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French agency for food, environmental and occupational health & safety

Investigate, evaluate, protect

Study plan on the carcinogenic potential of glyphosate

Anses Opinion
Collective expert appraisal report

March 2019 - Scientific Edition

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ANSES Opinion
Request No 2018-SA-0078

The Director General

Maisons-Alfort, 27 March 2019

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on "study plan on the carcinogenic potential of glyphosate"

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES's public health mission involves ensuring environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with the necessary information concerning these risks as well as the requisite expertise and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are published on its website. This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 27 March 2019 shall prevail.

On 28 March 2018, ANSES received a formal request from the Minister for Ecological and Inclusive Transition, the Minister for Solidarity and Health and the Minister for Agriculture and Food to undertake the following expert appraisal: study plan on the carcinogenic potential of glyphosate (Request No 2018-SA-0078).

1. BACKGROUND AND PURPOSE OF THE REQUEST

Commission Implementing Regulation (EU) 2017/2324 of 12 December 2017 renewed the approval of the active substance glyphosate for a five-year period.

However, differing conclusions have been reported regarding the assessment of this substance's carcinogenicity. On the one hand, the IARC (2015) concluded that glyphosate is probably carcinogenic to humans, and on the other hand, EFSA (2015), JMPR (2016), ECHA (2017) and the US EPA (2017) concluded that glyphosate is not carcinogenic. The ministers requested that a toxicological study be conducted to improve knowledge of the hazards associated with glyphosate.

The goals were to:

- Identify relevant objectives for one or more studies such as that or those described in this formal request;
- Examine the feasibility of undertaking a study compliant with the regulations on animal testing and rules of ethics in general;

- Propose one or more relevant study plans that could feasibly be implemented to meet the identified objectives.

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)".

The collective expert appraisal on study plan was undertaken by the Emergency Collective Expert Appraisal Group (GECU) on "Study plan to clarify glyphosate carcinogenic potential" between September 2018 and February 2019. This group met on 21 September, 22 October and 10 December 2018.

The work of the GECU was accepted by the Expert Committee (CES) on "Plant protection products: chemical substances and preparations" at its meeting on 19 February 2019.

ANSES analyses interests declared by experts before they are appointed and throughout their work in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals.
The experts' declarations of interests are made public via the ANSES website (www.anses.fr).

3. ANALYSIS AND CONCLUSIONS OF THE CES AND GECU

The GECU referred to the available assessments on the carcinogenic potential of glyphosate as well as to data from the literature to propose an integrated approach aiming to provide additional knowledge in this area, explore potential carcinogenic mechanisms of action and determine their relevance to humans.

The GECU did not choose to recommend a carcinogenicity study in rodents since numerous studies of this type are already available. It thus favoured a mechanistic approach that is expected to shed light on the results obtained in the studies already available and the various results observed, while limiting the use of laboratory animals and complying with rules of ethics. Indeed, the majority of the recommended studies are *in vitro* studies, and when an *in vivo* study is deemed necessary and recommended, the protocol states that as much information as possible should be collected from the same animal.

This integrated approach based on numerous parameters considered in various studies is particularly relevant, as it should enable a distinction to be made between genotoxic effects and epigenetic effects involved in carcinogenesis. This should make it possible to identify mechanisms of action and determine their relevance to humans.

Since endocrine disruption is considered as a possible mechanism of action in carcinogenesis in endocrine organs, the GECU is reporting on the EFSA (2015), US EPA (2015) and JMPR (2016) assessments, which concluded, based on the analysed data, that glyphosate has no endocrine-disrupting properties.

In order to determine whether glyphosate is likely to have effects that could be related to cellular stress, *in vitro* tests specifically focusing on cellular behaviour, oxidative stress and cellular differentiation will need to be conducted.

Moreover, potential longer-term effects (oxidative stress, inflammation, differentiation, cell death, induction of repair enzymes) will also need to be explored *in vitro*. Glyphosate's presumed toxic mechanisms of action involved in cellular responses will be examined through transcriptome and epigenome analyses (respectively analysing the expression of genes and their potential epigenetic modifications). The tests recommended by the GECU are described in detail in the report found in Annex 3. Their results will enable the other recommended studies (see below) to be better interpreted and will provide explanations for any differences in results observed between certain studies.

In light of the data currently available, the level of evidence regarding the genotoxicity of glyphosate in animals can be considered as relatively limited. It can be noted that almost all of the genotoxicity tests conducted *in vivo* have led to statistically and/or biologically non-significant results. Nonetheless, no *in vivo* comet assays are available, and yet when they have been conducted *in vitro,* comet assays have led to statistically significant results in various cell models. This biological endpoint, which enables DNA base alterations (DNA fragmentation) to be assessed, can therefore be considered as the most sensitive for demonstrating the potential genotoxicity of glyphosate. It would therefore be beneficial to combine an *in vivo* comet assay, in the stomach, duodenum/jejunum, liver, kidneys and pancreas, with a micronucleus assay in order to clarify the genotoxic potential of glyphosate. Details concerning this assay are described in the report in Annex 3.

Moreover, to reduce uncertainties as to the carcinogenic potential of glyphosate while complying with the 3R rule[1] for animal testing, the implementation of an *in vitro* cell transformation assay (CTA) combined with the transformics method would enable modes and mechanisms of carcinogenic action to be identified. Indeed, based on experimental evidence demonstrating that the cellular and molecular processes involved in *in vitro* cell transformation resemble those responsible for *in vivo* carcinogenesis and are the result of a comprehensive cellular response to direct or indirect DNA damage, the CTA has been proposed as a possible alternative to carcinogenicity studies conducted in animals. This multi-stage process models some stages of *in vivo* carcinogenesis. However, the CTA cannot be used as a stand-alone test to predict carcinogenesis and it is necessary to generate mechanistic data in parallel. Thus, in order to improve the use of the CTA in the integrated testing strategy for carcinogenesis, this assay should be combined with the transformics method, which uses transcriptomics (the study and analysis of the transcriptome, i.e. all of the messenger RNA (mRNA) from genome transcription, enabling active genes to be identified in particular) to demonstrate key molecular events leading to *in vitro* malignant transformation. These tests are described in detail in the report in Annex 3. If negative results are obtained with the CTA, an initiation-promotion assay should also be undertaken as indicated in the annexed report.

The results of the recommended studies should be available within 18 months of their initiation.

Genotoxicity studies must be conducted in good laboratory practice (GLP)-compliant conditions. For other tests, if they are not conducted in accordance with GLP, the traceability of the experimental procedures and results (raw data, standardised operating procedures, etc.) should be guaranteed.

[1] Reduce the number of animals used for testing, Refine the methodologies used, and Replace animal models.

In terms of quality, since glyphosate should be formulated in an appropriate vehicle/solvent and then diluted in the same vehicle, the homogeneity, concentration and stability of glyphosate in the vehicle (Principles of Good Laboratory Practice, OECD 1997, § 6.2 Characterisation) should be determined.

Similarly, in *in vivo* studies, since the exposure of target tissues should be verified, the concentration of glyphosate in biological samples (e.g. plasma) should be determined to demonstrate systemic exposure. This means that analytical methods (including validation parameters) should be developed (for the solvent/vehicle and biological matrices) and validated.

Thus, with regards to analysis and bioanalysis, the GECU recommends that a single laboratory (preferably GLP-compliant) take charge of the determination of glyphosate concentrations in samples used in *in vitro* and *in vivo* studies and in biological matrices used in *in vivo* studies. This enables improved comparison of the various studies' results since the same validated analytical methods and the same parameters for the samples (storage temperature, volume) will have been used.

Lastly, the GECU recommends entrusting the implementation of all of the studies to a consortium of competent laboratories in order to centralise information (in particular, the same concentrations should be tested in various studies), facilitate exchanges and adopt a comprehensive approach when interpreting the various tests' results.

4. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety endorses the recommendations of the GECU on "Study plan to clarify glyphosate carcinogenic potential" regarding studies to be implemented to clarify the carcinogenic potential of glyphosate. These recommendations involve an integrated approach that would shed light on glyphosate's possible mechanisms of carcinogenic action and enable their relevance to humans to be assessed.

The majority of the recommended studies are *in vitro* studies, limiting the use of laboratory animals. When an *in vivo* study is deemed necessary and recommended, the protocol states that as much information as possible should be collected from the same animal.

The GECU also recommends conducting GLP-compliant genotoxicity tests and ensuring the traceability of results and protocols for all studies. Lastly, the implementation of these studies should be entrusted to a consortium, which would facilitate the overall interpretation of all of the results.

ANSES recommends conducting the following tests[2], described in detail in the report given in Annex 3:

- *In vitro* tests to study cellular stress following exposure to glyphosate, identify the molecular pathways involved in the cellular response and assess the consistency and biological relevance of the generated data. These results could enable the other recommended tests to be better interpreted and provide explanations for the conflicting results observed in the literature.

[2] Based on the duration of the experimental phase of the tests and the preparation of study reports, it is estimated that these reports could be finalised within 18 months of the tests' initiation.

- An *in vivo* comet assay in the stomach, intestines, liver, kidneys and pancreas of rats and mice combined with a micronucleus assay, in order to clarify the genotoxic potential of glyphosate, in addition to the studies already available and those conducted by the NTP.
- A cell transformation assay combined with the transformics method, enabling glyphosate's potential carcinogenic modes and mechanisms of action to be identified *in vitro*.

Moreover, the Agency is continuing its work by mobilising its experts for the analysis of recently published epidemiological studies on the link between cancer and glyphosate, as well as studies on the endocrine-disrupting potential of this substance.

Dr Roger Genet

KEYWORDS

Glyphosate, cancérogénicité, protocole d'étude, génotoxicité, test de transformation cellulaire, tests *in vitro*

Glyphosate, carcinogenicity, study plan, genotoxicity, cell transformation assay, *in vitro* tests

ANNEX 1

Presentation of the participants

PREAMBLE: The expert members of the Expert Committees and Working Groups or designated rapporteurs are all appointed in a personal capacity, *intuitu personae*, and do not represent their parent organisation.

EMERGENCY COLLECTIVE EXPERT APPRAISAL GROUP (GECU) ON "STUDY PLAN TO CLARIFY GLYPHOSATE CARCINOGENIC POTENTIAL"

Chair

Mr Fabrice NESSLANY – Department head, Institut Pasteur in Lille (France) – Toxicology, genotoxicology

Members

Mr Robert BAROUKI – Professor, Paris Descartes University, Necker Hospital (France) – Toxicology, biochemistry

Ms Sylvie CHEVILLARD – Department head, Alternative Energies and Atomic Energy Commission (France) – Experimental carcinogenesis

Ms Annamaria COLACCI – Director of the Centre for Environmental Toxicology and Risk Assessment and Centre for Environment of the Health, Agency for Prevention, Environment and Energy (ARPAE) (Italy) – Toxicology, Cell transformation

Ms Christiane VLEMINCKX – Head of the Department of Risk and Health Impact Assessment, Sciensano (Belgium) – Toxicology, Genotoxicity, Carcinogenesis

……

EXPERT COMMITTEE

The work covered in this report was monitored and adopted by the following Expert Committee (CES):

CES on "Plant protection products: chemical substances and preparations" – 19/02/2019

Chair

Mr Eric THYBAUD – Head of division (French National Institute for Industrial Environment and Risks). Speciality: Ecotoxicology

Vice-Chair

Mr Christian GAUVRIT – Retired from the French National Institute for Agronomic Research. Specialities: Herbicides - Adjuvants - Coformulants - Agricultural practices - Resistance - Efficacy

Members

Mr Fabrice NESSLANY – Department head (Institut Pasteur in Lille). Specialties: Toxicology – Genotoxicity

Ms Jeanne STADLER – Toxicology Consultant, Retired from the Pfizer Research Centre. Speciality: Reproductive toxicology

Ms Sonia GRIMBUHLER – Researcher (Research Institute of Science and Technology for Environment and Agriculture). Specialities: Assessment of farm worker exposure - Agricultural machinery - Field measurement

Mr Jean-Ulrich MULLOT – Military Pharmacist (Military Health Service). Specialities: Toxicology, exposure and exposure metrology

Ms Brigitte FREROT – Research Engineer (French National Institute for Agronomic Research). Specialities: Physico-chemistry - Pheromones - Pheromone analysis

Ms Marie-France CORIO-COSTET – Research Director (French National Institute for Agronomic Research). Specialities: Efficacy - Fungicides - Vineyards - Resistance - Plant defence stimulators (PDSs)

Mr François LAURENT – Research Manager (French National Institute for Agronomic Research). Specialities: PPP metabolism - Soil - Plants - Animals - Pesticides - Fate of pollutants

Mr Jean-Pierre CUGIER – Retired from the Ministry of Agriculture, Senior Scientific Officer (European Food Safety Authority) until 30/09/2016. Specialities: Residues and food safety

Mr Marc GALLIEN – Project Officer (MSA). Specialities: Prevention - Chemical protection of farm workers

Ms Laure MAMY – Research Engineer (French National Institute for Agronomic Research). Specialities: Pesticides - Fate - Environment - Modelling

Mr Maurice MILLET – University Professor (University of Strasbourg). Specialities: Analytical chemistry - Physico-chemistry - Analysis (water, soil, air)

Mr Philippe BERNY – Teacher – Researcher (VetAgro Sup). Speciality: Ecotoxicology: birds and mammals

Ms Annick VENANT – Retired from ANSES. Specialities: Physico-chemistry - Regulations - Plant protection products - Analytical methods - Specifications

ANNEX 2

Request letter 2018-SA-0078

COURRIER ARRIVE
03 AVR. 2018
DIRECTION GENERALE

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

Ministère de la Transition écologique et solidaire

Ministère des Solidarités et de la Santé

Ministère de l'Agriculture et de l'Alimentation

2018 -SA- 0078

Paris, le 28 MARS 2018

Les ministres

A

Monsieur le Directeur général de l'Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail

Objet : Saisine de l'ANSES sur le cahier des charges d'une étude sur le potentiel cancérogène du glyphosate.

Le règlement d'exécution UE 2017/2324 du 12 décembre 2017 renouvelle l'approbation de la substance active phytopharmaceutique glyphosate pour une période de 5 ans.

Des incertitudes persistent néanmoins sur cette substance, en raison notamment des conclusions divergentes sur sa cancérogénicité. D'une part le CIRC (2015) a conclu à la cancérogénicité probable de la substance, et d'autre part l'EFSA (2015), le JMPR (2016), l'ECHA (2017) et l'US EPA (2017) ont conclu à l'absence de caractère cancérogène du glyphosate. En dépit des échanges entre experts des différentes agences, il n'a pas été possible pour l'instant d'établir un consensus sur l'origine de cette divergence.

Dans ce contexte, des parties prenantes ont mis en cause la nature et l'étendue des données prises en compte par les instances d'expertise, ainsi que l'indépendance de ces expertises par rapport aux porteurs d'intérêt.

C'est pourquoi il apparaît nécessaire qu'une étude toxicologique permettant d'améliorer les connaissances sur les caractères de danger du glyphosate, et en particulier sur sa cancérogénicité, puisse être menée en toute indépendance, grâce à un financement public dans ce cas précis.

Nous souhaitons que l'ANSES établisse le cahier des charges de cette étude, au regard d'une part des principales incertitudes manifestées sur la toxicité de la substance, et d'autre part des signaux qui, dans les travaux déjà réalisés et au regard de l'analyse qu'en a fait l'ANSES, demanderaient à être confirmés ou infirmés.

.../...

Vous proposerez un protocole d'étude fondé sur des lignes directrices adoptées au niveau européen ou international. Vous ferez également des propositions sur les modalités de mise en œuvre et de pilotage de ces travaux expérimentaux, en attachant une attention particulière au respect de la réglementation sur l'expérimentation animale et des règles éthiques en général. Vous préciserez le calendrier prévisionnel jusqu'à la remise du rapport final.

Vous voudrez bien nous adresser le cahier des charges de l'étude dans un délai de 6 mois.

Le ministre de la Transition écologique et solidaire	La ministre des Solidarités et de la Santé	Le ministre de l'Agriculture et de l'Alimentation
Nicolas HULOT	**Agnès BUZYN**	**Stéphane TRAVERT**

LETTER RECEIVED
03 APRIL 2018
DIRECTORATE GENERAL

Ministry for Ecological and Inclusive Transition

Ministry for Solidarity and Health

Ministry for Agriculture and Food

Paris, 28 March 2018

2018-SA-0078

The ministers
TO
The Director General of the French Agency for Food, Environmental and Occupational Health & Safety

Subject: ANSES request on a study plan on the carcinogenic potential of glyphosate

Commission Implementing Regulation (EU) 2017/2324 of 12 December 2017 renewed the approval of the phytopharmaceutical active substance glyphosate for a five-year period.

However, some uncertainties still remain with regard to this substance, due in particular to differing conclusions as to its carcinogenicity. On the one hand, the IARC (2015) concluded that glyphosate is probably carcinogenic, and on the other hand, EFSA (2015), JMPR (2016), ECHA (2017) and the US EPA (2017) concluded that it is not carcinogenic. Despite exchanges between experts from the various agencies, it has not been possible to reach a consensus regarding the origin of this difference of opinion.

In this context, stakeholders have called into question the nature and scope of the data taken into account by the expert appraisal agencies, as well as the independence of these expert appraisals in relation to the interested parties.

This is why it appears necessary to undertake an independent and publicly funded toxicological study to improve knowledge on the hazards associated with glyphosate.

We would like for ANSES to prepare a study plan for this study, considering on the one hand the main uncertainties expressed as to the toxicity of this substance, and on the other hand information that will need to be confirmed or disproven based on studies already undertaken and in light of ANSES's analysis.

You will propose a study protocol based on guidelines adopted in Europe or internationally. You will also make proposals regarding the implementation and steering of this experimental work, paying special attention to compliance with the regulations on animal testing and rules of ethics in general. You will provide a provisional timetable up to the delivery of the final report.

We would like for you to send us the study specifications within a six-month period.

The Minister for Ecological and Inclusive Transition	The Minister for Solidarity and Health	The Minister for Agriculture and Food
Nicolas HULOT	Agnès BUZYN	Stéphane TRAVERT

ANNEX 3

Report by the GECU on “Study plan to clarify glyphosate carcinogenic potential”

Study plan to clarify glyphosate carcinogenic potential

Request No 2018-SA-0078

Collective expert appraisal
REPORT

Expert Committee (CES) on "Phytopharmaceuticals: substances and products"

Working Group on "Study plan to clarify glyphosate carcinogenic potential"

February 2019

Key words

Glyphosate, carcinogenicity, study plan, genotoxicity, cell transformation assay, *in vitro* tests

Report: February 2019

Presentation of participants

PREAMBLE: The expert members of the Expert Committees and Working Groups or designated rapporteurs are all appointed in a personal capacity, *intuitu personae*, and do not represent their parent organisations.

WORKING GROUP

The work carried out as part of this report was performed by the following Working Group:

- "Study plan to clarify glyphosate carcinogenic potential"

Chairman

Mr. Fabrice NESSLANY – Head of department, Institut Pasteur de Lille (France) – Toxicology, Genotoxicity

Members

Mr Robert BAROUKI – Professor, Paris Descartes University, Necker Hospital (France) – Toxicology, Biochemistry

Ms Sylvie CHEVILLARD – Head of department, Commissariat à l'Energie Atomique (France) – Experimental carcinogenesis

Ms Annamaria COLACCI – Director of the Center for Environmental Health and Prevention Agency for Prevention, Environment and Energy (Arpae) (Italy) – Toxicology, Cell transformation

Ms Christiane VLEMINCKX – Head of Service Risk and Health Impact Assessment, Sciensano (Belgium) – Toxicology, Genotoxicity, Carcinogenicity

EXPERT COMMITTEE (CES)

The work carried out as part of this report was monitored and adopted by the following CES:

- "Phytopharmaceuticals: substances and products" – 19/02/2019

Chairman

Mr Eric THYBAUD – Head of division (Institut national de l'environnement industriel et des risques) - Ecotoxicology

Vice-chairman

Mr Christian GAUVRIT – Retired from INRA (Institut national de la recherche agronomique) - Herbicides - Adjuvants - Coformulants – Agricultural pratices - Resistance - Efficacy

Members

Mr Fabrice NESSLANY – Head of department (Institut Pasteur de Lille) - Toxicology, Génotoxicity

Ms Jeanne STADLER – Toxicology consultant, Retired from Pfizer research center.- Reprotoxicology

Mme Sonia GRIMBUHLER – Researcher (Institut de recherches en sciences et technologies pour l'environnement et l'agriculture). Exposure assessment - Agricultural Machinery– Field measurement

Mr Jean-Ulrich MULLOT – Military pharmacist (Service de santé des Armées) - Toxicology, Exposure

Ms Brigitte FREROT – Research Engineer (Institut national de la recherche agronomique). - Physico-chemistry - Pheromones – Pheromones analysis

Ms Marie-France CORIO-COSTET – Director of research (Institut national de la recherche agronomique) - Efficacy - Fungicides - Vines - Resistance – PDE (Plant Defense Elicitors)

Mr François LAURENT – Researcher (Institut national de la recherche agronomique).- PPP Métabolism - Soil - Plants - Animal - Pesticides – Pollutant fate

Mr Jean-Pierre CUGIER – Retired from Ministry of Agriculture, Senior Scientific Officer (EFSA) jusqu'au 30/09/2016 - Residues and food safety

Mr Marc GALLIEN – Project manager (Mutuelle Sociale Agricole) - Prevention – Chemical protection

Ms Laure MAMY – Research engineer (Institut national de la recherche agronomique) - Pesticides - Fate - Environment - Modelling

Mr Maurice MILLET – University Professor (Université de Strasbourg) – Analytical chemistry - Physico-chemistry - Analysis (water, soil, air)

Mr Philippe BERNY – Research fellow (Vetagro Sup) - Ecotoxicology : birds and mammals

Ms Annick VENANT – Retired from Anses - Physico-chemistry - Regulation – Plant protection products- Analysis methods – Specifications

MEETING WITH EXTERNAL EXPERTS

National Toxicology Program (NTP)

Mr Michael DEVITO, PhD., toxicologist

Mr Matt STOUT, PhD., toxicologist

Ms Kristina WITT, genotoxicologist

Ms Stephanie SMITH-ROE, PhD, genotoxicologist

Mr Scott MASTEN, PhD, toxicologist

CONTENTS

Acronyms and abbreviations

BfR: Bundesinstitut für Risikobewertung (German Federal Institute for Risk Assessment)
CLH: Harmonised Classification and Labelling
CTA: Cell Transformation Assay
ECHA: European Chemicals Agency
EFSA: European Food Safety Authority
EU: European Union
EURL ECVAM: European Union Reference Laboratory for alternatives to animal testing
FRAP: Ferric-reducing ability of plasma
GLP: Good Laboratory Practices
IARC: International Agency for Research in Cancer
JMPR: Joint FAO/WHO Meeting on Pesticide Residues
LD50: Lethal Dose 50
miRNA: Micro RNA
MTD: Maximum Tolerated Dose
NHL: Non Hodgkin Lymphoma
NTP: National Toxicology Program
OECD: Organisation for Economic Co-operation and Development
RAC: Risk Assessment Committee
RAR: Renewal Assessment Report
RMS: Rapporteur Member State
SOP: Standard Operating Procedure
TBARS: Thiobarbituric acid reactive substances
US EPA: United States Environmental Protection Agency
WG: Working group

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1 Background, purpose, and processing of the request

1.1 Background of the request

Glyphosate is a broad-spectrum, post-emergent, non-selective systemic herbicide. It is widely used and is the first herbicide in terms of sold quantity per hectare in France in 2016.

It was assessed by different agencies with conflicting conclusions regarding its carcinogenic potential. In 2015, IARC classified glyphosate in Group 2A, *probably carcinogenic to humans*.

In 2015, EFSA concluded that it was *unlikely to pose a carcinogenic hazard to humans*. In 2016, the JMPR concluded that it is *unlikely to pose a carcinogenic risk to humans via exposure from the diet*. In 2017, ECHA did not classify glyphosate for carcinogenicity. In 2017, the US EPA concluded that it is *not likely to be carcinogenic to humans*.

In this context, in March 2018, the French Ministries of Agriculture, of Health and of Ecology asked Anses to propose a study protocol to clarify glyphosate carcinogenic potential.

1.2 Purpose of the request

New studies are needed to fill the gaps in the knowledge of the toxicological behavior of glyphosate. These studies should be based on integrated approaches that both fulfil the need to reduce the animal experiments, and provide mechanistic-based information to support the need of the regulatory community.

The objectives of this expertise are the following:

- Identify relevant objectives of a study allowing clarifying glyphosate carcinogenic potential.
- Evaluate the feasibility of such a study in accordance with the goals expressed by the ministries in terms of time and costs and the regulation on animal welfare.
- Propose relevant study protocol(s) in order to answer the identified goals.

1.3 Processing of the request: means implemented and organisation

ANSES tasked the Working Group (WG) on “Study plan to clarify glyphosate carcinogenic potential” with carrying out the work to respond to this request.

The results of the WG expertise, described in this report, was presented to the Experts Committee (CES) “Phytopharmaceuticals: substances and products” on 19/02/2019.

The expert appraisal was carried out in accordance with French Standard NF X 50-110 “Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)”.

1.4 Prevention of conflicts of interest

ANSES analyses the links of interest declared by the experts prior to their appointment and throughout the work, in order to avoid potential conflicts of interest with regard to the matters dealt with as part of the expert appraisal.

The experts’ declarations of interests are made public via the ANSES website (www.anses.fr).

2 Existing evaluation on glyphosate

2.1 International Agency for Research in Cancer (IARC)

IARC is the specialized cancer agency of the World Health Organization. It produces monographs that identify environmental factors that can increase the risk of human cancer. The data set of these monographs consists in all pertinent epidemiological studies and cancer bioassays in experimental animals, mechanistic studies and other relevant data. Only reports published or accepted for publication in the openly available scientific literature are reviewed.

IARC classifies carcinogens in five categories[1]:

- Group 1: The agent is carcinogenic to humans.

This category is used when there is *sufficient evidence of carcinogenicity* in humans.

- Group 2A: Probably carcinogenic to humans

This category is used when there is *limited evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals.

- Group 2B: Possibly carcinogenic to humans

This category is used when there is *limited evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals.

- Group 3: Not classifiable as to its carcinogenicity to humans

This category is used when there is *inadequate evidence of carcinogenicity in humans* and *inadequate or limited evidence of carcinogenicity* in experimental animals.

- Group 4: Probably not carcinogenic to humans

This category is used when there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals.

In March 2015, IARC concluded that glyphosate was ***probably carcinogenic to humans*** *(Group 2A)*[2].

Regarding human carcinogenicity data, IARC found statistically significant increased risks of Non Hodgkin Lymphoma (NHL) and haematopoietic cancers in 2 large case-control studies from USA and Canada and 2 case-control studies from Sweden.

Regarding animal carcinogenicity, IARC considered:

- A positive trend in the incidence of renal tubule carcinoma and of renal tubule adenoma or carcinoma (combined) in one feeding study in male mice
- A significant positive trend in the incidence of haemangiosarcoma in the second feeding study in male mice
- A significant increase in the incidence of pancreatic islet cell adenoma in 2 feeding studies in male rats
- A significant positive trend in the incidence of hepatocellular adenoma in male rats in one of these studies

[1] https://monographs.iarc.fr/wp-content/uploads/2018/07/QA_ENG.pdf

[2] IARC Monographs on the evaluation of carcinogenic risks to humans. Some organophosphate insecticides and herbicides. Volume 112 (2016).
https://monographs.iarc.fr/wp-content/uploads/2018/06/mono112-10.pdf

- A significant positive trend in the incidence of thyroid C-cell adenoma in female rats in the same study as above

IARC considered that in addition to limited evidence for the carcinogenicity of glyphosate in humans and sufficient evidence for the carcinogenicity of glyphosate in experimental animals, there is strong evidence that glyphosate can operate through two key characteristics of known human carcinogens, and that these can be operative in humans.

Specifically:

- There is strong evidence that exposure to glyphosate or glyphosate-based formulations is genotoxic based on studies in human cells *in vitro* and studies in experimental animals.
- There is strong evidence that glyphosate, glyphosate-based formulations, and aminomethylphosphonic acid (AMPA) can induce oxidative stress based on studies in experimental animals, and on studies in human cells *in vitro*. This mechanism of action has been challenged experimentally by administering antioxidants, which abrogated the effects of glyphosate on oxidative stress. Studies in aquatic species provide additional evidence for glyphosate-induced oxidative stress.

2.2 ECHA and EFSA

Glyphosate was assessed by EFSA in view of its re-approval as a phytopharmaceutical active substance according to Regulation (CE) 1107/2009. The assessment was carried out by Germany. After the peer review process which took into account the IARC evaluation (potential carcinogenicity), EFSA laid down its final assessment in October 2015.

It was also assessed by ECHA in order to propose a harmonised classification and labelling at EU level according to Regulation (CE) 1272/2008. Germany submitted a CLH report. After the public consultation, the RAC opinion on the proposed harmonised classification and labelling was adopted on 15 March 2017.

Conclusions of both instances are based on the same corpus of toxicological and epidemiological data which consists in published and unpublished studies.

Regarding human data, it was concluded that epidemiological data does not provide convincing evidence that glyphosate exposure in humans might be related to any cancer type including NHL.

Regarding animal data, it was concluded that there is insufficient evidence to support a carcinogenicity classification in category 2 based on the evaluation of seven carcinogenicity studies in the rat and that findings in the individual mouse carcinogenicity studies were not by themselves strong enough to warrant classification. The lack of evidence in the mouse studies is based mainly on an evaluation of statistical significance (considering that pairwise comparisons with controls were all non-significant and only some of them were significant in trend test), biological relevance (absence of non-neoplastic lesions, doses exceeding the maximum tolerated dose (MTD)) and consistency of the findings, including comparison with historical control data and differences in findings between the sexes.

Regarding genotoxicity, it was concluded that based on a weight of evidence approach, glyphosate did not present *in vivo* genotoxic potential and no classification for germ cell mutagenicity is warranted.

Therefore, EFSA considered that *Glyphosate **is unlikely to pose a carcinogenic hazard to humans** and the evidence does not support classification with regard to its carcinogenic potential according to the Regulation (EC) No 1272/2008*[3].

ECHA also concluded that *Glyphosate **is unlikely to pose a carcinogenic hazard to humans** and the evidence does not support classification with regard to its carcinogenic potential according to the Regulation (EC) No 1272/2008*[4].

2.3 JMPR

In May 2016, the JMPR (Joint FAO/WHO meeting on Pesticide Residues) issued a monograph on glyphosate[5]. It evaluated all previously considered toxicological data in addition to new published or unpublished toxicological studies and published epidemiological studies on cancer outcomes. The evaluation of the biochemical aspects and systemic toxicity of glyphosate was based on previous JMPR evaluations, updated as necessary with additional information. The particular focus was on genotoxicity, carcinogenicity, reproductive and developmental toxicity and epidemiological studies on cancer outcomes. The scope was restricted to the active ingredient.

The JMPR concluded that, in view of the absence of carcinogenic potential in rodents at human-relevant doses and the absence of genotoxicity by the oral route in mammals, and considering the epidemiological evidence from occupational exposures, glyphosate is ***unlikely to pose a carcinogenic risk to humans via exposure from the diet***.

2.4 US EPA

In December 2017, the US EPA (Environmental Protection Agency) issued an evaluation of glyphosate carcinogenic potential[6].

The hazard and exposure of glyphosate was re-evaluated to determine its potential risk to human and environmental health using current practices and policies. The US EPA has performed a comprehensive analysis of available data from submitted studies using guidelines and from the open literature. This included epidemiological, animal carcinogenicity, and genotoxicity studies. This evaluation focused on studies on the active ingredient glyphosate

It concluded that the available data do no support a carcinogenic process for glyphosate and therefore US EPA considered glyphosate as "***not likely to be carcinogenic to humans***".

[3] Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA Journal 2015;13(11):4302. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2015.4302

[4] RAC opinion proposing harmonised classification and labelling at EU level of glyphosate (15 March 2017). https://echa.europa.eu/documents/10162/2f8b5c7f-030f-5d3a-e87e-0262fb392f38

[5] JMPR Monograph of glyphosate (GLYPHOSATE 89–296 JMPR 2016)
http://apps.who.int/pesticide-residues-jmpr-database/pesticide?name=GLYPHOSATE

[6] Revised Glyphosate Issue Paper: Evaluation of Carcinogenic Potential. EPA's Office of Pesticide Programs December 12, 2017
https://cfpub.epa.gov/si/si_public_file_download.cfm?p_download_id=534487

3 Proposed study plan

The expert group took into account the previous evaluations (see chapter 2) and the on-going work on glyphosate[7] in order to identify knowledge gaps and propose a study plan to fill them.

The group mainly focused on the mechanisms of action pointed by IARC as hypothetically involved in glyphosate carcinogenic potential and designed studies to further explore these mechanisms.

3.1 In vitro tests

3.1.1 Objectives

The global aim of these assays is to investigate whether glyphosate elicits cellular responses that could be related to cellular stress including genotoxicity.

Changes in cellular behaviour can be either the consequence of genotoxic stress or can mediate such a stress. Indeed, in response to a genotoxic stress, cells are able to adapt and to elicit a cellular response that is dependent on the intensity of the stress and the extent of damage. Conversely, cellular stress can itself elicit changes that, at certain doses, lead to a genotoxic stress.

For that, the following issues are proposed to be addressed:

- **Cell behaviour**: the effects of short and medium term exposure to glyphosate on cell behaviour related to genotoxicity need to be assessed. For this, it is possible to screen a large battery of human cell lines representative of organs that have been previously suspected of being the targets of glyphosate genotoxic hazards.
- **Effect of oxidative stress**: when glyphosate genotoxicity has been observed, it was often related to an oxidative stress which is known to be very deleterious to a variety of macromolecules, including DNA. Consequently, the oxidative stress and the effectiveness of the antioxidant defence system should be qualified and quantified.
- **Effect of cell differentiation**: the effect of glyphosate on cell differentiation which could influence its properties should be assessed. Cell lines which can be differentiated *in vitro* such as HepaRG cells should be used. Analysing putative epigenetic effects would be extremely relevant under these conditions.
- **Addressing the putative mechanisms of glyphosate action**: the aim is to identify molecular pathways involved in the cellular response by analysing the transcriptome and epigenome and eventually to identify the mechanisms of glyphosate genotoxic action. After a stress, cells are able to reprogram gene expression through epigenetic modifications, to adapt and respond to the stress (e;g; oxidative stress) Thus, it would be relevant to analyse the epigenome by characterizing miRNA expression, DNA methylation and histone acetylation in order to carry out global and integrative multi-omics analysis and thus to provide consolidated pathways reflecting the mode of action of glyphosate.

Results of these tests should provide an overview of possible cellular disruptions, which will be useful to fully interpret the results of the other recommended tests (see 3.2 and 3.3) and to eventually explain discrepancies observed in published studies.

[7] https://ntp.niehs.nih.gov/results/areas/glyphosate/index.html

- **Assessing potential long term effects**:

This can be assessed *in vitro* through several (up to 3) weeks' exposure to glyphosate of HepaRG.. Biomarkers can be assayed to evaluate the "long term" effects of glyphosate on several outcomes:

- Oxidative stress (induction of anti-oxidant enzymes for example)
- Inflammation (induction of cytokines)
- Differentiation (induction of liver specific markers)
- Cellular senescence and apoptosis
- Induction of repair enzymes and chaperones.

These markers will help identifying relatively moderate effects that could be triggered upon longer term treatment of cells.

3.1.2 Methodology

3.1.2.1 Cell lines

Human cell lines should be favoured in so far as they are "reliable", in the sense that tests on these cell lines have been previously shown to provide repeatable results, relevant for human hazard identification. However, in some cases, other cell lines (regardless of their origin) which have proven to be relevant could also be included. If possible, p53 wild type cell lines will be chosen. The following cell lines should be used as a priority: human kidney (Caki-1, Hek292), pancreas (as an example *EndoC*-β*H1* but see also ATCC annex) and liver (HepaRG and HepG2, kept more or less capacity of metabolism). In order to position in vitro tests with the NTP *in vitro* testing, TK6 lymphoblastoid cell line should be included in the panel. Furthermore, in order to have elements of comparison with the CTA (see paragraph 3.3), BALB/c 3T3 cell line should be included in the panel

3.1.2.2 Choice of concentrations

In a first attempt, a large range of concentrations should be tested and should include concentrations relevant for the human exposure (consumer exposure, occupational exposure). The highest dose actually tested should not exceed the dose that induces 30% toxicity.

It is recommended that common doses (ideally 3) will be tested across all types of tests described throughout the following paragraphs (genotoxicity, transformation assay, mutagenesis…) for bridging purpose.

3.1.2.3 Phenotypic alterations

Step 1: Cell impedance

A first screening assay by following modification of cell impedance, a non-invasive measurement which allows detecting changes in the cell behaviour integrating any modification/alteration in cell proliferation, cell death, cell membrane integrity, cell morphology and cell adherence. This high throughput screening test is performed on a 6x 96-plates format and it allows a real time monitoring of cellular responses, up to a 15-day after short- and long- term exposure (Asphahani, 2007).

According to data obtained by measuring cell impedance in responsive cell lines, glyphosate concentrations and duration of exposure should be chosen to deeply characterize glyphosate cell responses using a battery of *in vitro* tests characterizing phenotypic alterations related to genotoxic stress (see below Step 2). It should be noted that the cell lines that will be considered as responsive

are the ones that will have shown impedance profiles that differ statistically from the profiles of untreated control cell lines.

Step 2: Other endpoints

Cell survival and cell death

Cell survival, cell death and proliferation will be studied by flow cytometry (Chen, 2017).

Oxidative stress

The oxidative stress should be studied by flow cytometry using DCFDDA probe (Figueroa, 2018) integrity of mitochondria by flow cytometry and microscopy (MitoSOX (Kauffman, 2016), mitoTrackers (Puleston, 2015)) and antioxidant defence (RT-PCR) (Tahmasbpour, 2016).

DNA breaks, cell cycle and DNA ploidy

DNA breaks and DNA repair will be investigated by analysing shortly after exposure, gamma-H2AX signal by confocal microscopy and flow cytometry (Moche, 2017; Paget, 2014).

Cell cycle and DNA ploidy will be studied by flow cytometry

OMICS

Transcriptome

Transcriptomic analyses (RNAseq) will help identify mechanisms of toxicity, if any (Rempel, 2015). Analysis will be done both on responsive and a non-responsive cell lines using at least three concentrations and considering different durations of exposure including an early and a late time after initiation of exposure. Indeed, the putatively altered pathways will be further validated using specific cell lines in which a given gene could be invalidated or overexpressed in the same genetic background.

Epigenome will be studied by miARN expression (Chappell, 2016), DNA methylation (Ruiz-Hernandez, 2015) and global histone H3K9 acetylation (Chen, 2017).

System biology will be used to integrate OMICS and other phenotypic data, to assess the coherence and the biological relevance of the data and ultimately to provide an integrated view of glyphosate action.

3.2 Genotoxicity

In general terms, in order to adequately assess the genotoxic potential of any substance, it is necessary to evaluate different endpoints, namely the induction of gene point mutations and chromosomal aberrations (both structural and numerical), as each of these events has been involved in genotoxic carcinogenesis processes.

Adequate assessment of genotoxic potential can only be achieved by using several test systems, as no single method can provide simultaneously sufficient information on all these endpoints. It is therefore necessary to use a battery of tests measuring these different genetic events to cover the widest possible spectrum of genotoxicity.

Overall, *in vitro* tests which are considered as highly sensitive represent alerts for genotoxic/mutagenic potential and are useful to identify mechanistic pathways. Therefore, the investigation of the genotoxic and/or mutagenic potential always starts with *in vitro* tests (on bacterial and/or cellular test systems) while more specific *in vivo* testing may be performed for confirming or infirming alerts found in vitro, if any (follow-up studies).

For instance, the recommendations of the EFSA Scientific Committee (EFSA, 2011) make it possible to meet these basic requirements.

3.2.1 Current state of knowledge on mutagenicity and genotoxicity of Glyphosate

Glyphosate has already been extensively investigated for its mutagenicity and its genotoxicity, using regulatory (*i.e*. for which an OECD technical guidance exists) and not-standardized methodologies (mainly literature studies), both *in vitro* and *in vivo*. Some mechanistic studies are also available.

An exhaustive assessment of almost all these results was performed by IARC and by Rapporteur Member State (Germany and co-RMS: Slovakia) during the re-evaluation of Glyphosate at the European level (EU RAR, Renewal Assessment Report, and its Addendum, 2015).

Summaries of genotoxicity/mutagenicity results are given below.

3.2.1.1 Negative results in studies taken into account in the EU RAR

In vitro tests:

- No mutagenic effect in 16 validated Ames tests.
- No mutagenic effects in 3 gene mutation tests on mammalian cells: 2 studies on L5178Y mouse lymphoma cells (Jensen 1991, Clay 1996) and one study on CHO cells (Li 1983).
- No effect in the Rec assay, a test for determining the transcriptional induction of the SOS regulator in E.coli induced by direct or indirect perturbations of DNA replication (Akanuma, 1995).
- Lack of induction of unscheduled DNA synthesis (UDS test) in vitro with CHO cells (Li and Long 1988).
- No publication related the presence of DNA adducts (note that a structure-activity relationship assessment indicates that glyphosate has no direct binding effect on DNA).

In vivo tests:

- No effect in the dominant lethal mutation test in mice (Wrenn et al 1980, cited in EPA 1980) and in rats (Suresh 1992).
- No induction of chromosomal aberrations after single intraperitoneal exposure in rats (Li and Long 1988).
- No induction of micronuclei in mouse bone marrow after single intraperitoneal exposure (Rank et al 1993).
- No induction of chromosomal aberrations in plants (onion) after glyphosate exposure (Rank et al. 1993).
- No induction of micronuclei in plants (Vicia faba) (De Marco et al 1992).

3.2.1.2 Negative results in studies taken into account in the EU RAR and cited by IARC

In vitro tests:

- *in vitro* unscheduled DNA synthesis test (UDS test) on rat hepatocytes (Rossberger 1994).

- Chromosome aberration tests on mammalian cells and human lymphocytes (Van de Waart 1995, Wright 1996, Kyomu 1995, Clay 1996, Jensen 1991).

b) In vivo tests:

- Micronucleus tests on rodent bone marrow (Jensen 1991, Suresh 1993, Fox and MacKay 1996, Honarvar 2008, Patel 2012, Roth 2012, Flügge 2009, Carvalho and Marques 1999, Durward 2006, Costa KC 2008, 2010).

- Test for chromosomal aberrations on rodent bone marrow (Suresh 1994).

3.2.1.3 Positive results in studies cited by IARC and included in the EU RAR

In vitro tests:

- Induction of DNA strand breaks detected by the *in vitro* Comet assay (i) on different mammalian cell lines without metabolic activation (Mañas et al 2009a, Alvarez-Moya et al 2014, Monroy et al 2005, Koller et al 2012), with and without metabolic activation (Mladinic et al 2009), (ii) in various fish (Moreno et al 2014, Guilherme et al 2012, Lopes et al 2014, Alvarez-Moya et al 2014), and (iii) in plants (Alvarez-Moya et al 2011).

- Induction of chromosomal aberrations *in vitro* in bovine lymphocytes with metabolic activation (Lioi et al 1998) and in plants (Siddiqui et al 2012, Frescura et al 2013).

- Induction of micronucleus *in vitro* on CHO cells with metabolic activation (Roustan et al 2014).

- Induction of sister chromatid exchanges (SCE) in vitro in human lymphocytes (Bolognesi et al 1997). However, note that this endpoint has been removed from the OECD guidelines as it is now considered to be related to cytotoxicity rather than genotoxicity. In any case, the induction of sister chromatid exchanges is considered to have a low weight in the Weight of Evidence approach (compared to the induction of gene point mutations or chromosomal aberrations).

In vivo tests:

- DNA strand breaks (alkaline elution test) at 4 h (but negative at 24 h) in renal and hepatic cells of mice treated intraperitoneally at 300 mg/kg (Bolognesi et al 1997).

- Induction of micronuclei *in vivo* in the bone marrow after two intraperitoneal injections, 24 hours apart (Bolognesi et al 1997, Mañas et al, 2009a).

Note that these positive results were discussed in the EU RAR which modulates interpretation according to considerations of:

- The use of high concentrations inducing clear toxicity that may be at the origin of artifacts during the alkaline elution test,
- The failure to follow the OECD guidelines with more or less significant deviations.

3.2.1.4 Mechanistic studies

3.2.1.4.1 Oxydative stress and inflammation

It has been hypothesized that the induction of DNA strand breaks, as demonstrated by the in vitro comet assay, could be explained by the production of oxidative stress induced by glyphosate.

Significant results cited by IARC and retained by BfR

- Mladinic et al (2009) evaluated both the genotoxic and oxidative potential of glyphosate *in vitro* on human lymphocytes, with and without metabolic activation (S9). Different methods were implemented: FRAP[8] and TBARS[9] as well as the modified comet test by adding hOGG1. In this study, during the Comet assay, very slight and not dose-related increases in % Tail Intensity compared to control were observed, either with or without metabolic activation, without any real potentiation in the presence of hOGG1.

When using FRAP method, increases were noted only at the highest concentration of 580 µg/mL independently of metabolic activation, while TBARS values increased significantly. Regardless of the method used, no clear dose-dependent effects were observed.

- Astiz et al (2009) carried out an *in vivo* study in rats analyzing oxidative stress in various organs after intraperitoneal injection of glyphosate; indications of oxidative stress were found in the plasma, liver, brain and kidney of exposed rats.

3.2.1.5 Pro-inflammatory effect

Positive results were reported:

- Nakashima et al (2002): Exposure of peripheral human lymphocytes *in vitro* induced modification of pro-inflammatory cytokine profile.

- Kumar (2014): C57BL/6, TLR4$^{-/-}$, and IL-13$^{-/-}$ mice inhaled different doses of glyphosate (and ovalbumin). The cellular response, humoral response, and lung function of exposed mice were evaluated. Exposure to glyphosate to the lungs increased eosinophil and neutrophil counts, mast cell degranulation, and production of IL-33, TSLP, IL-13, and IL-5. In contrast, in vivo systemic IL-4 production was not increased. Co-administration of ovalbumin with glyphosate did not substantially change the inflammatory immune response. However, IL-13-deficiency resulted in diminished inflammatory response but did not have a significant effect on airway resistance upon methacholine challenge after 7 or 21 days of glyphosate exposure. Glyphosate was concluded to induce pulmonary IL-13-dependent inflammation and promote Th2 type cytokines, but not IL-4 for glyphosate alone.

Significant results cited by IARC but rejected in the EU RAR

Several articles rejected by the EU RAR due to the lack of data on the purity of the batch actually tested, or the use of a formulation and not the active substance or the absence of controls:

- Gehin et al (2005) and Kwiatkowska et al (2014) which did not indicate the purity of the glyphosate tested,

- Elie-Caille et al (2010) who did not quantify ROS-induced fluorescence on the fluorochrome. However, it should be noted that a paper from the same author in 2012 with a very different technique (AFM) led to the same conclusion of oxidative stress on cells grown in vitro,

- Astiz et al (2013) who studied the protective effect of lipoic acid (LA) against the intoxication by mixtures of pesticides including glyphosate by exploring inflammation in organs such as rat testicles after i. p. injection. The EU RAR indicates that glyphosate is used in combination with pesticides and not alone and invalidates the IARC conclusion.

[8] Ferric-reducing ability of plasma

[9] Thiobarbituric acid reactive substances

- Bolognesi et al (1997), publication not retained by the EU RAR.

Negative result cited by IARC and included in the EU RAR

- Chaufan et al (2014), but the purity of glyphosate was not reported.

Other publications that were not cited by IARC nor EU RAR

- Slaninova's (2009) review on oxidative stress generated by several groups of pesticides in fish mentions 2 studies conducted with Roundup that concluded that low oxidative stress was induced. The transcriptomics study by Uren Webster & Santos (2015) conducted in trout with glyphosate showed transcriptional changes in the liver, affecting components of the redox system and a number of proteins in response to stress, as well as the induction of compensation mechanisms.

- Ashan et al., 2008 showed by a proteomic approach that glyphosate induced oxidative stress in rice leaves.

3.2.2 Discussion on mutagenicity and genotoxicity of Glyphosate

To summarize, all genotoxicity tests performed *in vitro* and *in vivo* within the regulatory framework lead to negative results (see Renewal Assessment Report, and its Addendum, 2015). For illustrative purposes, *in vitro*, no mutagenic effect was noted in 16 validated Ames tests and in 3 gene mutation tests on mammalian cells and negative results were also reported for chromosomal aberration and micronunuclei.

However, the literature reports contradictory results *in vitro*, which can be partly explained by the use of a wide variety of test systems, cellular models, glyphosate concentrations, glyphosate with a different purity than the one considered for annex I inclusion, treatment durations, protocol quality, etc. For instance, chromosomal aberrations and micronucleus induction were observed in vitro (4 positive in vitro tests on human or bovine lymphocytes and on plants).

In particular, the *in vitro* Comet test showed statistically significant results in different cellular models. It should be stressed that this test is subject to strong variations related to the experimental conditions, was often performed on unconventional models (fish, plants...), at too high doses... Furthermore, such *in vitro* methodology is not validated (no OECD guideline) and it is considered to have a low weight in a Weight of Evidence approach (Brusick et al, 2016).

In the *in vivo* test systems used, there is no significant induction of chromosomal aberrations (micronuclei or structural abnormalities by metaphase analysis) in rodent bone marrow, no DNA adducts in post-labelling detection, and no effect in the rat dominant lethal mutation test.

In the literature, only the study by Bolognesi et al (1997), using mice treated intraperitoneally at a dose of 300 mg/kg, showed significant results in the alkaline elution test (positive 4 hours after treatment but negative after 24 hours) and only a very slight increase in micronuclei. This publication, which was not retained in the RAR, is questionable, in particular because of the maximum dose level used, which appears to be excessive in view of the reported LD50.

Overall, the level of evidence of glyphosate genotoxicity in animals can be considered relatively limited. Interestingly, while almost all *in vivo* tests led to non-statistically significant results, there are no *in vivo* Comet test results, which could be the most sensitive biological parameter.

The question of the induction of oxidative stress was raised. A non-dose dependent pro-oxidant effect of glyphosate was found in different *in vitro* systems on human cells but negative results were also reported. At the same time, only one article refers to this type of mode of action *in vivo* in rats, but the increase in oxidative stress was observed in association with cytotoxic/degenerative effects of the organs studied (Astiz et al 2009). At the same time, negative results were also reported.

Overall, there is not enough evidence to assert that an oxidative mechanism actually occurs *in vivo* after exposure to glyphosate, and that it is "intrinsic" (i.e. directly induced by glyphosate). At this stage, the available data are not sufficient to draw any firm conclusions and such a mechanism

cannot be totally excluded. Such an effect could trigger the induction of DNA strand breaks found in *in vitro* comet tests.

Finally, based on the data available in rodent studies, there is no clear evidence of an immunosuppressive effect of glyphosate.

3.2.3 Planned NTP research on glyphosate

NTP is currently pursuing glyphosate and glyphosate formulations research[10]. A meeting with the NTP was held with the expert group to discuss their ongoing work.

The specific aims of NTP include the following:

- Evaluate whether glyphosate is genotoxic (causes DNA damage)
- Evaluate whether glyphosate induces oxidative damage
- Identify data gaps on the effects of glyphosate [and glyphosate-based formulations] on human health outcomes other than cancer.

As part of this research plan, NTP will use *in vitro* and *in vivo* approaches to further investigate whether glyphosate (as well as glyphosate-based formulations and amino methyl phosphonic acid, AMPA, a metabolite of glyphosate) can induce genetic toxicity and/or oxidative stress. First, as detailed hereafter, a screening strategy using cellular assays will be used in order to identify test articles (i.e., glyphosate and/or glyphosate-based formulations) for potential follow-up experiments (that may include short-term animal studies).

3.2.3.1 *In vitro* screening assays

Glyphosate, positive and negative controls will be tested in in vitro screening assays. The battery of assays includes assays for **oxidative stress**, **DNA damage**, and **cell viability**. Furthermore, the effects of the test articles on multiple cellular pathways will be assessed using a **transcriptomics assay**.

Several human cell lines will be used for testing, including metabolically active liver derived cells (human HepaRG cells), a lymphoblastoid cell line derived from B cell lymphocytes (TK6 cells), and skin cells (a keratinocyte cell line called HaCaT). Robust dose-response data will be generated.

A benchmark dose analysis will be used to identify the concentration of test article at which biological effects first become evident, as well as the AC50.

3.2.3.2 Genetic toxicology testing

Glyphosate will be tested in the following *in vitro* genotoxicity assays, in the presence or absence of an exogenous rat liver metabolic activation system:

- Bacterial mutagenicity assays with *Salmonella typhimurium* tester strains TA100, TA98, TA97, TA1535, and Escherichia coli tester strain WP2.

- Micronucleus assay (TK6 cells)

Significant increases in chromosomal aberrations and micronucleus induction were obtained *in vitro* with 4 positive tests on human or bovine lymphocytes (and even on plants). Therefore, the completion of the micronucleus assay appears to be useful.

- Comet assay (TK6 cells)

[10] https://ntp.niehs.nih.gov/results/areas/glyphosate/index.html

Along the same line, as the *in vitro* Comet test showed statistically significant results in different cellular models, the completion of this test, even if it is not validated, is of interest. Furthermore, taking into account uncertainties regarding an oxidative mode of action of glyphosate, the modified comet assay using a DNA glycosylase to detect oxidized DNA bases is particularly relevant. As for micronucleus, the issue of the cell model used for such an endpoint is raised. If the use of lymphoblastoid TK6 human cells seems relevant, the comparison with other cell types (*e.g.*, human lymphocytes, other, *e.g.*, cells used in the screening step as HaCaT cells) may be difficult. There could be specific sensitivity (of fish and/or plant cells/organisms) of particular importance.

3.2.3.3 Discussion on NTP planned research

Overall, the *in vitro* testing strategy as proposed by the NTP appears to be relevant. Even if the implementation of the Ames test does not seem very timely for Glyphosate as no mutagenic effect was noted in 16 validated Ames tests, it is to be noted that one of the objectives of these mutagenicity tests is to compare glyphosate, AMPA and several formulations in the same experimental conditions.

Otherwise, the issue of the single cell model used for both the micronucleus and the Comet assays is raised: If the use of lymphoblastoid TK6 cells seems relevant (human, p53 efficient), the comparison with other cell types (*e.g.*, human lymphocytes or other cells like the ones used in the screening step such as HaCaT cells) may be difficult.

Furthermore, no *in vivo* tests will be systematically performed and the NTP will discuss the need for the in vivo comet assay depending on the results of their in vitro tests.

3.2.4 Proposal from the Experts Group

Based on the literature data, the expert group considered that an additional *in vivo* genotoxicity assay should be conducted to clarify glyphosate genotoxic potential. As a matter of fact, whatever the issue of the complementary *in vitro* assays performed under the aegis of the NTP, controversial results in the literature will still exist even if they can be (at least partly) explained by the use of a wide variety of test systems, cellular models, glyphosate concentrations, treatment durations, protocol quality, etc.

As recalled previously, the *in vitro* Comet test showed statistically significant results in different cellular models. Even if the relevance of such a methodology is questionable, it cannot be ignored and a confirmation using *in vivo* approaches is required. Yet, there are no *in vivo* Comet test results, which could be the most sensitive biological parameter.

Therefore, in order to remove any doubt concerning these *in vitro* 'alerts', additional *in vivo* genotoxicity studies combining the analysis of micronuclei in bone marrow with the comet assay in appropriately selected tissues in rodents should be considered. This approach was described by Vasquez (2010)[11] and is mentioned in the OECD guideline No. 489[12]. The possibility of coupling these 2 endpoints would also confirm the absence of induction of micronuclei in the bone marrow by the selected route of administration, while verifying systemic exposure, without using additional animals.

The regulatory recommendations (2011 EFSA Strategy and OECD guidelines No. 489 and 474) should be followed and the proposed combined study should be carried out in rodents. As several carcinogenic effects were noted in different target organs depending on whether the study was conducted in rats or mice, the Expert Group concluded that this study should be performed in both species, using both male and female in order to anticipate any interspecies and/or intersex differences.

[11] Vasquez M.Z. Combining the in vivo comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. Mutagen 25 (2010) 187-199

[12] OECD Guideline for the Testing of Chemicals No. 489: In vivo Mammalian Alkaline Comet Assay, July 29th 2016.

Animals should be exposed through the anticipated expected route of human exposure. Therefore, oral route (by gavage) should be preferentially chosen. In any case the route should be chosen to ensure adequate exposure of the target tissue(s) that should be checked (see EFSA opinion 2017, EFSA Journal 2017;15(12):5113).

Glyphosate should be formulated in an appropriate vehicle and then diluted with the same vehicle. Following Principles of GLP (OECD 1997: § 6.2 Characterisation)[13], as the test item should be administered in a vehicle, the homogeneity, concentration and stability of Glyphosate in that vehicle should be determined.

The OECD guidelines recommend that the test be carried out at the maximum tolerated dose (MTD) which is described as the highest dose which causes no mortality, but which may give rise to the appearance of signs of toxicity. For non-toxic products, the dose of 2000 mg/kg should be chosen as the maximum test dose, according to the OECD guideline and the joint directives of the Japanese Environmental Protection Agency (OECD No. 489, 2016; Hayashi et al, 1994)[14],[15]. In any case, a preliminary test should be performed using the oral route. Groups of male and female rats/mice should receive a series of doses of Glyphosate, chosen in accordance with available toxicological data in order to determine or to check and to specify the MTD which should be actually tested for genotoxicity. From the results of the preliminary toxicity assay, three dose levels should be selected for the main genotoxicity assay (usually MTD, MTD/2 and MTD/4).

For the *in vivo* comet assay, the choice of target organs is of particular importance. In general, sites of direct contact, major systemic organs of metabolism and possible target organ(s) in carcinogenesis may be investigated. Regarding specifically Glyphosate administered orally, the following organs should be investigated: the glandular stomach and duodenum/jejunum[16] (as sites of direct contact), the liver (a positive trend of hepatocellular adenoma was noted in Sprague-Dawley male rats in one carcinogenicity study)[17], kidney (a positive trend was noted for renal tumours in male mice)[18], and pancreas (a significant difference was observed in Sprague-Dawley male rats in 2 carcinogenicity studies)[19]. The Expert Group concluded that all these organs should be investigated both in mice and rats even if they are not necessarily target organs in both species, but it could highlight a possible species susceptibility (for ethical reasons, the study should be carried out on the same animals for all organs).

Furthermore, a provision to allow processing of tissue sample for histopathological assessment should be included and may be analyzed if it will be considered helpful to the interpretation of any positive effects (*e.g.*, to detect a false positive result due to cytotoxicity).

In addition, considering the possible mode of genotoxic action via oxidation, the assay should also be conducted as a modified comet assay in which a DNA glycosylase should be added to detect the presence of oxidized DNA bases (*e.g.*, 8-OH-dG) in order to increase the sensitivity of the test to this type of lesion.

Finally, according to the RAR (2015), there is considerable evidence suggesting interference with cytotoxic phenomena, rather than interaction with DNA. Thus, particular attention should be paid to the assessment of cytotoxicity in order to avoid any interference with these phenomena on the genotoxic response. In the same way, hedgehogs (also known as clouds or ghost cells) which are morphological indications of highly damaged cells (often associated with severe genotoxicity and/or necrosis and/or apoptosis) should be independently reported.

[13] OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17;

[14] Hayashi M., Tice R.R. and MacGregor J.T. Report of the International Workshop on Standardization of Genotoxicity Test Procedures (6th ICEM, Melbourne 1993): In vivo Rodent Erythrocyte Micronucleus Assay. Mutation Res. 312 (1994), 293-304

[15] OECD Guideline for the Testing of Chemicals No. 489: In vivo Mammalian Alkaline Comet Assay, July 29th 2016.

[16] ECHA recommended the investigation of at least 2 organs of the gastrointestinal tract when the oral route is used (ECHA, 2016).

[17] Stout et al (1990)

[18] Knezevich et al (1983)

[19] Stout et al (1990) et Lankas et al (1981)

3.3 Cell transformation assay (CTA)

3.3.1 The use of the CTA in the regulatory context

In recent years, the European Union (EU) has promoted the development of alternative methods, with the aim to achieve the replacement, reduction and refinement of animal experiments, according to the 3Rs Principles (Annys et al, 2014; Mascolo et al, 2018). Similarly in the US, there is a strong drive to move to more in vitro and high throughput chemical testing as part of the 21st century vision of toxicology.

Carcinogenesis is a field where the demand for alternative methods is particularly high (Annys et al, 2014). Moreover, carcinogenicity testing has been recognised as the area with the most relevant needs for harmonisation amongst the different regulatory approaches (Annys et al, 2014). The standard rodent carcinogenicity bioassay (RCB) requires an extensive use of animals. Apart from animal welfare considerations, RCB show several limitations, particularly due to the high costs, the prolonged duration (2 years), and the scarce mechanistic information, which can make it difficult to completely understand the human relevance (Corvi et al, 2017; Paparella et al, 2017). Further, international work is ongoing with the aim to review the uncertainty and complexity of the RCB-based assessments. This shall contribute to revisiting RCB reference data evaluation and to improve the definition of acceptable performance of in vitro approaches (Jacobs et al, 2016; Corvi et al, 2017; Paparella et al, 2017).

Among in vitro tests, the cell transformation assay (CTA) has been proposed as a possible alternative to animal models based on some experimental evidence that cellular and molecular processes involved in in vitro cell transformation seem to be similar to those sustaining *in vivo* carcinogenesis, and occur as a result of comprehensive cellular response to direct and indirect damage to DNA (Combes et al, 2007; Corvi and Vanparys, 2012; Lilienblum et al., 2008; Mascolo et al., 2010; Rohrbeck et al, 2010; Vanparys et al., 2012; Vasseur and Lasne, 2012). CTA measures the morphological transformation of cells, as transformed colonies or foci derived from a single cell. It is supposed to involve a multistage process that closely models some stages of in vivo carcinogenesis.

Several models have been developed and implemented since the early ‘60s. Each model offers advantages and disadvantages (Table 1)

Table 1: Description of available CTA models

CTA model	Pros	Cons
SHE	Normal, diploid cells. Primary cells offering the opportunity to explore the entire process of transformation. Validated Protocol & OECD Guidance Document	Difficulty in correctly identifying the morphological endpoint. Not established cells. The test reduce but not replace the use of animal tests No Test Guideline
BALB/c 3T3	Established cell line offering a morphological end-point easy to be identified. The cells retain enough metabolic competence to avoid	Not diploid cells (hypotetraploid cells) No OECD Guidance Document or TG

	the use of exogenous metabolic systems. 3Rs -complying ECVAM Validated Protocol The protocol can be easily adapted to explore initiation/promotion properties of the tested chemicals	
Bhas-42	Established cell line offering a morphological end-point easy to be identified Validated protocol – OECD Guidance Document Initiation/promotion protocol	Cells contain multiple copies of activated H-Ras, integrated into the genome (initiated cells). The model can highlight the role of chemicals only in the late steps of the carcinogenesis process. The initiation/promotion protocol does not reflect the two-step in vivo carcinogenesis model

The Syrian Hamster Embryo (SHE) cells were first used to set up a model for studying the cell transformation in vitro. The SHE cells are normal diploid, metabolically and p53-competent primary cells, which retain the ability to biotransform a wide range of xenobiotics as evidenced by studies with substances requiring metabolic activation (OECD ENV/JM/MONO(2015)18). The SHE cells are primary cells that form aberrant colonies of fusiform disoriented cells derived from single parental cells, after the treatment with potential carcinogens. One of the main advantages in using the SHE model is represented by the possibility to study the very early steps of the process leading to malignancy, since SHE cells are not established cells. This could be also regarded as a limitation, since in the 3R perspective to replace, refine and reduce animal studies, the SHE model can be regarded as a possibility to reduce (but not replace) the use of in vivo studies. SHE cells are, indeed, obtained by pregnant hamsters, which are, for this purpose, euthanized. However, one hamster may provide enough cells to perform 50-100 CTAs, provided that the cells are adequately stocked and preserved. Another possible weak point of the SHE model is represented by the need to perform the test at different pHs. The original protocol was developed by performing the test at a neutral pH (7.2 – 7.3). The SHE CTA was then modified by performing the test in an acid environment (pH 6.7) to increase the sensitivity of the assay and cells responsiveness to different chemicals. It has been not possible so far to provide sufficient evidence of clear differences in performing the assays at neutral or acid pH. For those chemicals that had been tested under both experimental conditions no significant differences were observed. However, the performance of the test appears slightly improved at pH 6.7. Recently, the SHE CTA has been postulated to be a promising tool for the identification of non-genotoxic carcinogenic compounds (Colacci et al., 2014, ENV/JM/TG/RD, 2014).

The BALB/c 3T3 model was the first model to be developed by using established cells.

Initially developed to study virus-induced cell transformation, it was the first transformation assay to be set up to examine the tumour promotion in vitro (Schechtman, 1985).

BALB/c3T3 cells are embryonic mouse fibroblasts, which undergo transformation, following the chemical treatment, with cells escaping the contact-inhibition and piling up randomly. The endpoint of transformation is represented by the formation of foci of altered multi- layered, disorganized, anchorage-independent cells forming on a monolayer background of confluent contact-inhibited cells. The transformed cells from malignant foci are tumorigenic and metastatic when injected into

suitable host animals and acquire invasive properties in vitro (Adatia et al., 1993; Colacci et al., 1993; Melchiori et al., 1992).

The first BALB/c 3T3 model was developed by using the clone A-31 from BALB/c mouse strain, established in 1968. A second clone BALB/c 3T3 A31-1-1 was established in 1980, after what was initially described as a procedure of subcloning of BALB/c 3T3 A31, stocked in JCRB (Japanese Collection of Research Bioresources Cell Bank) and distributed to several researchers. The BALB/c 3T3 A31-1-1 clone was established to improve the cell sensitivity to carcinogens, by using polycyclic aromatic hydrocarbons as the reference chemicals. Contrary to other established cell lines (Jacobs et al., 2013), BALB/c 3T3 cells still retain enough metabolic activity to support both phase-1 and phase-2 metabolic activation of procarcinogens (Colacci et al., 2011).

Recently, the National Institute Biomedical Innovation, Health and Nutrition (NIBIHN) has performed genotyping survey on mouse cell lines in JCRB, revealing that 14 cell lines among 94 mouse cell lines (16 %) were misidentified (Uchio-Yamada et al. 2016), including BALB/3T3 clone A31-1-1 cell lines, which were not derived from BALB/c but Swiss mouse. However, it was concluded that the occurred misidentification did not affect the inherent properties of these cell lines which are useful for performing the CTA, such as the sensitivity to contact inhibition and the susceptibility to chemically-induced transformation.

BALB/c 3T3 CTA can be performed by using the standard protocol, which has been validated by ECVAM and included in the list of methods for REACH (method B-21), or a modified protocol to reduce the cytotoxicity and improve the specificity of the test (Vaccari et al, 1999).

Bhas 42 cells have been established by Sasaki et al from BALB/c 3T3 A31-1-1 cells through the transfection with a plasmid containing v-Ha-ras gene (Sasaki et al., 1988). Untransformed Bhas 42 cells grow to confluence forming a contact-inhibited monolayer. They are not tumorigenic upon transplantation in vivo. After exposure to carcinogenic agents, Bhas 42 cells form transformed foci, rising from morphologically altered cells, which acquire the ability of invading the surrounding non-transformed contact-inhibited monolayer.

Since Bhas 42 cells express an activated v-Ha-ras oncogene, they are regarded as already initiated cells, according to the two-stage paradigm of genotoxic carcinogenesis (Sasaki et al., 1990; Sasaki et al., 2015). A high-throughput version of the assay has also been established (Arai et al., 2013).

The recent discussion (2016-2017) on the origin of the cell lines provides an opportunity to reconsider the role of Bhas 42 CTA within an integrated approach to testing and assessment (IATA) for strategies to explore non-genotoxic carcinogens.

All CTA models provide an easily detectable endpoint of oncotransformation, which can be used to anchor the exposure to the acquisition of the malignant phenotype.

However, the subjectivity in identifying morphologically transformed foci or colonies has often indicated as one of the main limitation of the CTAs (EURL ECVAM, 2011). To overcome this limitation, a photo-catalogue was provided as a visual aid for the identification and the scoring of foci in the conduct of the assay (Sasaki, 2012). Automated imaging tools for the scoring of foci were also proposed, mostly to support naïve laboratories to set up CTA protocols (Callegaro et al, 2015).

3.3.2 Implementing the use of CTA in the regulatory context: the transformics assay

The international validation studies carried out on CTAs, including BALB/c 3T3 and Bhas 42 models, suggest that these assays could be considered as scientifically valid for assessing the carcinogenic potential of hazardous compounds (Sakai et al., 2011; Vanparys et al., 2012) and may provide suitable alternatives to the in vivo RCB (Vanparys et al., 2011).

The CTA is listed among the accepted methods for the evaluation of toxicological properties of chemicals under the REACH regulation (EU, 2008). In 2007, the Organisation for Economic Co-operation and Development (OECD) proposed to use CTA as a second-level screening for

carcinogens and as a screening test of choice for non-genotoxic carcinogens, which are not detected in standard mutagenicity assays (OECD, 2007). Recently, however, OECD concluded that the use of all CTA models as stand-alone assays to predict carcinogenesis cannot be considered into the regulatory framework. CTA should be used together with other experimental results, such as genotoxicity data, structure-activity analysis and pharmaco-toxicokinetic information, as part of a testing strategy and/or in a weight-of-evidence approach. In the development of an Integrated Approach to Testing and Assessment (IATA) for non-genotoxic carcinogens, for example, the CTA has been proposed to provide data on the endpoint related to the morphological transformation (Jacobs et al., 2016).

One of the main criticisms, which prevents the use of the CTA as a stand-alone-test in the regulatory context, is based on the lack of mechanistic information to understand the key events leading to the oncotransformation (Mascolo et al 2018).

To overcome this limitation and to improve the use of this assay in the integrated testing strategy for carcinogenesis, the transformics method has been recently developed, which combines the cell transformation assay and transcriptomics, to highlight the molecular steps leading to in vitro malignant transformation (Mascolo et al 2018). Since BALB/c 3T3 CTA is currently the only CTA model accepted for the evaluation of toxicological properties of chemicals under the REACH regulation, it was used to develop the transformics method. Two reference chemicals, 3-Methylcholanthrene and Benzo(a)pyrene, have been tested so far, following the protocol validated by ECVAM integrated with transcriptomics in the transformics method (Mascolo et al, 2018, Mascolo et al, 2018; ALTEX, under review). The transformics method has already been incorporated in the ongoing work on the IATA for non-genotoxic carcinogenesis.

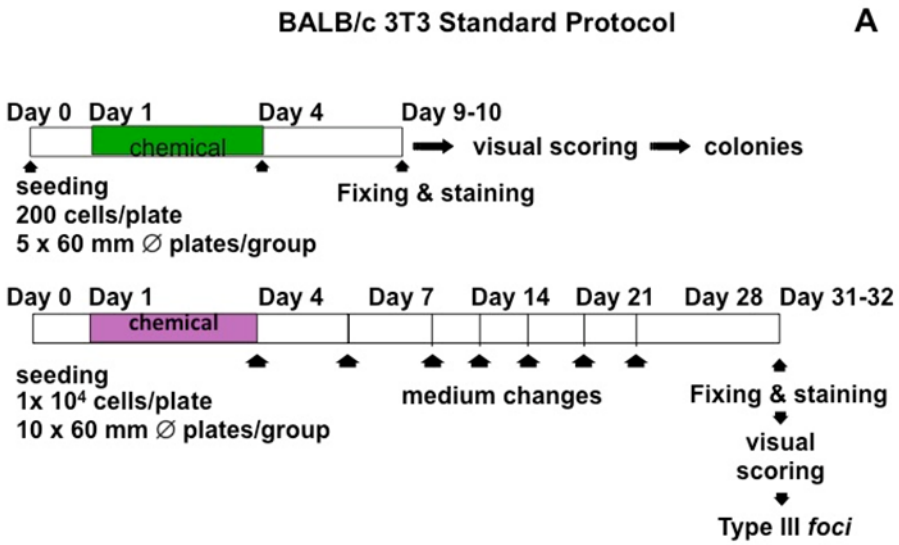
Transformics is able to describe the entire carcinogenesis process, anchoring the molecular machinery to the oncotransformation biological endpoint, and as such can be regarded as a useful tool in the integrated testing strategy for carcinogenesis chemical hazard assessment. With respect to genotoxicity tests, which only highlight DNA damage, transformics offers the possibility to understand the biological significance of the genotoxic events along the multistep carcinogenesis process. Indeed, toxicogenomics has been described as the tool to bridge both genotoxicity and non-genotoxicity events to carcinogenesis (Mascolo et al, 2018). Indeed, , cancer may be the consequence of non-genotoxic mechanisms, supported by the induction of tissue inflammation and by the stimulation of the immune response, fostering the biological conditions for the occurrence of DNA damage in tissues injured by a prolonged exposure to stressors. Transformics can highlight the gene pathways involved in the non-genotoxic carcinogenic process at both the molecular and cellular levels and predict the late stages leading to the adverse outcome (Mascolo et al, 2018).

3.3.3 The proposed CTA model and protocols

Based on the considerations reported above (see Table 1), the BALB/c 3T3 model seems to be the most appropriate CTA model to investigate the *in vitro* genotoxic and non-genotoxic carcinogenic potential of glyphosate. This model has recently been implemented to develop the transformics assay, an integrated approach adding information at both the molecular and cellular levels to understand the mechanism(s) leading to the in vitro oncotransformation and possibly identify events initiating the transformation process.

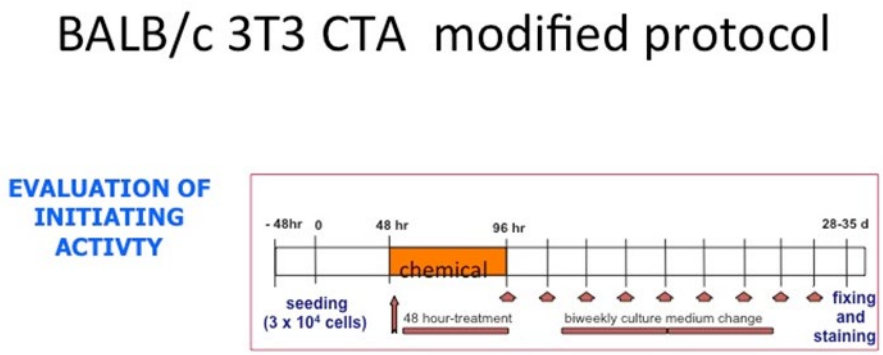
A validated protocol is available for the BALB/c 3T3 CTA, which can be used for testing chemicals within the regulatory context. The protocol is shown in Figure 1:

Figure 1: Description of the BALB/c 3T3 validated protocol



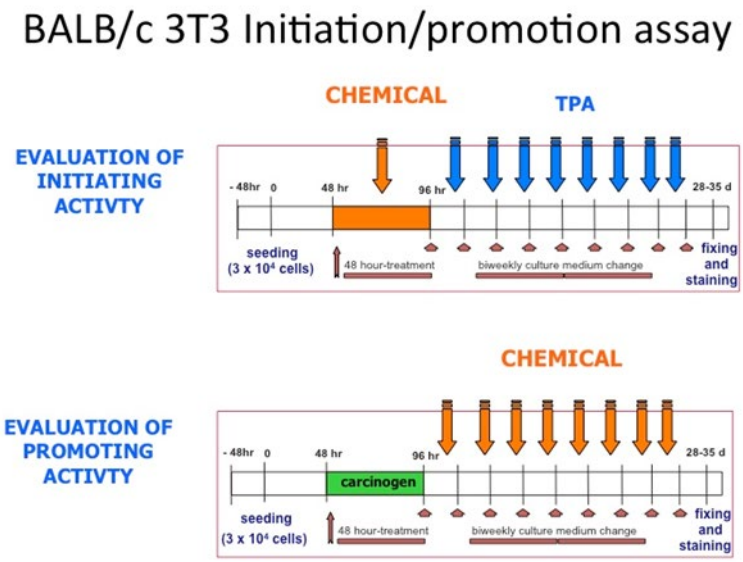
Since the validated protocol requires the use of toxic doses to facilitate the formation of malignant foci, including a top dose, which often exceeds the maximum tolerated dose and induces up to 90% cell loss, a modified protocol should be used as well. This modified protocol reduces the uncertainty related to the increased sensitivity of the surviving cells to the chemical and increase the specificity of the test (Figure 2).

Figure 2: Description of the BALB/c 3T3 modified protocol



The initiation/promotion test should be performed in case the standard test does not give positive results and/or the Transformics Assay provides evidence of gene/pathway involved in non-genotoxic carcinogenesis (Figure 3)

Figure 3: Description of the BALB/c 3T3 two-step protocol



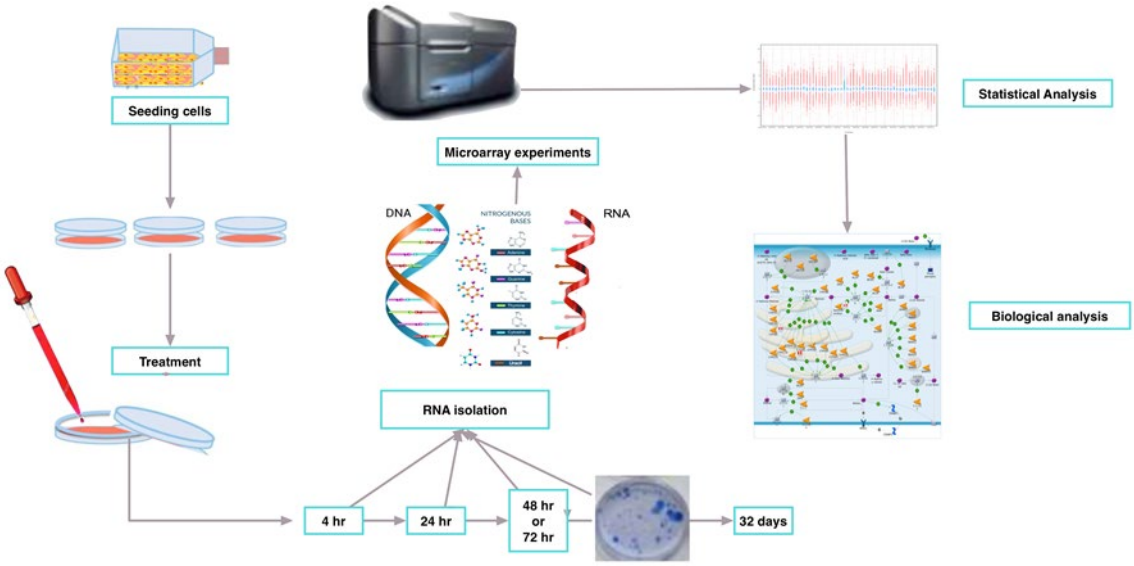
The Transformics Assay should be performed by using both CTA protocols. The transformics assay will provide information at the cellular and molecular levels to understand the mechanism(s) sustaining the oncotransformation, in case of positive results in the CTAs and to identify the

thresholds. In case of negative results from the CTAs, the Transformics Assay will provide useful information to highlight other possible mode(s) or mechanism(s) of action of glyphosate leading to different adverse outcomes.

Furthermore, as recalled previously considering the possible mode of genotoxic action via oxidation, a modified protocol of the Transformics Assay should also be developed to highlight the earliest molecular changes possibly related to the oxidative stress, one of the few recognized molecular main initiating events in the pathway leading to the adverse outcome.

The modified transformics protocol to explore this hypothesis is showed in Figure 4

Figure 4: The Transformics Assay - modified protocol



3.3.4 Proposed study design

A possible study design should include the following steps

1. Preliminary cytotoxicity assays to test the glyphosate toxicity on the chosen cell system and the proper working concentrations to be used in the CTA and in the Transformics Assay. The concentrations range should include concentrations relevant for the environmental exposure. Higher concentrations should be included according to the standard and modified CTA protocol. Lower concentrations should be included to investigate the effects at the molecular level, to discriminate among adaptive and adverse responses, and to identify possible thresholds. The choice of concentration should also take into account the range of concentrations used for other endpoints, particularly the concentrations chosen in the *in vitro* tests (see 3.1.2).

2. A CTA performed on the BALB/c 3T3 cell system, following the ECVAM validated protocol, including 9 (nine) working concentrations, chosen on the basis of the results from the preliminary cytotoxicity test.

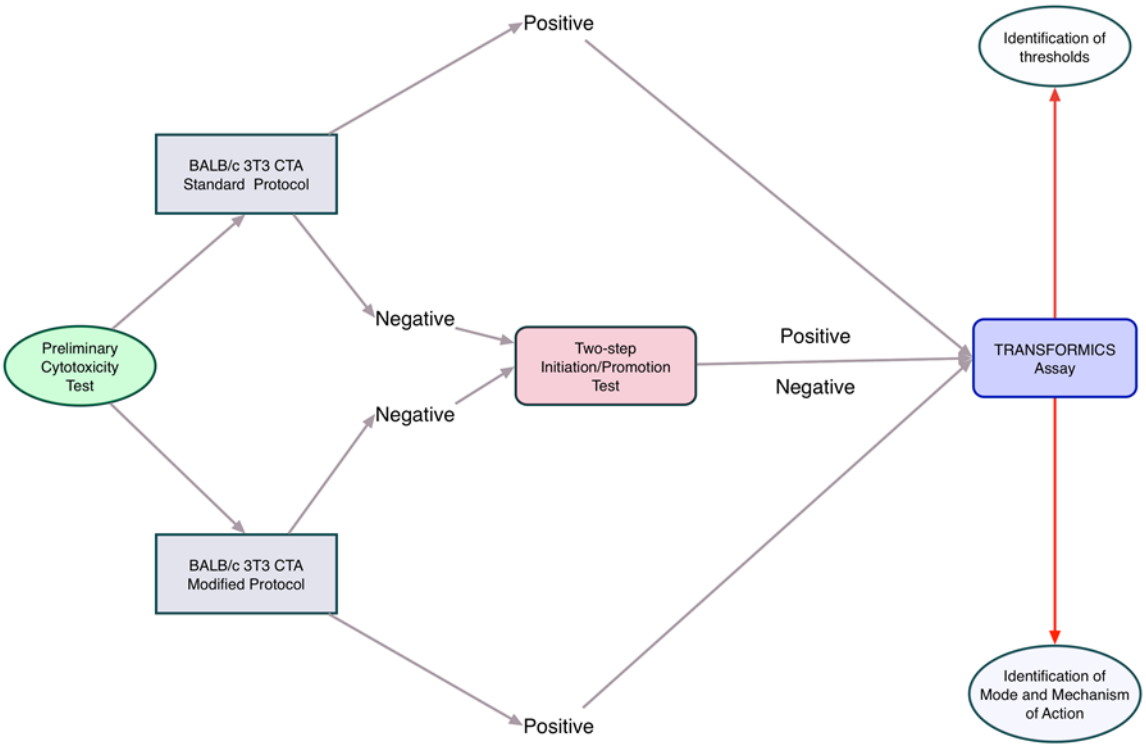
3. A CTA performed on the BALB/c 3T3 cell system, following the modified protocol, including 5 (five) working concentrations, chosen on the basis of the results from the preliminary cytotoxicity protocol

4. An initiation/promotion study to be performed on the BALB/c 3T3 cell system, in case of negative results in both CTAs.

5. A Transformics Assay performed to reveal the early molecular events related to adverse outcomes, to detect the mode and mechanisms of action of glyphosate, and to identify the threshold

The graphic representation of the study design is shown in Figure 5

Figure 5: Flow-chart of the integrated approach to highlight the toxicological behaviour of glyphosate



3.4 Endocrine disrupting activity

Since the endocrine disrupting activity is considered as a possible mechanism of action in the carcinogenesis of endocrine organs, it was deemed necessary to summarise the regulatory assessments conducted by several international instances (EFSA[20], USEPA[21], JMPR[22]) mainly based on the same corpus of data. These data consisted in studies submitted by the industry to national regulatory authorities for the approval of glyphosate in the European Union, USA and Japan as well as published studies considered of sufficient quality.

More specifically, the available information considered in determining the potential interaction of glyphosate with the endocrine pathways includes:

- A full battery of the Tier I screening assays generated according to the Endocrine Disruptor Screening Programme (EDSP) of the US Environmental Protection Agency. This Tier 1 assay battery was designed to provide the necessary empirical data to evaluate the potential of chemicals to interact with the estrogen (E), androgen (A) or thyroid (T) signalling pathways. This interaction includes agonism and antagonism at estrogen and androgen receptors as well as at the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–thyroid axes, and altered steroidogenesis.
- All other scientifically relevant information that may be suitable to address the potential endocrine activity (especially Level 4 and 5 assays indicated in the OECD Conceptual Framework)
- Published studies of sufficient quality selected after a scientific peer-review of the recent open literature regarding this topic. EFSA conducted a public literature search according to the EFSA Guidance on application of systematic review approach (EFSA Journal 2010; 8(6):1637). Briefly, the search performed on databases Embase, Medline and Scopus retrieved 116 publications. Amongst them, 12 publications were considered relevant for the evaluation of ED properties of glyphosate according to the following established criteria:
    1. Observations, examinations/analysis performed or necropsy are sufficiently well described;
    2. Endpoints addressed should be according to or equivalent to tests listed under EDSP or OECD conceptual framework;
    3. Testing results have to be based on the active ingredient rather than products/formulations

The European food Safety Agency (EFSA), the United States Environmental Protection agency (US EPA) and the Joint FAO/WHO meeting on Pesticide Residues (JMPR) used a weight of evidence (WoE) analysis for their scientific assessments of endocrine disruption potential of glyphosate. US EPA used the principles, criteria and approach in the WoE determination on the potential of a substance to interact with endocrine-related processes described in the WoE guidance document (USEPA, 2011). Regarding EFSA, their scientific assessment of the endocrine disruption potential of glyphosate was based on the EFSA Scientific Committee opinion on the hazard assessment of endocrine disruptors (EFSA Scientific Committee, 2013) and the testing strategy indicated in the OECD Conceptual Framework (OECD, 2012). JMPR has re-examined the data evaluated by the EDSP of the US EPA in addition to all toxicological data on the scope of endocrine disruption available on previous JMPR evaluations, updated as necessary with additional information.

[20] Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate (EFSA Journal 2017;15(9):4979) and addendum 2 to the RAR : assessment of potential endocrine disruption properties of glyphosate (2015).

[21] EDSP: weight of evidence analysis of potential interaction with the estrogen, androgen or thyroid pathways : glyphosate (USEPA 2015).

[22] JMPR Monograph of glyphosate (GLYPHOSATE 89–296 JMPR 2016)

Summary of the endocrine-mediated effects of glyphosate observed in the overall database on glyphosate

In this context, Annexe 2 presents the overall studies according to the OECD conceptual framework for testing and assessment of endocrine disrupters:

- Level 2: *In vitro* assays providing data about selected endocrine mechanism(s) pathways
- Level 3: *In vivo* assays providing data about selected endocrine mechanism(s)/pathways
- Level 4: *In vivo* assay providing data on adverse effects on endocrine relevant endpoints
- Level 5: *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant end-points over more extensive parts of the life cycle of the organism

Estrogen pathways

No evidence of potential interaction of glyphosate with the estrogen pathway was demonstrated in the Level 2 *in vitro* assays (i.e, ER binding, ER transactivation assay (ERTA), aromatase and steroidogenesis assays were negative). While glyphosate was reported to show estrogen-receptor agonism *in vitro* with estrogen-dependent human breast cancer cells (Thongprakaisang et al., 2013), other *in vitro* estrogen receptor studies with glyphosate did not demonstrate an interaction (e.g. Kojima et al., 2004). In addition, glyphosate was negative in the Level 3 or 4 mammalian assays (i.e. uterotrophic or female pubertal assays) and there were no treatment related effects on female reproductive parameters in the existing Level 5 mammalian studies (two generation or developmental toxicity studies).

In the fish short-term reproduction assay (FSTRA), a decrease in vitellogenin (VTG) was seen only at mid-treatment; however this effect was observed in isolation in the absence of any treatment-related effects in the other estrogen-related endpoints such as gonado-somatic index (GSI), gonadal staging, fecundity and fertilization. In addition, there was no notable gonadal histopathology (Schneider et al. 2012). In the open literature, glyphosate did not increase plasma VTG in juvenile rainbow trout (Xie et al, 2005)

Androgen pathways:

No evidence of interaction of glyphosate was observed in the level 2 *in vitro* [i.e, androgen receptor (AR) binding and steroidogenesis assays were negative] or level 3 or 4 in vivo mammalian assays (i.e., Hershberger and male pubertal assays were negative in the absence of overt toxicity). In addition, glyphosate was negative in an AR transactivation assay (Kojima et al., 2004). However, evidence for the aromatase and steroidogenesis assays is conflicting. Indeed, these assays were negative for glyphosate alone in the US EPA evaluation[23] (aromatase and H295R steroidogenesis test) and a murine *in vitro* model (Forgacs et al., 2012), but positive for the coformulants in another laboratory (Benachour et al., 2007; Defarge et al., 2016), with mechanistic underpinning via both the regulatory steroidogenic acute regulatory protein (StAR) and the P450scc cleavage enzyme first shown by Walsh et al. (2000).

In apical mammalian studies (level 4 and 5 of the OECD Conceptual Framework), the only treatment-related effects observed in the absence of overt toxicity were decreases in sperm count in the subchronic rat study (1678 mg/kg bw per day) and a delay in preputial separation (PPS) at 1234 mg/kg bw per day in the post-1998 two-generation reproduction study in rats. However, the delay in PPS was not reproduced in the second generation (F2 generation) of the same study or in another study investigating the same endpoint. Both effects were observed at a dose that was above the limit dose (1000 mg/kg bw per day) for those studies and general toxicity has been shown at this dose level in other developmental toxicity studies (reduced parental and offspring's body weight).

[23] EDSP program : weight of evidence analysis of potential interaction with the estrogen, androgen or thyroid pathways : glyphosate (USEPA 2015)

No androgen-related effects were seen in the wildlife toxicity studies (decreases in offspring body weight observed in one avian reproduction study).

Thyroid pathways:

No treatment related effects on thyroid hormones (thyroxine (T4) and thyroid-stimulating hormone (TSH)), thyroid weights or thyroid histopathology in the male pubertal assay were observed in the absence of overt toxicity. Additionally, there were no thyroid-related effects observed in the female pubertal assay.

In addition, there was no thyroid-related effect noted in any of the level 4 or 5 studies (Subchronic and chronic toxicity and developmental toxicity studies). However, it should be noted that these studies were performed according to old versions of their respective OECD guidelines. These old versions do not integrate the additional endocrine disruption relevant endpoints (such as circulating hormone measurements (T4, T3, TSH…), sperm parameters or oestrous cycle assessments…) included in the updated versions of these guidelines. Thus, only limited information regarding endocrine endpoints were available in these studies.

In the amphibian metamorphosis assay (AMA), there were no developmental effects or alterations in thyroid histopathology.

Other endocrine-related pathways

According to the JMPR assessment, there is little information about any endocrine-mediated effects of glyphosate, for example, in relation to retinoids, vitamin D receptors, metabolic syndrome, obesogens, glucocorticoids…

In the literature, there are some studies assessing the effect of glyphosate on endocrine-related pathways other than EATS. Two endocrine relevant pathways have been reported in non-mammalian models: retinoic acid-signalling pathway dysfunction in studies conducted in *Xenopus laevis* and chicken embryos (Paganelli et al., 2010) and inhibition of cortisol response in fish (Koakoski et al. 2014). However, it should be mentioned that both studies were performed with glyphosate commercial formulations and not the active substance alone. Other receptor-mediated pathways reported in the literature, including aryl hydrocarbon receptors, peroxisome proliferator-activated receptors and pregnane X receptor (PXR), were all negative (Takeuchi et al. 2008; Kojima, Takeuchi & Nagai, 2010).

Conclusions reached by EPA/EFSA/JMPR

Following a weight of evidence analysis of the overall data regarding endocrine mediated effects, the US EPA concluded that glyphosate demonstrates *"no convincing evidence of potential interaction with the estrogen, androgen or thyroid pathways in mammals or wildlife".*

A similar approach has been used by The European Food Safety Authority (EFSA), which concluded that "*the weight of evidence indicates that glyphosate does not have endocrine disrupting properties through oestrogen, androgen, thyroid or steroidogenesis mode of action based on a comprehensive database available in the toxicology area. The available ecotox studies did not contradict this conclusion"*

Similarly the JMPR concluded that: "*the studies considered as adequate by JMPR for the evaluation demonstrate no interaction with estrogen or androgen receptor pathways or thyroid pathways*.

4 Conclusion

Based on the available assessment of glyphosate carcinogenic potential, the expert group proposed an integrative approach in order to fill the gaps of knowledge, explore potential carcinogenic mechanisms of action and determine their relevance to humans.

Most of the recommended studies are *in vitro* studies in order to limit the use of animals. When an *in vivo* study is recommended, the protocol is designed to provide a maximum of information on the same animals.

In order to elucidate whether glyphosate elicits cellular responses that could be related to cellular stress *in vitro* tests exploring cell behaviour, oxidative stress and cell differentiation should be conducted. Potential long-term effects should also be addressed. An analysis of the transcriptome and the epigenome should be conducted in order to identify molecular pathways involved in the cellular response. System biology should be used to integrate OMICS and other phenotypic data, to assess the coherence and biological relevance of the data and ultimately to provide an integrated view of the mechanism(s) of toxic action of glyphosate. Details regarding the assays recommended by the experts group are provided in section 3.1. Results of these assays will be useful to fully interpret the results of the other recommended tests (see below) and to eventually explain discrepancies observed in published studies.

Regarding genotoxicity, in view of the overall existing results, the level of evidence of glyphosate genotoxicity in animals can be considered relatively limited. Interestingly, while almost all *in vivo* tests led to statistically and/or biologically non-significant results, there are no *in vivo* Comet test results, which could be the most sensitive biological parameter. A first step would thus be to conduct an additional *in vivo* comet assay combined with a micronucleus test to clarify glyphosate genotoxic potential. Details regarding the *in vivo* Comet assay recommended by the experts group are provided in section 3.2.

In order to reduce uncertainties regarding mechanisms of action, a cell transformation assay (CTA) combined with a transformics assay should be conducted to allow the identification of mode and mechanism of action. Cell transformation assay (CTA) has been proposed as a possible alternative to animal models based on some experimental evidence that cellular and molecular processes involved in in vitro cell transformation seem to resemble those sustaining *in vivo* carcinogenesis, and occur as a result of comprehensive cellular response to direct and indirect damage to DNA. It is supposed to involve a multistage process that closely models some stages of in vivo carcinogenesis. However, the CTA cannot be used as a stand-alone test to predict carcinogenesis and mechanistic information should be provided. Transformics couples CTA with transcriptomics, overcoming the limitations of the CTA alone and linking the key events at the molecular level with the phenotypic endpoint of onco-transformation. Details regarding the CTA assay recommended by the experts group are provided in section 3.3. In case of negative results in the CTA, a two-step initiation/promotion test should also be realized as detailed in sections 3.3.3. and 3.3.4.

The results of the recommended studies should be available 18 months after their initiation

Regarding Good Laboratory Practices (GLP) compliance, it should be mandatory for genotoxicity tests. For the other tests, if there are not conducted under GLP, the traceability of the studies should be guaranteed (raw data, SOPs…).

In this quality context, as Glyphosate should be formulated in an appropriate solvent/vehicle and then diluted with the same vehicle, the homogeneity, concentration and stability of Glyphosate in that vehicle should be determined (Principles of GLP; OECD 1997: § 6.2 Characterisation).

In the same way, in the *in vivo* studies, as exposure of the target tissue(s) should be checked, the concentration of Glyphosate in biological samples (*e.g.*, plasmas) should be determined in order to demonstrate systemic exposure.

This implies that analytical methods (including validation parameters) should be developed (in solvent/vehicle and in the proper biological fluid).

Therefore, for analysis and bioanalysis, the group of experts recommends that a single analytical lab (preferentially GLP-compliant) takes in charge the determination of the concentrations of Glyphosate in samples used in all *in vitro* and *in vivo* studies (even if not GLP-compliant) and in the proper biological fluid in the in vivo studies. This will allow a better comparison between the different studies by using the same validated analytical methods, the same parameters for aliquots of dosing samples (temperature of preservation, volume needed…).

Finally, the group of experts recommends that the call should be answered by a consortium of proficient labs in order to centralize information (in particular, common concentrations should be tested across all types of tests for bridging purpose), to facilitate the exchanges and finally to have an overview when interpreting results from all the tests in an integrated approach.

Date of validation of the collective expertise report by the working group and the expert committee: 19 February 2019

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ANNEXES

Annex 1: Request letter

COURRIER ARRIVE
03 AVR. 2018
DIRECTION GENERALE

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

Ministère de la Transition écologique et solidaire | **Ministère des Solidarités et de la Santé** | **Ministère de l'Agriculture et de l'Alimentation**

Paris, le 28 MARS 2018

2018 -SA- 0078

Les ministres

A

Monsieur le Directeur général de l'Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail

Objet : Saisine de l'ANSES sur le cahier des charges d'une étude sur le potentiel cancérogène du glyphosate.

Le règlement d'exécution UE 2017/2324 du 12 décembre 2017 renouvelle l'approbation de la substance active phytopharmaceutique glyphosate pour une période de 5 ans.

Des incertitudes persistent néanmoins sur cette substance, en raison notamment des conclusions divergentes sur sa cancérogénicité. D'une part le CIRC (2015) a conclu à la cancérogénicité probable de la substance, et d'autre part l'EFSA (2015), le JMPR (2016), l'ECHA (2017) et l'US EPA (2017) ont conclu à l'absence de caractère cancérogène du glyphosate. En dépit des échanges entre experts des différentes agences, il n'a pas été possible pour l'instant d'établir un consensus sur l'origine de cette divergence.

Dans ce contexte, des parties prenantes ont mis en cause la nature et l'étendue des données prises en compte par les instances d'expertise, ainsi que l'indépendance de ces expertises par rapport aux porteurs d'intérêt.

C'est pourquoi il apparaît nécessaire qu'une étude toxicologique permettant d'améliorer les connaissances sur les caractères de danger du glyphosate, et en particulier sur sa cancérogénicité, puisse être menée en toute indépendance, grâce à un financement public dans ce cas précis.

Nous souhaitons que l'ANSES établisse le cahier des charges de cette étude, au regard d'une part des principales incertitudes manifestées sur la toxicité de la substance, et d'autre part des signaux qui, dans les travaux déjà réalisés et au regard de l'analyse qu'en a fait l'ANSES, demanderaient à être confirmés ou infirmés.

.../...

Vous proposerez un protocole d'étude fondé sur des lignes directrices adoptées au niveau européen ou international. Vous ferez également des propositions sur les modalités de mise en œuvre et de pilotage de ces travaux expérimentaux, en attachant une attention particulière au respect de la réglementation sur l'expérimentation animale et des règles éthiques en général. Vous préciserez le calendrier prévisionnel jusqu'à la remise du rapport final.

Vous voudrez bien nous adresser le cahier des charges de l'étude dans un délai de 6 mois.

Le ministre de la Transition écologique et solidaire	La ministre des Solidarités et de la Santé	Le ministre de l'Agriculture et de l'Alimentation
Nicolas HULOT	**Agnès BUZYN**	**Stéphane TRAVERT**

Annex 2 : Summary of glyphosate ED studies

Endocrine disruption potential studies in rodents

Study type and acceptability	**Doses/conc.**	**Effects observed**	**Reference**
OECD Level 5 In vivo comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism			
2-generation reproductive toxicity , rat (SD), oral (diet) TG416 (2001), study acceptable, performed according to current standards, i.e. investigating oestrus cycles, sperm parameters, sexual maturation (last update TG416: 22/01/2001)	0, 1500, 5000 and 15000 ppm	NOAEL parental, offspring & reproductive: 5000 ppm (351 mg/kg bw per day) **Delayed sexual maturation: delayed preputial separation in F1 M at 15000 ppm** (1000 mg/kg bw per day), but no impact on subsequent reproductive performance	Dhinsa et al., 2007 (addendum 2 on glyphosate ED properties; Germany, 2017b);
2-generation reproductive toxicity , rat (Wistar), oral (diet) TG416 (1983) Study supplementary	0, 10, 100, 1000 and 10000 ppm	NOAEL: 10000 ppm (700-800 mg/kg bw per day) Sexual maturation was not examined. Negative for ED	Suresh, 1993
2-generation reproductive toxicity , rat (SD), oral (diet) TG416 (1983) Study acceptable	0, 1000, 3000 and 10000 ppm	NOAEL parental & offspring: 3000 ppm (197 mg/kg bw per day) NOAEL reproductive: 10000 ppm (668 mg/kg bw per day) No effect on preputial separation Negative for ED	Brooker et al., 1992
2-generation reproductive toxicity , rat (SD), oral (diet) Study in accordance with TG416 (1983) Study acceptable	0, 2000, 10000 and 30000 ppm	NOAEL parental, offspring & reproductive: 10000 ppm (720-760 mg/kg bw per day) Sexual maturation was not examined.	Reyna, 1990

Deviations: no data on food efficiency; no details on fertility indices, number of live births and post-implantation loss, number of pups with grossly visible abnormalities.		Negative for ED	
2-generation reproductive toxicity , rat (Wistar), oral (diet) Study acceptable TG416, US EPA (1998)	0, 1000, 3000 and 10000 ppm	NOAEL parental & offspring: 3000 ppm (293 mg/kg bw per day) NOAEL reproductive: 10000 ppm (985 mg/kg bw per day) No consistent toxicologically-significant effects on female oestrous cycles No impact on sexual maturation observed. Negative for ED	Moxon, 2000
2-generation reproductive toxicity , rat (SD), oral (diet) TG416 (1981) Study acceptable	0, 1200, 6000 and 30000 ppm	NOAEL parental & offspring: 6000 ppm (417 mg/kg bw per day) NOAEL reproductive: 30000 ppm (>2000 mg/kg bw per day) Sexual maturation (preputial separation, vaginal opening) was not examined. Negative for ED	Takahashi, 1997
Overall conclusion for Level 5: negative **Overall no fertility impairment or endocrine-related findings in 5/6 studies**			
OECD Level 4 In vivo assays providing data on adverse effects on endocrine relevant endpoints			
Studies on short-term toxicity: 7 90-day dietary studies in rats, 2 90-day dietary studies in mice, 6 studies in dogs Studies performed between 1979 and 1993 and therefore not in compliance with last update of TG408 (25/06/2018) or TG409 (last update: 21/09/1998)		No endocrine effect ➔Negative for ED	(DAR Germany, 2015)

Important deviation: No measurement of thyroid hormones			
Chronic toxicity and carcinogenicity studies in rats (last update TG453: 27/06/2018)			
2-year rat (Wistar), oral (diet) Study acceptable TG453 (1981) Deviations: Individual animals exceed the 20% range in body weight; organ weights were not determined for all animals; weights of heart, spleen and (para)thyroids are missing	0,100, 1000, 10000 ppm	NOAEL: 100 ppm (60 mg/kg bw per day) Negative: pancreas, thyroid, liver, kidney, testes and mammary gland No endocrine effect Negative for ED	Suresh, 1996
2-year rat (SD), oral (diet) Study acceptable According to US EPA (1982)	0, 10, 100, 300, 1000 mg/kg bw per day	NOAEL: 10 mg/kg bw per day Non stat.signif. top-dose increase of thyroid follicular adenoma in males Negative: pancreas, liver, kidney, testes and mammary gland No endocrine effect Negative for ED	Atkinson et al., 1993
2-year rat (SD), oral (diet) Study acceptable US EPA (1982) in general accordance with OECD TG453 Deviations: only 10 rats/sex for interim sacrifice; overall survival at termination was below 50%	0, 2000, 8000, 20000 ppm	NOAEL: 2000 ppm (89 mg/kg bw per day) Non stat.signif. top-dose increase of thyroid C-cell adenoma in females and non-dose-related increase pancreas adenoma in males Negative:liver, kidney, testes and mammary gland No endocrine effect Negative for ED	Stout and Ruecker, 1990
26-month rat (SD), oral (diet) Supplementary study In general accordance with OECD 453 (1981)	M: 0, 3, 10, 31 mg/kg bw per day, F: 0, 3, 11 and 34 mg/kg bw per day	NOAEL: 31 mg/kg bw per day Non stat.signif. increase thyroid adenoma in males and carcinoma in females, non-dose-related increase pancreas adenoma in males non stat. signif. top dose increase liver adenoma in females and benign testes tumors	Lankas, 1981

		Negative: kidney and mammary gland No endocrine effect Negative for ED	
2-year rat (Wistar), oral (diet) TG453 (1981)	0, 1500, 5000 and 15000-24000 ppm	NOAEL: 5000 ppm (285 mg/kg bw per day) Non stat.signif. top-dose increase of benign mammary gland tumors Negative: pancreas, thyroid, liver, kidney and testes No endocrine effect ➔Negative for ED	Wood, 2009
2-year rat (Wistar), oral (diet) TG453 (1981)	0, 2000, 6000 and 20000 ppm	NOAEL: 6000 ppm (361 mg/kg bw per day) Stat. signif. top dose increase liver adenomas in males Negative: pancreas, thyroid, kidney, testes and mammary gland. No endocrine effect ➔Negative for ED	Brammer, 2001
2-year rat (SD), oral (diet) TG453 (1981)	0, 3000, 10000 and 30000 ppm	NOAEL: 3000 ppm (104 mg/kg bw per day) Non stat.signif. top-dose increase of benign kidney tumors in males Negative: pancreas, thyroid, liver, testes and mammary gland No endocrine effect ➔Negative for ED	Enomoto, 1997
12-month rat (Wistar), oral (diet)	0, 2000, 8000, 20000 ppm	NOAEL: 2000 ppm (141 mg/kg bw per day) No endocrine effect ➔Negative for ED	1996
Carcinogenicity studies in mice (last update TG451: 27/06/2018)			
2-year mouse (CD-1), oral (diet) Study acceptable	0, 100, 300 and 1000 mg/kg bw per day	NOAEL: 1000 mg/kg bw per day)	Atkinson et al., 1993

TG451 However not according to last update TG451 (27/06/2018)		Non-stat. signif. increase haemangiosarcoma in top-dose males, w/i HC Negative: lymphoma, kidney, liver and lung No endocrine effect Negative for ED	
2-year mouse (CD-1), oral (diet) Study acceptable TG451	0, 1000, 5000, 30000 ppm	NOAEL: 1000 ppm (157 mg/kg bw per day) Non-stat.signif. increase kidney carcinoma in top-dose males Negative: lymphoma, haemangioma, liver and lung No endocrine effect Negative for ED	Knezevich and Hogan, 1983
18-month mouse (CD-1), oral (diet) TG451 (1981)	0, 500, 1500 and 5000 ppm	NOAEL: 5000 ppm (810 mg/kg bw per day) Stat.signif. increase lymphoma in top-dose males, w/i HC Non stat.signif. top dose increase lung carcinoma in males, w/i HC Negative: kidney, haemangioma and liver No endocrine effect ➔Negative for ED	Wood, 2009
18-month mouse (Swiss albino), oral (diet) TG451 (1981)	0, 100, 1000 and 10000 ppm	NOAEL: 1000 ppm (151 mg/kg bw par day) Non-stat.signif. increase lymphoma in top-dose males and females, w/i HC Non-stat. signif increase kidney adenoma in females, w/i HC Stat signif top dose increase in haemangioma in females Negative: liver and lung No endocrine effect ➔Negative for ED	Kumar, 2001
18-month mouse (CD-1), oral (diet) TG451 (1981)	0, 1600, 8000 and 40000 ppm	NOAEL: 1600 ppm (153 mg/kg bw per day)	Sugimoto, 1997

		Non-stat.signif. increase lymphoma in top-dose males, non-stat.signif. increase kidney adenoma in males Non--stat.signif. increase haemangiosarcoma in males and females Negative: liver and lung No endocrine effect ➔Negative for ED	
18-month mouse (Balb/c), oral (diet) Study not acceptable (no. animals too small)	0, 75, 150, 300 ppm	Negative for ED	Bhide, 1988
18-month mouse (CFLP/LAT1), oral (diet) Study not acceptable (no. animals surviving too small)	0, 100, 300 ppm	Negative for ED	Vereczkey and Csanyi, 1982
No increased incidence of hormone-sensitive tumors in rodents			
Developmental toxicity studies (last update TG414: 27/06/2018)			
Developmental toxicity rat (CD), gavage Study acceptable TG414 (1981) Important deviation: No measurement of thyroid hormones as request in last update	0, 300, 1000, 3500 mg/kg bw per day d 6-15	NOAEL mat. & dev.: 300 mg/kg bw per day Negative for ED	Brooker et al., 1991
Developmental toxicity rat (CD), gavage Study acceptable pre-guideline; satisfies in general the requirements of OECD TG414 (1981) Important deviation: No measurement of thyroid hormones as request in last update	0, 300, 1000, 3500 mg/kg bw per day d 6-19	NOAEL mat. & dev.: 3500 mg/kg bw per day Negative for ED	Tasker and Rodwell, 1980
Developmental toxicity rat (Wistar), gavage Study acceptable (limited test)	0, 1000 mg/kg bw per day d 6-15	NOAEL mat.: 1000 mg/kg bw per day NOAEL dev.: <1000 mg/kg bw per day Negative for ED	Suresh, 1991

Developmental toxicity rat (CFY), Diet Study supplementary	0, 22, 103 and 544 mg/kg bw per day d 6-18	NOAEL mat. & dev.: 544 mg/kg bw per day Negative for ED	Anonym, 1981
Developmental toxicity rat (Wistar), gavage Study acceptable TG414 (2001) Important deviation: No measurement of thyroid hormones as request in last update	0, 250, 500 and 1000 mg/kg bw per day d 7-16	NOAEL mat. & dev.: 1000 mg/kg bw per day Negative for ED	Moxon, 1996 + 2002
Developmental toxicity rat (CD), gavage Study acceptable TG414 US EPA (1984) Important deviation: No measurement of thyroid hormones as request in last update	0, 30, 300 and 1000 mg/kg bw per day d 6-15	NOAEL mat. & dev.: 300 mg/kg bw per day Negative for ED	Hatakenata, 1995
Developmental toxicity rabbit (NZW), gavage Study acceptable TG414 (1981) Important deviation: No measurement of thyroid hormones as request in last update	0, 10, 100 and 300 mg/kg bw per day d 6-18	NOAEL mat.: 100 mg/kg bw per day NOAEL dev.: 300 mg/kg bw per day Negative for ED	Hojo, 1995
Developmental toxicity rabbit (NZW), gavage Study acceptable TG414 (1981) Important deviation: No measurement of thyroid hormones as request in last update	0, 50, 200 and 400 mg/kg bw per day d 6-18	NOAEL mat. & dev.: 50 mg/kg bw per day Negative for ED	Coles and Doleman, 1996
Developmental toxicity rabbit (NZW), gavage Study acceptable TG414 (1981) Important deviation: No measurement of thyroid	0, 100, 175 and 300 mg/kg bw per day d 8-20	NOAEL mat.: 100 mg/kg bw per day NOAEL dev.: 175 mg/kg bw per day Negative for ED	Moxon, 1996

hormones as request in last update			
Developmental toxicity rabbit (NZW), gavage Study supplementary TG414 (1981) Important deviation: No measurement of thyroid hormones as request in last update	0, 20, 100 and 500 mg/kg bw per day d 6-18	NOAEL mat.: 20 mg/kg bw per day NOAEL dev.: 100 mg/kg bw per day Negative for ED	Suresh, 1993
Developmental toxicity rabbit (NZW), gavage Study supplementary TG414 (1981) Important deviation: No measurement of thyroid hormones as request in last update	0, 125, 250 and 500 mg/kg bw per day d 6-15	NOAEL mat. & dev.: 250 mg/kg bw per day Negative for ED	Bhide and Patil, 1989
Developmental toxicity rabbit (Dutch Belted), gavage Study supplementary pre-guideline; satisfies in general the requirements of OECD TG414 (1981) Important deviation: No measurement of thyroid hormones as request in last update	0, 75, 175 and 350 mg/kg bw per day d 6-19	NOAEL mat.: 75 mg/kg bw per day NOAEL dev.: 175 mg/kg bw per day Negative for ED	Tasker and Rodwell, 1980
Other studies			
1-generation reproductive toxicity (range-finding) , rat (SD), oral (diet) Study supplementary TG415 (last update: 26/05/1983)	0, 1000, 3000, 10000 and 30000 ppm	LOAEL maternal: <3000 ppm (<236 mg/kg bw per day) LOAEL offspring: <3000 ppm (<368 mg/kg bw per day) Negative for ED	Brooker et al., 1991
A pubertal development and thyroid function assay in female rats; gavage, acceptable even though not OECD agreed guideline (EPA OPPTS)	0, 100, 300 and 1000 mg/kg bw/d	Significantly lower percentage of females regularly cycling at the end of the study based on a limited number of animals but study not appropriate for addressing this endpoint (sexual immaturity of animals at end of study)	Stump, 2012

		No convincing impairement of sexual development in intact females	
A pubertal development and thyroid function assay in male rats, gavage acceptable even though not OECD agreed guideline (EPA OPPTS)	0, 100, 300 and 1000 mg/kg bw/d	Overall, the study is considered negative because isolated effects were either not significant or within the performance standards set in respective EPA guideline Overall, no convincing impairement of sexual development in intact males	Stump, 2012
OECD Level 3 In vivo assays providing data about selected endocrine mechanism(s) / pathway(s)			
Hershberger assay, M rat, gavage; acceptable TG441 (last update 7/09/2009)	0, 100, 300 or 1000 mg/kg bw/d 10 days	No significant effect on sex accessory gland weights in castrated males Negative	Stump, 2012
Uterotrophic assay; ovariectomized F rats, gavage acceptable TG440 (last update 16/10/2007)	0, 0, 100, 300 or 1000 mg/kg bw/d 3 days	No significant effect on sex accessory gland weights in OX females Negative	Stump, 2012
Effect of glyphosate on reproductive organs in male SD rat; gavage, supplementary non-guideline study	0, 5, 50, 500 mg/kg bw/d, 5 weeks	Significantly decreased absolute but not relative weight of seminal vesicle gland and coagulating gland. Total sperm count was significantly decreased at a dose of 500 mg/kg bw, the highest dose tested. No significant effects were detected on immuno histochemistry of androgen receptor (AR), testosterone-, oestradiol- or progesterone concentration and oxidative stress parameters	Dai et al., 2016
OECD Level 2 In vitro assays providing data about selected endocrine mechanism(s) / pathways(s)			
Oestrogen receptor transcriptional activation (human cell Line (HeLa9903)) screening assay; acceptable TG455	10^{-10} to 10^{-3} M	No agonism at hERa receptor Negative	Willoughby, 2012

Oestrogen receptor binding (rat uterine cytosol) screening assay; acceptable (EPA OPPTS)		No competition with E2 to ER of rat uterine cytosol Negative	Willoughby, 2012
Androgen receptor binding (rat prostate cytosol) screening assay; acceptable (EPA OPPTS)		No competition with methyltrienolone to AR of rat prostate cytosol Negative	Willoughby, 2012
Human recombinant aromatase assay; acceptable (EPA OPPTS)		No inhibiting effect on CYP19-adrostenedione metabolism Negative	Wilga, 2012
H295R steroidogenesis assay; Acceptable TG456 (last update 28/07/2011)		No reduction of neither oestradiol nor testosterone in H295R cells Negative	Hecker et al.,
OECD Level 2 In vitro (non-guideline studies)			
Glyphosate induces human breast cancer cells growth via oestrogen receptors; study supplementary		Glyphosate showed some oestrogenic activity in T47D cells under the conditions of this test	Thongprakaisang et al., 2013
Development of a recombinant human ovarian (BG1) cell line containing oestrogen receptor alpha and beta for improved detection of oestrogenic/ antioestrogenic chemicals; study supplementary		Glyphosate did not show any oestrogenic activity Glyphosate has no hERα, hERβ agonistic activities, in vitro under the conditions of this test	Brennan et al., 2016
Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells (JEG3) below toxic levels; study supplementary		The reported data showed that glyphosate did not significantly inhibit aromatase activity at non-cytotoxic concentrations	Defarge et al., 2016
Differential effects of glyphosate and roundup on human placental cells and aromatase; study supplementary		For the active substance, no effects were described giving evidence for endocrine disruption. As in several other published papers, however, the pesticide formulation Roundup seemed to have an array of toxic effects	Richard et al., 2005

BLTK1 murine Leydig cells: a novel steroidogenic model for evaluating the effects of reproductive and developmental toxicants; study supplementary		Glyphosate was negative in this non-guideline steroidogenesis assay	Forgacs et al., 2012
Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells in vitro; study supplementary		Proliferation of granulosa cells was impaired and at the same time E2 production inhibited in a non-dose-dependent manner by an unknown mode of action	Perego et al., 2016
Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines (HepG2); study supplementary		The data confirm that formulations are more toxic than the active substance. Some of them seem to have antiandrogenic properties. This cannot be confirmed to the same extent for the active substance, however, a non-dose-dependent reduction of transcriptional activity at the androgen receptor was observed	Gasnier et al., 2009

Endocrine disruption potential of glyphosate towards wildlife (additional information):

- OECD Level 3:
    - Amphibian metamorphosis assay (TG231): glyphosate was not found to interfere with the normal function of the hypothalamic-pituitary-thyroid (HPT) axis of African clawed frog tadpoles in this study (Schneider et al., 2012)
    - Fish short-term reproduction assay (TG229): glyphosate is concluded to not impact the function of the hypothalamus-pituitary-gonadal (HPG) endocrine axis in fathead minnows (Schneider et al., 2012)
- OECD Level 5:
    - Fish full life cycle test with fathead minnow (EPA OPPTS) : no indication of endocrine disruption

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