals (Scopus) as well as four text books on genetic toxicology. He has a Hirsch-index of 52, and was cited > 9,000 times. Currently SK is editor of the journal "Mutation Research – Genetic Toxicology" and co-editor of the journal of "Food and Chemical Toxicology".

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