Comments concerning the mutagenic/genotoxic properties of glyphosate

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Preface

This document describes our scientific opinion concerning the genotoxic properties of glyphosate. It is based on the evaluation of individual industrial reports, our own findings and on articles which were published in scientific journals.

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

Armen Nersesyan

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1. Introduction

This document concerns the question whether the classification of glyphosate (GLY) by health authorities (Bundesinstitut für Risikobewertung, BfR, and European Food Safety Authority, EFSA) as non-mutagenic is justified on the basis of the existing scientific evidence. The first part contains a general description of tests/methods which are used to assess genotoxic properties of chemicals, the second concerns the evaluation of industrial studies provided by EFSA which were performed by toxicological laboratories for producers. The third part contains a discussion concerning misinterpretations of the conclusions reached by health authorities and suggestions for further investigations which are needed to clarify the question if GLY causes damage of the genetic material. The last section contains some statements concerning the evidence of effects in humans.

At present genotoxicity studies are routinely conducted with new chemicals as is known that DNA damage leads to adverse health effects. Already in 1973 Ames [1] postulated that mutagenic chemicals cause cancer ("Mutagens are Carcinogens"). This hypothesis was confirmed in last decades by numerous investigations and it is known that damage of the genetic material is a hallmark of human cancer [2-4]. In addition, there is some evidence that DNA damage plays a role in neurodegenerative diseases and accelerates aging [5]. Damage of germ cells (sperm and eggs) leads to infertility and heritable diseases in the offsprings [6].

2. Genotoxicity testing of chemicals

Not all new chemicals can be tested in carcinogenicity studies as they are time consuming and very costly. Therefore, most of them are studied first in genotoxicity experiments; only when positive results are obtained, further studies with rodents are conducted. Important

pharmaceutical drugs and chemicals which are produced in large amounts and are released into the environment are tested a priori in long-tern carcinogenicity studies.

The mutagenic properties of GLY in rodents and the outcome of cancer studies are still controversially discussed [7, 8]. Therefore, the question if the compound causes damage of the genetic material is of high relevance as it may contribute substantially to the ongoing controversies concerning its carcinogenic properties. Positive results in genotoxicity experiments would strongly support the assumption that this chemical causes cancer.

Different genotoxicity tests were developed in the last decades which are used for the investigation and classification of chemicals. They comprise experimental models with bacterial indicator cells (bacterial DNA has a similar structure as that of higher organisms). Furthermore, cultured cells from vertebrates (including humans) as well as *in vivo* experiments with laboratory rodents are frequently performed. Human studies are not conducted to classify new chemicals but can provide valuable information about potential occupational and life style related effects.

In vivo experiments with laboratory rodents (mice and rats) are regarded in general as more reliable as *in vitro* experiments with cultivated cells and bacteria. The most widely *in vitro* test is at present the Salmonella/microsome assay (also commonly known as the “Ames test”) in which different bacterial strains are used to find out if chemicals cause mutations in specific genes (which encode for histidine auxotrophy). As a consequence of mutations in specific genes, cells become “histidine independent” and form colonies on Petri dishes (containing medium without histidine). In mammalian cells, chromosomal aberrations are often monitored, since they are difficult to score, a method was developed which detects chromosomal damage in form of so-called “micronuclei” which contain either entire chromosomes or chromosomal fragments. The most widely performed test with laboratory animals and new chemicals is the so-called “bone marrow micronucleus test” in which blood cells (polychromatic erythrocytes) are evaluated in

cells from the bone marrow of chemically treated mice and rats for induction of micronuclei which can be detected under the microscope.

The single cell gel electrophoresis assay (SCGE) is a relatively new procedure assay which is based on the measurement of DNA migration in an electric field. It reflects single and double strands breaks and apurinic sites of the DNA. These lesions lead to formation of "comet"-shaped images which can disappear due to repair processes, while chromosomal and gene mutations are stable and irreversible.

To control the correctness of routine genotoxicity tests, international guidelines were developed, they contain precise descriptions of quality criteria which must be fulfilled when chemicals are tested. The most important guidelines for European countries are those which were published by the Organization for Economic Co-operation and Development (OECD). Only when the quality criteria which are described in the guidelines are fulfilled, the results of genotoxicity tests can be regarded as reliable. The methods which are included in the OECD guideline have been standardized and were validated by international expert panels in regard their predictive value for the detection of carcinogens. In addition, several methods which are less standardized/validated are used in genetic toxicology, but no guidelines are available. These approaches can be used to obtain "supporting evidence" and may provide relevant information if a compound causes damage of the genetic material. Apart from the OECD guidelines also guidelines from other organizations are available, for example from the European Chemical Agency (ECHA). Also national mutation societies (for example, the United Kingdom Environmental Mutagen Society (UKEMS), the US Environmental Protection Agency (US EPA) and US Food and Drug Administration (US FDA) published guidelines for routine mutagenicity testing.

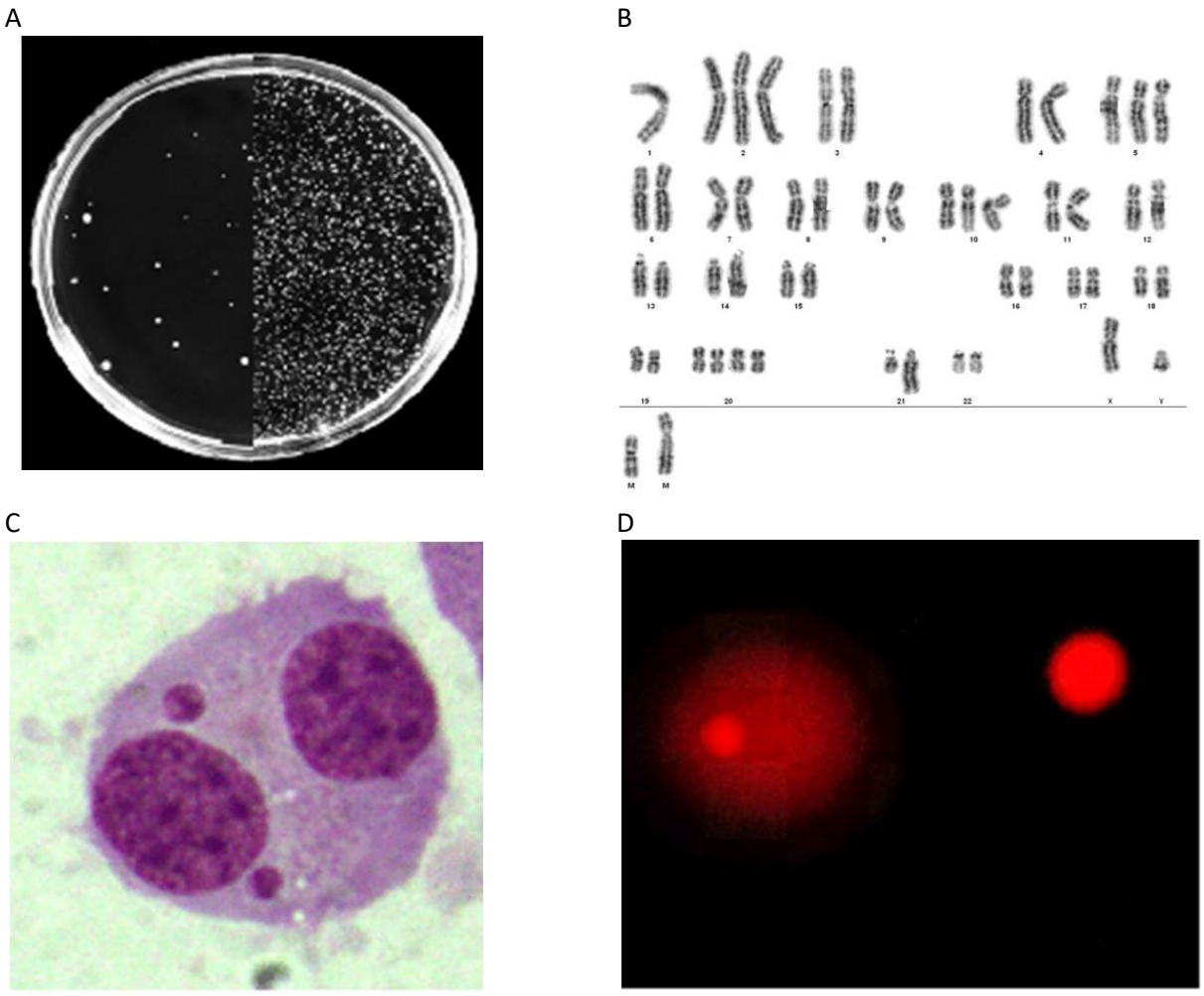


Fig. 1. Frequently used methods for routine testing of chemicals. Induction of mutations in bacteria leads to his^+ colonies in the "Salmonella/microsome test" (A). Induction of structural and numerical chromosomal aberrations can be monitored *in vivo* in bone marrow and in peripheral human lymphocytes (B). Micronuclei reflect broken or entire chromosome and can be tested in cell lines but also in lymphocytes in humans (C). The formation of comets by use of SCGE experiments reflects single- and double-strand breaks and apurinic sites (D). This test can be conducted with cells from a variety of inner organs and also with blood cells.

3. The EFSA files

In December 2019 the EFSA forwarded the results of industrial studies to the NGO “Sumofus”. The results of these investigations are the basis for the classification of the mutagenic properties of the herbicide by BfR and EFSA. Both agencies classified GLY as “non-mutagenic” while the International Agency for Research on Cancer (IARC) came to the conclusion that the herbicide is mutagenic.

In total, 53 documents were sent which were evaluated by S. Knasmueller and A. Nersesyan in regard to their scientific quality and compliance with the current OECD and other guidelines (e.g. from the UK EMS) and with the recommendations of international expert groups. Prof. S. Knasmueller is currently head of the Environmental Toxicology Group of the Institute of Cancer Research, Medical University of Vienna and Editor-in-Chief of the journal “Mutation Research - Genetic Toxicology and Environmental Mutagenesis”, which is focuses on studies concerning the genotoxic effects of chemicals. He is also co-editor of the journal “Food and Chemical Toxicology”, one of the most cited toxicological journal worldwide.

3.1. Individual studies

In total 54 studies were analyzed. They comprise the results of 24 bacterial tests (Ames test), 2 studies with the bacterial Rec-A test (one of them was not evaluated by BfR), 3 studies with so-called *Hprt* assay in mammalian cells, one DNA repair test in rat primary hepatocytes, 5 investigations with chromosomal aberration analyses in mammalian cells, 2 concerned dominant lethal mutations (DLM) tests with mice, 17 *in vivo* experiments with rodents (15 were micronucleus assays and 2 chromosomal aberrations analyses in bone marrow cells of mice).

Notably, the files contain also 2 studies which did not concern GLY (surfactants that are contained in GLY formulation). These studies are irrelevant for the assessment of properties of the herbicide itself.

Results of two studies were provided which concern formulations. In this case it can be not excluded that compounds other than GLY caused biologically relevant effects.

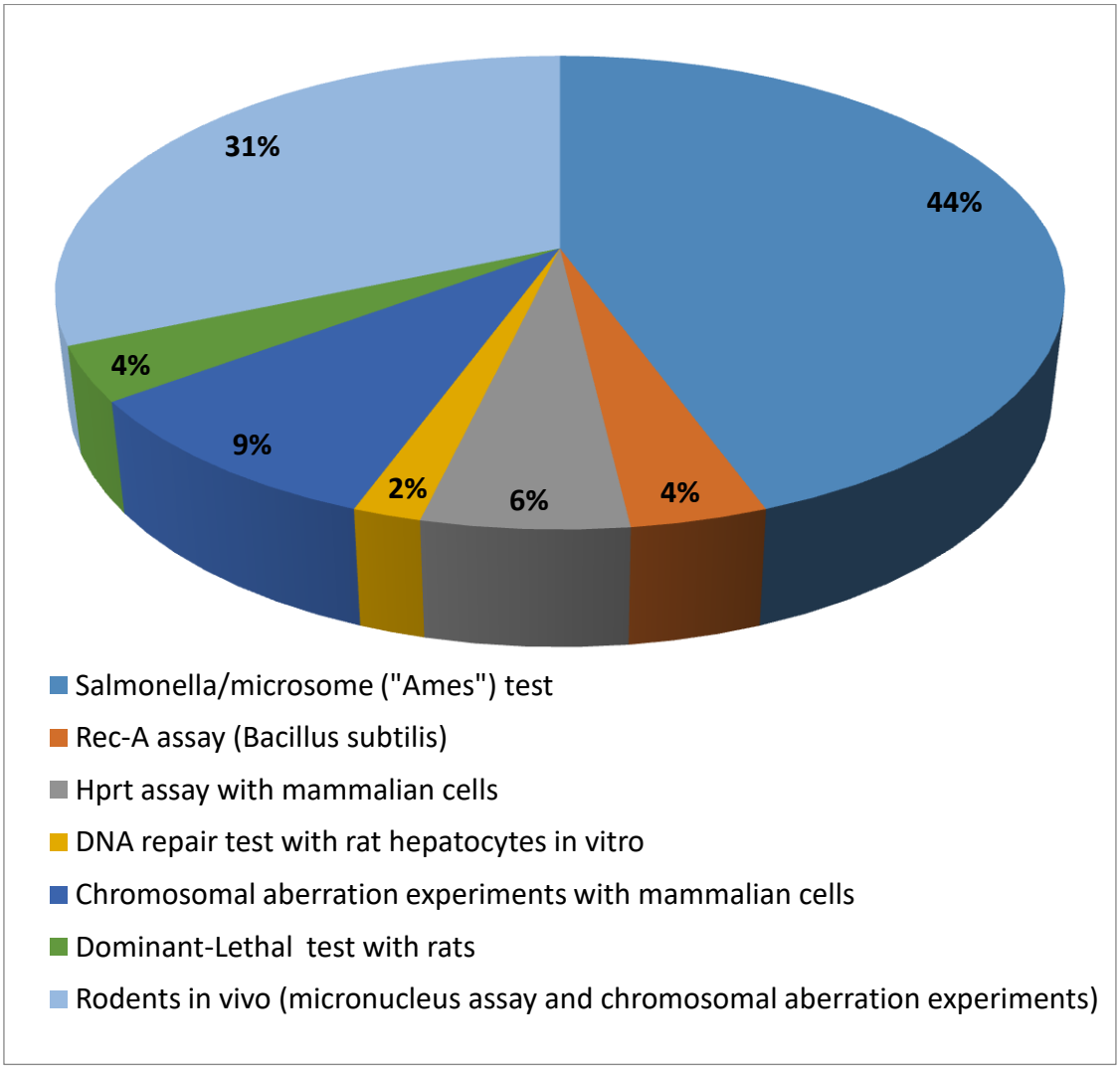


Fig 2. Numbers of assays described in the EFSA files. Hprt - hypoxanthine phosphorybosyl transferase

3.2. Quality criteria

It is mandatory that reports which are performed in toxicological test laboratories and are sent from the producers to health authorities have a sufficient scientific quality in order to ensure that they are reliable. Important general issues which are also mentioned in respective guidelines are 1) information concerning the purity of the test compounds; 2) adequate selection of the tested doses; 3) inclusion of repetition experiments (non-mandatory for all test procedures); 4) adequate background values, i.e. the number of spontaneous gene mutations or chromosomal aberrations in untreated cultures or in cells from untreated animals should be in a certain range; 5) inclusion of positive control chemicals which show that the experiment worked; 6) evaluation of the results of the studies with adequate statistical methods (non-mandatory for bacterial tests but mandatory for other procedures); 7) in animal experiments: justification of the route of administration. It is mentioned in many guidelines that a result in a specific test is only valid when the values are outside of historical control data (obtained with untreated cultures or animals). Therefore, the laboratories should also provide results of earlier investigations.

3.2.1. Evaluation of the quality of the Salmonella/microsome assays

The test is included in the OECD guidelines. In total 24 studies were forwarded to us. The Salmonella/microsome assay is one the oldest mutagenicity tests and was used for the testing of more than 15,000 individual chemicals. According to all international guidelines, the test has to be conducted at present with 5 different bacterial strains which detect different groups of genotoxic carcinogens, therefore, assays with a lower number of strains are not acceptable. At least 3 doses of a chemical should be tested, per dose 3 parallel plates should be used. Untreated (negative) and positive controls must be included in all individual experimental series. When a negative result is obtained, a further test has to be performed. If it is not performed, a justification

should be provided. All experiments are conducted with and without liver homogenate which mimics the metabolism of chemicals in the human body. Information about historical controls should be provided according to the current OECD guideline. Fig. 3. shows how Salmonella/microsome ("Ames test") is performed.

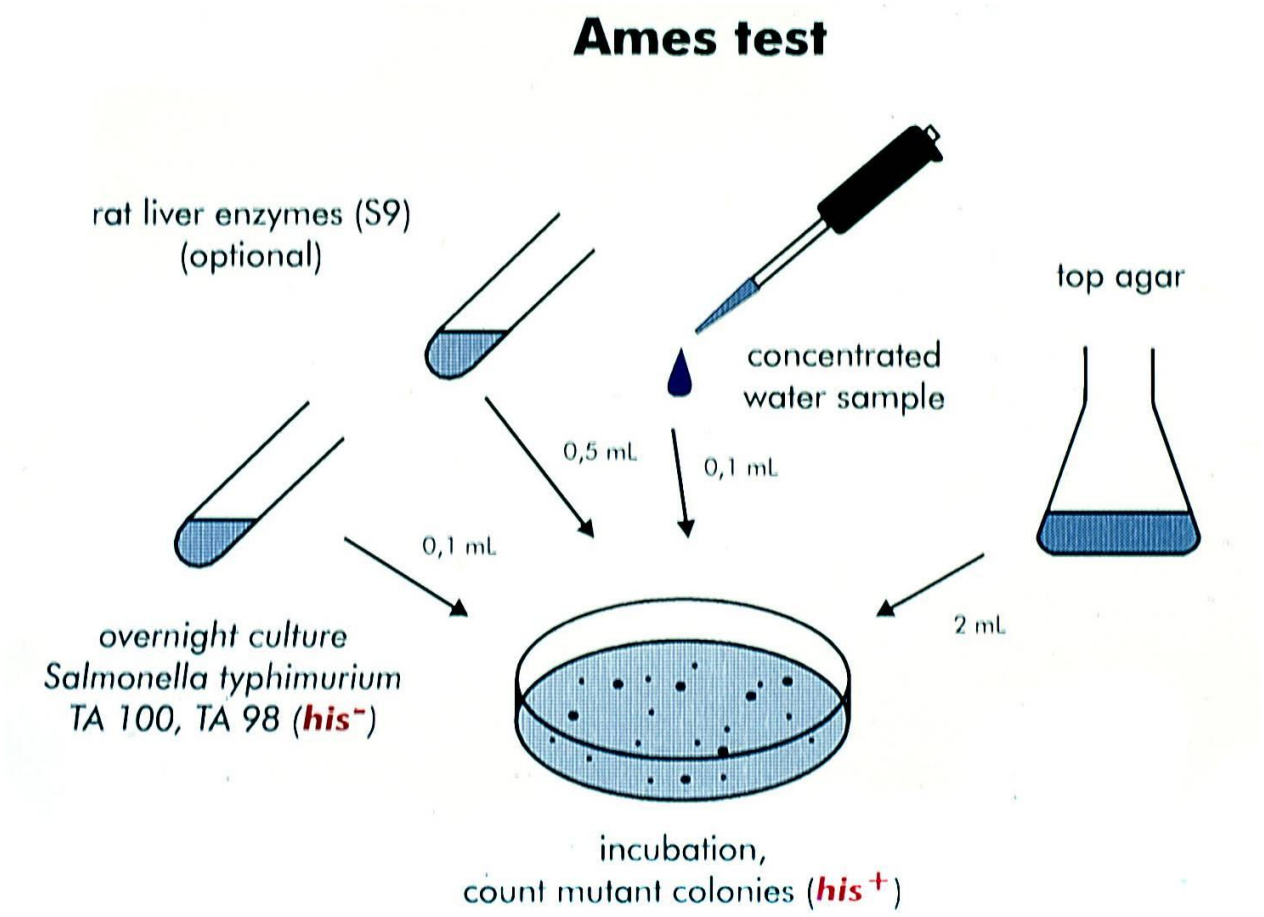


Fig. 3. Scheme of the Salmonella/microsome assay. Bacterial strains that need the amino acid histidine to grow are incubated with a test compound in presence and absence of liver homogenate which contains activating enzymes. If a compound induces mutations enabling the cell to produce histidine, they form colonies.

Our evaluation showed that 12 out of 24 studies have severe shortcomings; the results of these investigations are not reliable. Various reasons make the results inconclusive. For example, the number of strains **was not adequate (in 7 trials)**; inadequate positive controls were used in 1 report; inadequate negative controls are reported in 2 studies. Also the remaining studies are only in partial agreement with the current OECD and other guidelines. In some studies historical control data are not provided.

3.2.2. Evaluation of the "Rec-A" test with *Bacillus subtilis*

The test is not included in the OECD guidelines. This bacterial test is based on the comparison of the viability of two bacterial strains which differ in DNA repair capacity after treatment with test chemicals. When the strains are treated with acute toxic compounds, the survival of both strains will be equally reduced. If a compound causes DNA damage, the survival of the strains without intact repair will be lower as that of the wild type (repair proficient) strain. It is notable that the Rec-A test was only partly validated in regard to its usefulness for the detection of genotoxic carcinogens and is not included in the OECD guidelines.

Results of two tests were provided by the producers (one was not evaluated by BfR). Both of them have severe methodological shortcomings. One was performed without activation mix (liver enzyme homogenate) which is mandatory according to the standard protocol. In the second study it is unclear why only a relatively low dose was used.

Conclusions: the results are completely irrelevant for the classification of GLY.

3.2.3. Gene mutation tests with mammalian cells

The test is included in the OECD guidelines. The experiments are based on measurements of induction of gene mutations in mammalian cells which lead to resistance towards specific toxic

compounds (antimetabolites). As a consequence of mutations in specific genes the cells become resistant, they survive and form colonies (similar as in bacterial mutagenicity tests).

Results of three studies were provided and none of them contains completely reliable results: 1) in two investigations a moderate effect was seen with individual doses; results of statistical analyses are not provided; 2) historical control data (from earlier investigations concerning the effects of untreated cultures) are not described. Two studies were classified by us as partly reliable and one as inconclusive.

3.2.4. DNA repair test with liver cells

The test is not included in the OECD guidelines.

One study was provided. It is not acceptable from a methodological point of view as only one dose of the compound was tested and no statistical analyses are described. At present the method is outdated and not used in genetic toxicology.

3.2.5. Chromosomal aberration experiments with mammalian cells

The test is included in the OECD guidelines.

Five individual studies were provided by the industry. Two of them are completely irrelevant as the number of scored cells is too low in all of them, three are partly relevant. Three studies do also not provide historical control data.

3.2.6. Chromosomal aberration experiments with rodents

The test is included in the OECD guidelines.

Two studies were provided by the industry. The results are not relevant as the number of evaluated cells is in both cases insufficient (50 cells instead of requested 200). The studies have

also additionally other shortcomings (e.g. lack of acute toxicity experiments to identify suitable doses before the main experiment).

3.2.7. Micronucleus assays with rodent bone marrow cells

The test is included in the OECD guidelines. This test is the most widely used *in vivo* procedure with laboratory rodents for routine screening of new chemicals and it is based on the evaluation of the formation of extra-nuclear bodies (so-called micronuclei which contain chromosomal fragments and/or entire chromosomes). Micronuclei can be scored under a light microscope. All criteria to perform micronucleus experiments are clearly defined by different guidelines. It is mandatory to test at least 3 doses and use at least 5 animals per dose. Furthermore, it is mandatory to use a specific treatment schedule. Negative and positive controls have to be included and adequate statistical analyses have to be performed. The results of these studies are of high relevance for the classification of chemicals by the health authorities as their weight of evidence is regarded as higher as that of *in vitro* experiments (for example with bacteria and mammalian cells) [9].

Only two studies out of 15 are partly in agreement with the OECD guidelines (partly reliable), 13 of them are completely inconclusive. In one of these latter studies, no plausible ratio of polychromatic erythrocytes to normochromatic ones was found indicating a compromised health status of the animals (mice). In 6 studies only one dose was tested (not 3 as requested by the international guidelines). In 7 studies the compound was given intraperitoneally. This route of administration is largely irrelevant as in humans inhalation and oral uptake are the common exposure pathways. One study did not concern GLY, two studies are inconclusive due to several severe mistakes (for details see the full report).

3.2.8. Dominant lethal tests with mice

An OECD guideline is available for the dominant lethal test.

These experiments are very important as they detect mutations in germ cells (sperm) which cause reduced fertility. Male animals are treated with test chemicals and mated subsequently with virgin females.

An increase of dead implants per female in the treated group over the dead implants per female in the control group reflects chemically-induced post-implantation loss. The post-implantation loss is calculated by determining the ratios of dead to total implants in the treated groups compared to the ratio of dead to total implants in the control group. Preimplantation loss can be estimated by comparing *corpora lutea* counts minus total implants or the total implants per female in treated and control groups. Such a situation is indicative for induction of lethal mutations in the sperm cells.

Two studies were submitted. One study is completely irrelevant (no preliminary experiment was realized to define the highest suitable dose in the main experiment), no historical control data are provided, no statistical analyses were performed which are mandatory in the current guidelines. The second study was initially sent to us initially in truncated form, subsequently a complete report was sent. Notable, a moderate effects was seen in this study with certain doses. No historical control data are provided in this study.

4. Comparisons of the evaluation of the studies by the BfR (RAR) and of the evaluation of the studies by Prof. S. Knasmüller

The Renewal Assessment Report was prepared by the German Bundesinstitut für Risikobewertung (BfR), Umweltbundesamt (UBA) and Julius Kühl Institut (JKI) of Federal Research Center for Cultivated Plants between May 2012 and December 2013, was revised on

29th January 2015 and 31st March 2015 and was peer-reviewed afterwards by EFSA and the Member States authorities. It is a relevant document (4,322 pages) concerning the classification of the carcinogenic and mutagenic properties of glyphosate and the studies which were performed for producers are basis for the judgement of the genotoxic properties of the herbicide.

Ninety three pages of this document (pages 305 – 397) concern the genotoxic properties of glyphosate and 53 studies were analyzed; individual studies are mentioned and statements are provided by the authority in which were classified as "acceptable" (or valid), "not acceptable" or "supplementary". With one exception (recA-assay; Report No.: ET-78-241 of 1978; study No. 14) all studies, which were forwarded to SK by EFSA, are included in the RAR.

Table 1 gives an overview on the results of this evaluation and comparisons are shown with the results of the evaluation by SK.

Different reasons account for the discrepancies in the evaluation. The quality of the studies should be judged on the basis of criteria, which are defined in international guidelines for routine mutagenicity testing of chemicals that were valid at the time of publication of the final (revised) version of the RAR in 2015. Such guidelines were published by different organizations such as US EPA, US FDA, UK EMS, the Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), and by the OECD. For the European market, the OECD guidelines are the most relevant documents.

Many studies which were classified in the RAR as acceptable or valid are not in agreement with the criteria which were defined by the aforementioned organizations. Furthermore, the BfR accepted studies in which the methodology was outdated and/or which were not included at all in international guidelines. One assay (OECD #482, 1986; DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells in vitro) was removed from the guidelines in April 0f 2014 due to severe limitations but was included in the RAR evaluation.

Here are some examples for discrepancies between the BfR evaluations and our evaluation:

1) BfR/RAR assessed in total 6 *Salmonella*/microsome assays in which only 4 tester strains were used. All of them were classified as acceptable. Each of the bacterial strains detects specific modes of action and the strains are complementary in regard to the detection of mutagens. In 1975 several strains were available and it was recommended to use 4 of them for routine testing. Due to severe criticism from the scientific community, further strains were developed which detect oxidative damage. One of these strains (TA 102 or TA104 or alternatively an *E.coli* strain) has to be included in the standard battery according to all currently relevant guidelines (so the total number of strains has to be five). Alternatively also *E.coli* strains can be replaced by TA102 or TA104. The strains detect different groups of mutagens. It is assumed, that one of the important modes of action, by which glyphosate may cause DNA-damage and/or cancer (IARC, 2012), is the generation of reactive oxygen species which cause oxidative damage. Therefore, it is not acceptable when *Salmonella*/microsome tests are only performed with strains which are insensitive towards this type of damage. All studies which were performed with the 4 initial strains, are, therefore, inacceptable. In some bacterial tests (in total 2) the number of background revertant colonies in untreated cultures was not in normal range. This indicates that the genetic background has been changed and the results of such experiments cannot be regarded as acceptable.
2) The BfR/RAR included in its evaluation results of the so-called “recA-assay” with *Bacillus subtilis* and also a test concerning DNA-repair measurements with mammalian cells. Both methods are not included in the current international guidelines and are only partly validated.

a. The recA-assay (with bacteria) detects only compounds which activate a specific DNA-repair pathway. It was therefore never included in any international guideline. It is notable that several compounds, which cause DNA-damage do not induce this specific pathway, therefore, they can be not detected.

b. The DNA-repair assay with mammalian cells (DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells *in vitro*) was initially included in a OECD guideline (#482, 1986) which was removed in 2014, as the test was considered to be inadequately validated and outdated. It is nevertheless considered in the RAR document and classified as "acceptable".

3) Many studies were accepted by BfR/RAR in which the number of tested doses is inadequate and/or in which the number of evaluated cells was not sufficiently high. In some cases older OECD guidelines accepted lower cell numbers, but in 2014 new guidelines and recommendations were published for certain test systems (before the final version of the RAR came out). On the basis of these OECD guidelines the testing strategy of many glyphosate studies is inadequate and not acceptable. It is not acceptable that studies are classified as valid, when only half of the cells which are required to obtain reliable results were counted. Furthermore, it is also not acceptable that in some studies only one dose was tested (without justification) instead of 3 which are requested in the respective guidelines.

4) Two "Dominant Lethal Tests" were classified as acceptable by BfR/EFSA. The test detects mutations in male germ cells. Both studies do not meet the criteria defined by the OECD.

5. Does GLY cause DNA damage in multiple inner organs of rodents?

The classification of BfR/EFSA is predominantly based on the results of negative bacterial *in vitro* experiments (Salmonella/microsome assays) and of bone marrow micronucleus tests.

There is a general consensus that *in vivo* experiments with rodents are more relevant as *in vitro* studies with cultivated cells which do not reflect the metabolism of chemicals in the human body in an ideal way. Therefore, the health authorities (BfR and EFSA) assumed that the submitted experiments with rodents have more weight as finding from *in vitro* studies (for example, results of bacterial mutagenicity tests).

Negative results from micronucleus experiments with bone marrow cells of rodents were repeatedly found in most investigations. However, it cannot be excluded that the herbicide causes damage in inner organs other than the bone marrow. Experimental evidence for this assumption comes from several independent investigations.

i) In a number of *in vitro* investigations with liver-derived cells, clear positive results were obtained (for example, in the human-derived liver cell line HepG2 evidence for induction of DNA damage was observed in two studies [10, 11]). These cells are a stable line which reflects the metabolism of chemicals better than other currently used *in vitro* tests since the metabolic pathways are represented in a better way.

ii) Several investigations with rodents found positive effects of GLY in the liver [12, 13] and kidneys [14]. These findings were not taken into consideration by the health authorities. It is stated in reviews by authors that were paid by Monsanto that these investigations are methodologically incorrect. The main reason for this criticism was that acute toxicity was not monitored in these studies. It was argued that it cannot be excluded that cell killing may have led to false positive results and may have mimicked genotoxic activities of the compound. These statements were obviously

accepted by the health authorities and the respective investigations were not taken into consideration.

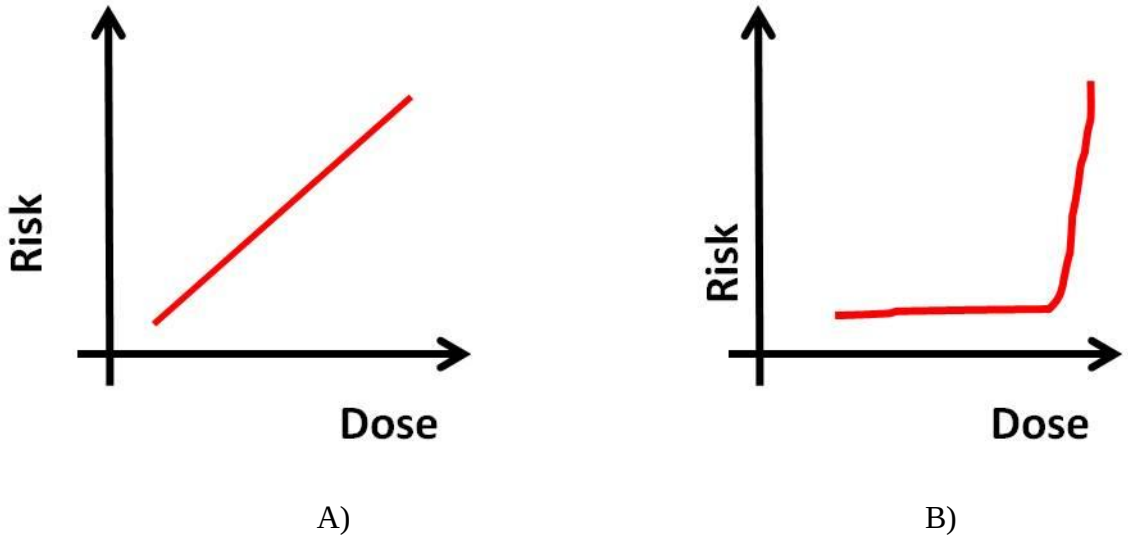
iii) Many studies with non-mammalian species (fish, amphibians, reptiles) reported positive results with GLY in different inner organs but the health authorities did not take these findings into consideration. Obviously, they anticipate that rodent data are more reliable and convincing.

iv) We performed recently a study in which we analyzed induction of DNA damage by GLY in multiple inner organs of mice. We found in single gel electrophoresis (or "comet", SCGE) assays which were conducted according to the OECD guidelines (see chapter 1) comet formation in testes, colon, liver and kidneys but not in bone marrow and brain of the animals. In contrast to earlier investigations, we monitored additionally acute toxic effects (in histopathological examinations of different organs) and the results show clearly that we can exclude that the positive findings of the genotoxicity measurements are due to cell death. Our results showed that the herbicide causes genotoxicity in the liver (which was also reported earlier by two less well controlled studies). We analyzed in our study also micronucleus formation in bone marrow as well as comet formation in this tissue and obtained consistently negative results (which confirm the results of earlier studies). Our new data indicate that the bone marrow is not a target organ for induction of DNA damage by GLY, but clearly show that other organs are affected which were not included in the evaluation by the health authorities. It is stated in a publication of an expert panel [9] concerning the weight of evidence of different study types which were used to evaluate the genotoxic properties of GLY that bacterial tests and micronucleus assays with bone marrow have more relevance as SCGE assays (which we performed with different inner

organs). Part of the experts of this panel were paid by the industry (see Conflict of Interest Statements). However, newer studies [15, 16] showed that the sensitivity of the method for the detection of genotoxic carcinogens which we used for the detection of genotoxicity (the single cell gel electrophoresis, SCGE technique) is higher than that of micronucleus assays with bone marrow on which the classification of herbicide by EFSA and BfR is mainly based).

Our findings indicate that the classification of GLY by IARC as a mutagen is correct, and that the classification by BfR/EFSA has to be reconsidered.

v) It is notable that not all carcinogenic chemicals cause damage of the genetic material; some of them can also act via different other modes of action. The classification of carcinogens as genotoxic or mutagenic is of high relevance for risk assessment as it is known that very low or no threshold doses exist for genotoxic carcinogens. In other words, also low doses of such compound pose a risk and the effects are in general additive and linear over a broad range. This is relevant when millions of humans are exposed to low doses of a chemical. A typical example for a genotoxic carcinogenic factor is ionizing radiation: also long-term exposure to relatively low doses is dangerous for humans. On the contrary, threshold doses (below which no adverse effects take place can be defined for non-genotoxic carcinogens; typical example: hormones).



A) B)

Fig. 4. Dose-response characteristics of carcinogens; A) Genotoxic carcinogens are dangerous even when humans are exposed to low doses (no or only very low threshold doses); B) for non-genotoxic carcinogens threshold doses can be defined.

6. Potential genotoxic effects of GLY in occupationally exposed humans

It is important to note that the risks of humans that are exposed to carcinogens depend strongly on the dose. In the case of GLY the highest exposure occurs probably in agricultural workers and in individuals who are exposed at production sites.

In this context it is notable that none of the animal studies which were submitted by the industry were performed with inhalative exposure. It is well known that the route of exposure to toxins has a substantial impact on the outcome of toxicological studies.

It is possible to study damage of the genetic material in environmental and occupationally exposed humans with different methods. It is known that certain forms of damage, i.e. induction of chromosomal aberrations and micronuclei in lymphocytes are reliable biomarkers for increased cancer risks of humans [17, 18]. Also micronucleus induction in cells upper respiratory

tract (mouth and nose) may be indicative for increased cancer risks, but this approach is less well validated. This also true for formation of comets (monitored in SCGE assays; see previous chapter) which can be monitored in electrophoresis experiments with different cell types.

In 2015 Kier [19] evaluated for Monsanto the results of 19 genotoxicity studies with humans in which induction of DNA damage was investigated in agricultural workers and environmentally exposed groups. Notably, not a single study was performed with humans who are exclusively exposed to GLY at production sites. The study groups for which data were available are mainly farmers; furthermore, a few studies were performed with individuals living in areas where GLY formulations are sprayed. Thirteen out of 19 studies reported positive results and no newer investigations were performed in the last 5 years.

Overall, the results of the human studies are inconclusive since the majority of workers were exposed additionally to a number of other chemicals. However, it is clear that the high number of investigations in which positive results were obtained cannot be ignored and used as an argument that GLY is “not mutagenic”! There is a clear need to conduct well-controlled studies with humans who are solely exposed to GLY formulations or to pure GLY. My research group published in 2012 an interesting study [20] in a leading toxicological journal in which we investigated the effects of GLY and Roundup in a human buccal-derived cell line (*in vitro*). We found clear induction of DNA damage in the cells with very high dilutions which are sprayed on the fields indicating that direct inhalative exposure of cells causes adverse effects. In our opinion this indicates an urgent need to conduct meaningful human investigations since millions of workers and home gardeners are exposed to the herbicide.

6. Summary

6.1. Summary I

In total, 54 individual industrial studies were forwarded from EFSA which concern the mutagenic properties of GLY. All of them except one were evaluated by the BfR. Most of them are not in agreement with the current OECD and other guidelines for routine testing of chemicals which define criteria which have to be fulfilled to obtain reliable results. Out of 24 mutagenicity tests with bacteria 11 have severe shortcomings and can be not used as the findings are irrelevant. The remaining 13 reports have moderate shortcomings and the findings are consistently negative. Only one study fulfills all quality criteria of the OECD.

Also two of *in vitro* chromosomal aberration tests with mammalian cells are not in agreement with the guidelines (three are partly reliable out of 5 studies).

Two studies with rodents *in vivo* (chromosomal aberration test) are not in agreement with the OECD guideline and the results of them are not reliable.

Of particular relevance are experiments with mice as it is generally assumed that the weight of evidence of results from animal experiments is higher as that of *in vitro* experiments with cell lines and bacteria. Fifteen micronucleus assays (which reflect chromosomal aberrations) with bone marrow cells of mice are contained in the EFSA files, out of these only two contain partly relevant data, all others can be not accepted due to methodological shortcomings.

Two important reports concerning induction of mutations in sperm cells of mice (dominant lethal test) are also inconclusive due to methodological mistakes and an outdated statistical analyses.

6.2. Summary II

The classification of BfR/EFSA is predominantly based on results of *in vitro* bacterial mutagenicity tests and *in vivo* micronucleus experiments with bone marrow cells. The results of the latter experiments in the reports which were provided by the industry are consistently negative and are highly relevant for the classification of GLY as non-mutagenic by the agencies. The evaluation of the literature provided evidence that cells from organs other than bone marrow are targets for induction of DNA damage by GLY. These findings were not taken into consideration by the health authorities. (1) *In vitro* experiments with cultured liver-derived cells indicate that GLY causes damage in these cells. (2) Results of several studies which were (in contrast to the industrial reports) published in peer reviewed scientific journal showed that GLY causes DNA damage in the liver of rodents (one of them found also damage in the kidneys). The test system which was used in two investigations was the single cell gel eletrophoresis assay which is based on the measurement of migration of the DNA in an electric field and is part of the OECD guidelines. Studies which found evidence for DNA damage in organs other than the bone marrow, were criticized by Monsanto paid experts who stated that the positive results may be a misinterpretation due to acute toxic effects. Indeed, the aforementioned studies do not contain information about acute toxicity. These studies were not taken into consideration in the classification of BfR/EFSA, which also ignored positive results in several inner organs of non-rodents (reptiles, fish, amphibians).

A recent study by the authors of this document showed that GLY does not induce micronuclei and DNA damage (in SCGE experiments) in bone marrow cells but indicated that it causes DNA damage in the liver, kidneys, testes and colon. The study was realized in strict agreement with the current OECD guideline. To exclude acute toxicity which may lead to false-

positive results, additional histopathological analyses were conducted which yielded consistently negative results.

Taken together, there is growing evidence that GLY causes damage of the genetic material in multiple inner organs and that the bone marrow is not a target for induction of DNA damage by the herbicide. A re-evaluation of the genotoxicity of GLY is strongly warranted in the light of this situation.

6.3. Summary III

The inhalation of GLY and its formulations by agricultural workers and of workers at GLY factories is probably the most relevant form of exposure of humans. In the last decade reliable genotoxicity tests were developed which enable the prediction of human cancer risks caused by occupational exposures. It was stated by the IARC that exposure to herbicides and pesticides poses a cancer risk for humans [21]. Currently available results of genototoxicity in humans which were exposed to GLY and to its formulations do not allow to draw firm conclusions since in the majority of studies the participants were exposed additionally to multiple other chemicals. No studies were performed with workers from production sites (according to our knowledge). Furthermore, no reliable inhalation studies have been conducted with laboratory rodents. Results of *in vitro* experiments with human revived cells from the respiratory tract [20] indicated that extremely low dilution of GLY and Roundup cause damage of the genetic material which is a key mechanism leading to induction of cancer in humans [22]. Due to the fact that millions of agricultural workers and home gardeners are inhalatively exposed to GLY, meaningful and well-controlled human studies are warranted.

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Table 1. Evaluation of the results of industrial studies on genotoxicity of glyphosate by RAR and S. Knasmueller (SK)

Study type	BfR/RAR assessment				SK assessment			
	Acceptable/ Valid	Supplementary	Limited value	Not acceptable	Reliable	Partly reliable	Not reliable	Not in OECD
All mutagenicity tests (n=53)	**45**	**5 (2 also are considered as acceptable)**	**2**	**3**	**2**	**17**	**35***	**2**
Bacterial mutagenicity tests (n=24)	**22**	**2 (also considered as acceptable; #14 and #21)**	**1**	**1**	**2**	**10**	**12**	**0**
RecA assay (n=1)	**0**	**1**	**0**	**0**	**0**	**0**	**2***	**1**
Gene mutations in mammalian cells *in vitro* (n=3)	**3**	**0**	**0**	**0**	**0**	**2**	**1**	**0**
DNA repair assay (n=1)	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**1**
CA *in vitro* (n=5)	**4**	**1**	**0**	**0**	**0**	**3**	**2**	**0**
CA *in vivo* (n=2)	**2**	**0**	**0**	**0**	**0**	**0**	**2**	**0**
MN *in vivo* (n=15)	**11**	**1**	**1**	**2**	**0**	**2**	**13**	**0**
DLM assay (n=2)	**2**	**0**	**0**	**0**	**0**	**0**	**2**	**0**

*one of RecA studies was not evaluated by BfR