Comparison of the Genotoxicity of Glyphosate, (Aminomethyl)phosphonic Acid, and Glyphosate-Based Formulations Using *In Vitro* Approaches

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Abstract

Glyphosate-based herbicides are used globally for weed control. The use of glyphosate, which is applied in the form of glyphosate-based formulations (GBFs), increased dramatically with the introduction of crops that were genetically engineered for resistance to glyphosate. Public health agencies including the USEPA and the European Food Safety Authority (EFSA) have concluded that glyphosate does not pose a carcinogenic risk for humans. However, the International Agency for Research on Cancer (IARC) labeled glyphosate as a "probable human carcinogen" and included GBFs in their hazard characterization. The IARC report also cited supporting mechanistic information from genotoxicity studies. A National Toxicology Program (NTP) Toxicity Report on glyphosate published in 1992 indicated no genotoxicity and few systemic effects in rodents administered high doses (grams/kg) of glyphosate in feed for 13 weeks. However, GBFs are complex mixtures containing surfactants and detergents, and in some cases additional herbicides. There are few direct comparisons of the genotoxicity of glyphosate versus GBFs in the literature. NTP is evaluating glyphosate, glyphosate isopropyl amine, (aminomethyl)phosphonic acid (AMPA), 9 agricultural-use GBFs, 4 residential-use GBFs, and herbicides present in GBFs other than glyphosate using *in vitro* genotoxicity assays, including the MultiFlow® DNA Damage Assay, *in vitro* micronucleus (MicroFlow®) assay, and bacterial mutagenicity assays. Preliminary results indicate that glyphosate and AMPA are negative in the *in vitro* assays, while some GBFs and active ingredients other than glyphosate show genotoxic activity. These results suggest that while glyphosate alone lacks genotoxic activity, the genotoxicity of GBFs may require further evaluation. Supported by NIEHS/NTP contract HHSN273201300009C.

Introduction

Glyphosate is the most widely used herbicide in the United States and worldwide. It is applied as a formulation with other substances that help plants to absorb glyphosate. Glyphosate acts as an herbicide by preventing susceptible plants from making proteins that are needed for growth. Over the past 25 years, use of glyphosate has risen dramatically due to development of glyphosate-resistant genetically modified crops. Most people are exposed to residual amounts of glyphosate by ingestion of food or water. Individuals who regularly handle glyphosate products in occupational or residential use may experience higher exposures.

In NTP's Toxicity Report No. 16 (1992), rodents exposed to glyphosate in feed showed little evidence of toxicity, and there was no evidence of glyphosate causing damage to DNA[1]. Several public health agencies have since reviewed the scientific literature to learn whether exposure to glyphosate is a cancer hazard for humans.

- In March 2015, the International Agency for Research on Cancer (IARC) concluded that glyphosate is a likely human carcinogen based on studies in humans and animals. They also reported that glyphosate-based formulations are often more toxic than glyphosate alone[2].
- In November 2015, the European Food Safety Authority (EFSA) concluded that glyphosate is unlikely to pose a carcinogenic hazard to humans[3].
- In May 2016, the Joint Food and Agricultural Organization of the United Nations/World Health Organization Meeting on Pesticide Residues concluded that glyphosate is unlikely to pose a carcinogenic risk to humans from exposure in the diet[4].
- The United States Environmental Protection Agency (EPA) is completing a new human health risk assessment on glyphosate, including an evaluation of its cancer-causing potential.

Due to the multiple interpretations of evidence on the potential health risks of glyphosate exposure, major public concern about exposure risks, and reported differences in the toxicity of different glyphosate products, NTP is conducting additional research on glyphosate and GBFs. Glyphosate, GBFs, other herbicides in GBFs, and AMPA, a microbial metabolite of glyphosate, are being tested *in vitro* for potential mutagenicity and induction of chromosomal damage, as well as for potential clastogenic or aneugenic mechanism of action.

Materials and Methods

Preparation of Dosing Solutions:
Compounds and GBFs were provided by the NTP. Test articles were handled and stored in accordance to their MSDS and/or provided literature. Dosing solutions were prepared fresh each day of use at concentrations such that the final vehicle volume in the treated cultures was 10% (water vehicle) or 1% (DMSO vehicle).

GBFs – Formulations were diluted 1:10 with sterile water and the pH of this solution was adjusted to 7-7.6 using 2.5 N sodium hydroxide (NaOH). Serial dilutions (1:2 for Ames assays and 1:1.22 to 1:1.41 for MultiFlow® and *in vitro* micronucleus assays) were prepared using sterile water.

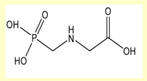
Compounds – Chemicals were weighed to the nearest 0.1 mg and dissolved or suspended in vehicle (DMSO for metolachlor and sterile water for the rest). Aqueous solutions were pH-adjusted to 7-7.6 using 2.5 N NaOH. Serial dilution was used to prepare the remaining dosing solutions for each assay.

MultiFlow® DNA Damage Assay:
Logarithmically growing human lymphoblastoid TK6 cells were exposed to 20 concentrations of test articles in 96-well plates in single wells (4 wells each for vehicle and positive controls). TK6 cells at 2.0 ± 0.25 x 10^5 cells/mL were plated into 96-well plates. For exposures +S9, the final S9 concentration was 0.5%. Treated cells were incubated at 37 ± 1° C in a humidified atmosphere with 6 ± 1% CO_2 in air and sampled at 4 and 24 h after initiation of exposures. Cells exposed with S9 were washed and resuspended in fresh culture medium prior to the 4 h sampling. At each sampling time, 25 µL of cell culture were mixed with 50 µL/well of prepared MultiFlow® Kit (Litron Laboratories, Rochester, NY) reagent in a new 96-well plate, then incubated at room temperature for at least 30 min. The cells were analyzed using a FACSCantoII™ flow cytometer equipped with a BD™ High Throughput Sampler (BD Biosciences, San Jose, CA). Raw data files from the flow cytometer were sent to Litron Laboratories for quality control and endpoint analyses.

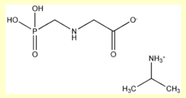
Bacterial Mutagenicity Assay:
Each test article was tested in a bacterial reverse mutation assay, including a range-finder and two independent mutation assays, in five test strains: *Salmonella typhimurium* strains TA98, TA97a, TA1535, TA100, and in *Escherichia coli* WP2 *uvrA* pKM101. Bacterial cultures were exposed to 5-7 doses of the test article, or to positive and negative controls, in triplicate, with and without 10% phenobarbital/benzoflavone-induced rat liver S9 mix using pre-incubation at 37° C for 20 minutes. The pre-incubated mixes were combined with top agar containing the appropriate amino acids (histidine/biotin for *Salmonella* strains and tryptophan for *E. coli*) and poured onto the surface of a minimal glucose agar plate. Plates were incubated at 37 ± 1 ° C for 48 ± 2 h. The number of revertant colonies was counted using the Sorcerer plate counter and Ames Study Manager software (Perceptive Instruments, Surrey, UK).

***In Vitro* Micronucleus Assay:**
TK6 cells were exposed to the test articles in 96-well plates in triplicate wells for 4 ± 0.5 h (±S9) and 24 ± 1 h (–S9) at 37 ± 1 ° C in a humidified atmosphere with 6 ± 1 % CO_2 in air. Following 4 h exposures, cells were washed and placed back into incubation in fresh culture medium. At the end of the culture period, passage through at least 1.5 cell cycles was confirmed and the cells were analyzed for cytotoxicity and micronucleus induction by flow cytometry. The flow cytometry-based high content cytotoxicity and micronucleus assay was performed using the *In Vitro* MicroFlow® kit (Litron Laboratories, Rochester, NY). Sample preparation, staining, and other methods were performed according to manufacturer's instructions and ILS SOPs. The data were collected using a Becton-Dickinson FACSCantoII™ flow cytometer equipped with a BD™ High Throughput Sampler (BD Biosciences, San Jose, CA). Unless limited by cytotoxicity, 5,000 (± 800) cells from each sample were analyzed for the frequency of micronuclei. Relative survival (measured as nuclei:bead ratios compared to the corresponding vehicle controls) and % apoptotic/necrotic cells were also determined.

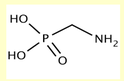
Chemical Structures



Glyphosate (N-(phosphonylmethyl) glycine)



Glyphosate, isopropyl amine salt



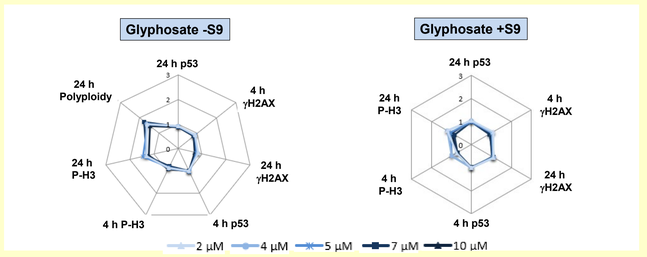
(Aminomethyl)phosphonic acid (AMPA)

Table 1: Summary of MultiFlow® Analysis of Glyphosates and Related Chemicals

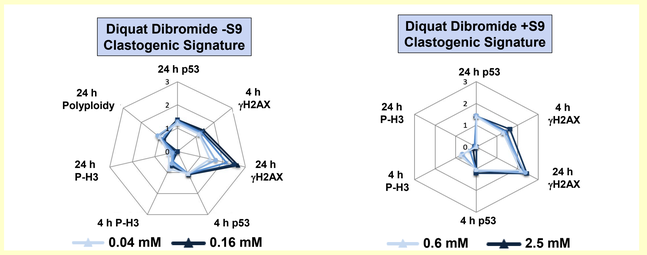
Name	-S9	Dose Range	+S9	Dose Range
Agricultural A	Clastogen (both)	1:1131 - 1:400 (ML) 1:800 - 1:400 (GEF) (28.6%)	NG	
Agricultural B	NG		NG	
Agricultural C	NG		NG	
Agricultural D	NG		NG	
Agricultural E	NG		NG	
Agricultural F	NG		NG	
Agricultural G	NG		NG	
Agricultural H	NG		NG	
Agricultural I	Clastogen (ML)	1:400 - 1:282 (48.7%)	Clastogen (GEF)	1:800 - 1:100 (31.8%)
Residential J	NG		NG	
Residential K	NG		NG	
Residential L	NG		NG	
Residential M	NG		NG	
Glyphosate acid	NG		NG	
Glyphosate isopropyl amine	NG		NG	
(Aminomethyl)phosphonic acid (AMPA)	NG		NG	
Diquat dibromide monohydrate	Clastogen (ML)	0.055-1.56 mM (29.5%)	Clastogen (both)	0.31-2.5 mM (ML) 0.078-2.5 (GEF) (34.2%)
Metolachlor	Clastogen (both)	0.31-0.62 mM (26.7%)	NG	
Mesotrione	NG		NG	

Numbers in parentheses = %RNC at highest positive exposure level; NG = non-genotoxic
ML = machine learning model prediction; GEF = global evaluation factor prediction; both = predicted by both models

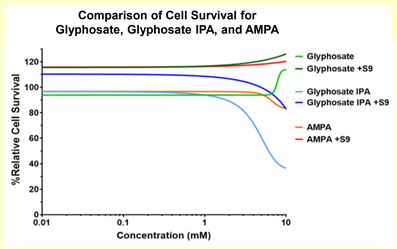
Figure 1: Clastogenicity and Cytotoxicity of Glyphosates and Related Chemicals from the MultiFlow® Assay



Radial plots of a typical non-genotoxic response in the MultiFlow® DNA Damage Assay. Glyphosate, glyphosate IPA, and AMPA were identified as **non-genotoxicants,** +/- S9.

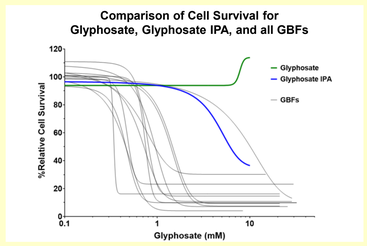


Radial plots of a typical clastogenic response in the MultiFlow® DNA Damage Assay showing activation of γH2AX (4 & 24 h), translocation of P53 (4 & 24 h), and modest attenuation of p-histone H3.



Glyphosates, including the metabolite AMPA, show little cytotoxicity in TK6 cells, especially +S9. Curves were generated using a nonlinear regression curve fitting function in GraphPad Prism.

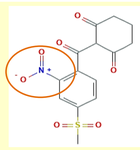
GBFs show very steep cytotoxicity curves and cytotoxicity is due to components other than glyphosate. Curves were generated using a nonlinear regression curve fitting function in GraphPad Prism.



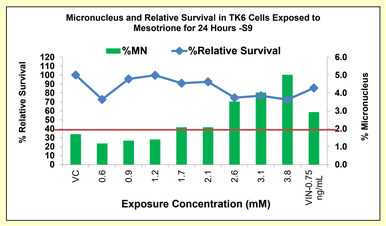
Results of Genotoxicity Testing - Mesotrione

Test Strain	Range of Responding Doses in µg/plate (Highest Fold Increase)			
	–S9		+S9	
	Assay 1	Assay 2	Assay 1	Assay 2
TA97a	500-6000 (10.5)	1500-6000 (2.7)	3000-6000 (2.7)	1500-6000 (3.1)
TA100	1500-6000 (3.4)	1500-6000 (2.5)	6000 (2.1)	NA
TA98	500-6000 (16.0)	500-6000 (11.1)	3000-6000 (3.0)	6000 (2.5)
TA1535	3000-6000 (6.6)	3000-6000 (5.2)	6000 (2.5)	NA
E. coli WP2	NA	NA	NA	NA

Table 2: Bacterial Mutagenicity Assay
Positive in TA98 and TA97a (+/-S9)
Positive in TA100 and TA1535 (-S9); magnitude of response in these strains is greatly reduced +S9
Negative in *E. coli uvrA* WP2 pKM101 (+/-S9)



The aromatic nitro group is a structural alert for mutagenicity in Leadscope



Note: Red line denotes cytotoxicity limit of 40% survival (60% cytotoxicity)

Figure 2: *In Vitro* MN Assay
Weak positive response following exposure for 24 hours –S9.

Table 3: Results Summary

Name	Constituents or Purity (%)	Ames		MultiFlow®		MicroFlow®		
		-S9	+S9	-S9	+S9	4h -S9	4h +S9	24h -S9
Glyphosate acid	95.2	negative	negative	negative	negative	negative	negative	negative
Glyphosate isopropyl amine	93.2[1]	negative	negative	negative	negative	negative	negative	negative
(Aminomethyl)phosphonic acid (AMPA)	99.8	negative	negative	negative	negative	negative	negative	negative
Agricultural A	glyphosate 20.5%; metolachlor 20.5%; mesotrione 2.05%	negative	negative	Clastogen	negative	In Progress		
Agricultural B	41% glyphosate IPA	negative	negative	negative	negative			
Agricultural C	41% glyphosate IPA	negative	negative	negative	negative			
Agricultural D	41% glyphosate IPA	negative	negative	negative	negative			
Agricultural E	44.9% glyphosate, K salt	negative	negative	negative	negative			
Agricultural F	48.7% glyphosate, K salt	negative	negative	negative	negative			
Agricultural G	48.8% glyphosate, K salt	negative	negative	negative	negative			
Agricultural H	50.2% glyphosate, dimethylamine salt	negative	negative	negative	negative			
Agricultural I	53.8% glyphosate IPA	negative	negative	Clastogen	Clastogen			
Residential J	1.92% glyphosate IPA	negative	negative	negative	negative			
Residential K	18% glyphosate IPA; diquat dibromide 0.73%	negative	negative	negative	negative			
Residential L	41% glyphosate IPA	negative	negative	negative	negative			
Residential M	50.2% glyphosate IPA	negative	negative	negative	negative			
Diquat dibromide monohydrate	99.5	negative	negative	Clastogen	Clastogen	+	negative	+
Metolachlor	98	negative	negative	Clastogen	negative	negative	negative	negative
Mesotrione	98	+	+	negative	negative	negative	negative	+

[1]Certificate of Analysis: chromatographic purity 98.5% minus water content of 5.3%

Discussion and Conclusions

- Glyphosate, glyphosate IPA, and AMPA did not show genotoxic activity and were not cytotoxic to human lymphoblastoid TK6 cells
  - Based on this data set, it is unlikely that any genotoxic activity of GBFs is due to glyphosate
- Some GBFs showed genotoxic activity in the MultiFlow® assay; GBFs were not active in bacterial mutagenicity assays
- Three other herbicides in GBFs (diquat dibromide, metolachlor, and mesotrione) showed genotoxic activity in different tests
- Genotoxicity of one GBF (Agricultural I) could not be attributed to a known active

References

1. National Toxicology Program Technical Reports Series Number 16 NTP Technical Report on toxicity studies of glyphosate (CAS No. 10371-83-6) administered in dosed feed to F344/N rats and B6C3F1 mice. NIH Publication 92-3135, July 1992.
2. Some organophosphates insecticides and herbicides. International Agency for Research on Cancer (IARC) Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 112. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Lyon, France, March 2015.
3. EFSA (European Food Safety Authority), 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA Journal 2015;13(11):4302, 107 pp. doi:10.2903/j.efsa.2015.4302.
4. Pesticide residues in food 2016. Special Session of the Joint FAO/WHO Meeting on Pesticide Residues, Geneva, Switzerland, May 2016.

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