Effects of Glyphosate and its Formulations on Markers of Oxidative Stress and Cell Viability in HepaRG and HaCaT Cell Lines

J. R. Rice, P. Dunlap, *S. Ramaiahgari*, *S. Ferguson*, *S. L. Smith-Roe*, and *M. DeVito*.
Division of the National Toxicology Program, Research Triangle Park, NC..

Abstract

Glyphosate (GLY) is the active ingredient found in herbicide formulations worldwide. GLY is toxic to plants by disrupting the shikimate amino acid synthesis pathway. The present day intensive use of GLY began with the introduction of GLY-resistant crops in the late 1990s. Although GLY has a low toxicity profile for humans and mammals, conflicting reports exist as to whether it poses a cancer risk for humans. The USEPA and European regulatory agencies have described GLY as unlikely to pose a carcinogenic hazard to humans. However, the International Agency for Research on Cancer (IARC) has classified GLY as “probably carcinogenic to humans”.

IARC proposed that oxidative stress may be a mechanism by which GLY could potentially cause cancer. To address this hypothesis, we are testing GLY in human cell lines using several assays that detect reactive oxygen species (ROS) or their effects. Studies were designed to compare the point of departure for the effects of GLY on cell viability (CellTiter-Glo assay) to the point of departure for effects in oxidative damage assays. We also directly compared the effects of GLY versus GLY salts, as well as GLY and adjunct active ingredients versus formulations. We used a high content, 384-well plate approach to generate extensive dose-response curves for multiple comparisons.
Assays (CellTiter-Glo, ROS-Glo, and JC10) were performed after 1 or 24 h of exposure to test articles. GLY and GLY isopropylamine decreased cell viability and altered mitochondrial membrane potential (MMP) at ≥ 10 mM, but did not affect ROS production. The formulations were more potent than GLY alone. Cell viability and MMP were significantly altered at 1 h by the formulations. Based on GLY concentrations, these mixtures were over 1000x more potent than GLY alone. In contrast to the robust induction of ROS by positive controls at both time points, formulations had no effect on ROS at 1 h and showed a marginal increase in ROS at 24 h. These data suggest that GLY does not induce oxidative stress. In addition, the formulations marginally increased oxidative stress only after significant loss of cell viability. The results were very similar for both HepaRG and HaCaT cell lines, suggesting that xenobiotic metabolism has little impact on cell viability and oxidative stress induced by these chemicals.

Introduction

GLY (*N*-(phosphonomethyl) glycine) was first discovered in 1970 and is the most widely used herbicide in the USA and worldwide, mainly because many staple crops have been genetically modified to be resistant to GLY. It is applied to plants as a formulation with other substances that promote absorption of GLY. In plants and some microorganisms, the molecular target of GLY is enolpyruvyl shikimate-3-phosphate synthase, an enzyme that is required for aromatic amino acid synthesis. Molecular targets of GLY in mammals have not yet been identified . Observational studies suggest that GLY and GLY formulations may cause oxidative stress and damage to DNA. Oxidative stress is an imbalance caused when free oxygen radical production (superoxide, hydrogen peroxide) exceeds the body's ability to neutralize the free radicals with antioxidants. This can result in oxidative damage to proteins, lipids, and DNA. Subsequently, this may lead to chronic diseases such as diabetes, atherosclerosis, rheumatoid arthritis, cancer, and chronic inflammation.
IARC has proposed that there is strong evidence that GLY and GLY formulations induce oxidative stress and that this may be involved in their potential carcinogenicity. In the current study, we assessed whether GLY and GLY formulations induce oxidative stress which can cause genotoxicity and also whether the GLY formulations are more effective than GLY alone in producing cytotoxicity and inducing oxidative stress.

Objective

To determine if either glyphosate or its formulations induce oxidative stress in two different cell lines, HaCaT and HepaRG.

To compare these effects with those of positive controls for oxidative stress and relate these effects to cell viability.

Test Articles

7 Glyphosate formulations

- 6 – farm use products labeled A-F
- 1 – residential use product

Positive Controls

- Antimycin
- Diquat Dibromide
- Etoposide
- Menadione
- Tertbutyl hydroperoxide

5 Actives

- Aminomethylphosphonic Acid (AMPA)
- Glyphosate
- Glyphosate Isopropyl ammonium salt (IPA)
- Metolachlor
- Mesotrione

Formulations range from 1.92 - 50.2% glyphosate. Glyphosate and glyphosate isopropylamine are the two forms of glyphosate used in these products. AMPA is a bacterial metabolite of glyphosate. In addition to glyphosate, one of the products contains diquat dibromide and another contains both mesotrione and metalochlor. All solutions of test articles were pH adjusted to ~7.2.

Methods

- Culturing and Plating HaCaT Cells
  HaCaT cells were obtained from AddexBio Technologies, Inc. (San Diego CA) .These cells were chosen because IARC cited studies by Gehin et al (2005 and 2006) and Hue et al (2012) as studies that indicated strong evidence of glyphosate induced oxidative stress that used these cells. Cells (10^7 cells/mL) were removed from frozen storage, thawed, and transferred to a 50ml centrifuge tube filled with HaCaT media (500ml DMEM (Gibco Cat #11965-092, Gaithersburg, MD), 50ml Fetal Bovine Serum (Gibco Cat # 10438034, Gaithersburg, MD), and 2.5ml Pen/Strep (Sigma Cat # P0781; St Louis, MO)) and spun down at 2000 rpm for 2 min. The supernatant is removed and cells are suspended in 5 ml HaCaT media. Cells were then transferred to a T25 flask and placed in the incubator at 37ºC. Cells are passed when nearly confluent (roughly every other day. Once expanded, cells were treated with 0.05% trypsin as described above and were diluted to $2X10^5$ cells/ml with HaCaT media and 50ul of this solution was added to each well with a final cell concentrations were 10,000 cells/well.

- Culturing and Plating HepaRG Cells
  HepaRG™ cells were chosen due to their xenobiotic metabolism activity (Ramaiahgari et al 2017). No-Spin HepaRG™ cells were obtained from (Triangle Research Laboratory, RTP, NC). Cells were removed from frozen storage, thawed, and transferred to a 50ml centrifuge tube filled with Thawing and Plating Media (500 mL of Williams E Media (Life Tech/Gibco, Gaithersburg, MD; Cat# A12176-01) plus Thawing and Plating media supplements (Triangle Research Laboratory, RTP, NC; Cat. MHTAP) and 25 U/ml penicillin and 25 µg/ml streptomycin (Sigma Cat # P0781; St Louis, MO). Once thawed, cells were diluted to $5x10^5$ cells/ml with Thawing and Plating media and 50ul of this solution was added to each well. Final cell concentrations were ≈25,000 cells/well. Cells were allowed to attach overnight and the Thawing and Plating media was removed and replaced with Pre-Induction/Tox Supplements (Triangle Research Laboratory, RTP, NC; Cat. MHPIT). Cells were incubated at 37ºC for 5 days with media changes occurring every 2-3 days prior to exposure to chemical.

- 10 dose concentrations in duplicate, 7 formulations or 5 actives and 7 positive or negative controls per plate.
  - ✓ Third log dosing regime
  - ✓ 1 and 24 h exposures
  - ✓ Image plates at time 0 and 24 h using an IncuCyte imaging system (Essen BioScience, Inc.; Ann Arbor MI)
  - ✓ Perform assays at 1 and 24 h

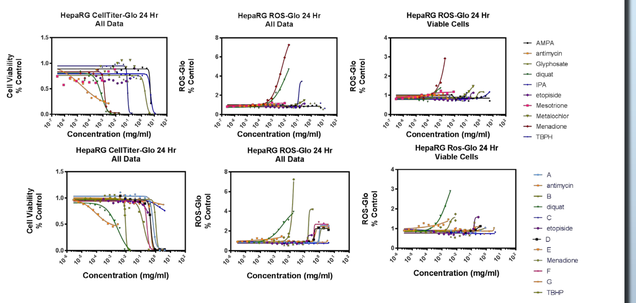
- Cytotoxicity assay
  CellTiter-Glo (Promega; Madison WI) was performed according to manufactuer’s protocol and data were captured using a ClarioStar plate reader (BMG LabTech; Cary, NC).

- Mitochondrial Membrane Potential (MMP) assay
  JC10 (Enzo Life Sciences, Farmingdale, NY) was performed by adding 30ul of a 10uM solution of JC10 in KREBS buffer (Sigma Aldrich, St. Louis MO) to cells, incubating for 1h, and data collecting data at 2 wavelengths using a ClarioStar plate reader. The result was the ratio of λ520/λ570.

- Reactive Oxygen Species assay
  ROS-Glo (Promega; Madison WI) was performed according to manufacturer's protocol on spent media (duplexed with CellTiter-Glo assay).

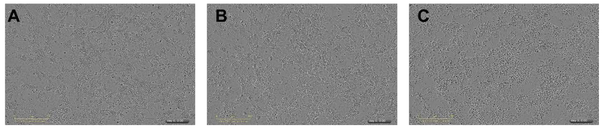
- Data Analysis
  All dose response data were fit to a Hill model using non-linear regression analysis (Prism 7.0 for Windows).

Figure 1. HepaRG 24h Results



Data is presented from one replicate study (n=2) and is representative of the other two replicates.
Note: Glyphosate concentrations ranged from 0.035-33 mM. Formulations A-F ranged from 0.014-16 mM while Formulation G ranged from 0.0007-0.66 mM

Phase Contrast Imaging of control and glyphosate formulation treated wells



A - Control HepaRG cells: Cells are a mixture of hepatocyte-like cells that form a cord like architecture with a cobblestone appearance with cholangiocytes that are flat and diffuse cells with poorly defined nuclei.
B - Mid dose level of formulation A (24 h exposure): Focal areas of the culture appear to be pulling apart from one another. Other areas of the culture appear normal. Little evidence of dead cells. Cellular ATP is approximately 50% of controls.
C - High dose formulation A (24 h exposure). The hepatocytes display a condensed phenotype, while the cholangiocytes appear to have detached from the plate. ATP is depleted to < 10% of control.

These images are examples of the relationship between cell morphology and the corresponding cell viability, as measured by ATP-depletion. In cultures where cellular ATP levels were less than 10% of the controls, there is considerable evidence of cell death based on morphology (B). In cultures where cellular ATP is approximately 50% of controls, cells demonstrate modest levels of stress (C).

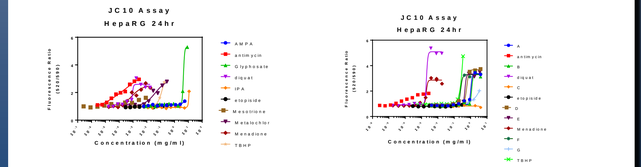
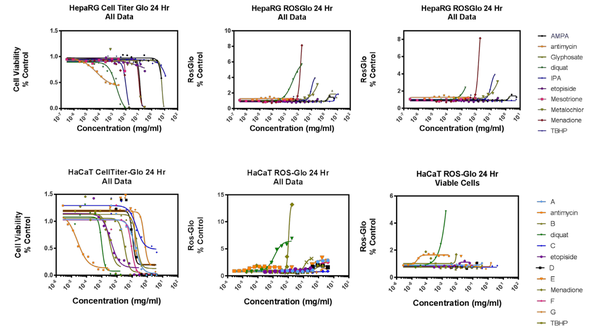


Figure 3. HaCat 24h Results



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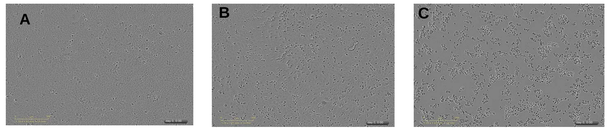

A - Control HaCaT cells: Cells human keratinocytes that form a monolayer of small cells.
B - Mid dose level of formulation A (24 h exposure): Focal areas of the culture appear to be pulling apart from one another. Other areas of the culture appear normal. Some evidence of dead cells. Cellular ATP is approximately 50% of controls.
C - High dose formulation A (24 h exposure). The HaCaT cells display a condensed phenotype, and a large number of cells appear to have detached from the plate. ATP is depleted to < 10% of control.

These images are examples of the relationship between cell morphology and the corresponding cell viability, as measured by ATP-depletion. In cultures where cellular ATP levels were less than 10% of the controls, there is considerable evidence of cell death based on morphology (B). In cultures where cellular ATP is approximately 50% of controls, cells demonstrate modest levels of stress (C).

EC_{50}s for Positive Controls

HaCat EC_{50}s

		Cell Titer Glo EC_{50}s		ROSGlo EC_{50}s	
Compound	MW	mg/ml	uM	mg/ml	uM
Gly	169	2.05000	12.13018	1.646	9.73964
Antimycin	548.63	0.00002	0.00003	0.00001657	0.00003
Diquat	362.06	0.00114	0.00315	0.0013375	0.00369
Etoposide	588.56	0.00894	0.01520	0.026655	0.04529
Menadione	172.18	0.00228	0.01323	0.010955	0.06363
TBHP	90.12	0.02181	0.24201	0.080025	0.88798

HepaRG EC_{50}s

		Cell Titer Glo EC_{50}s		ROSGlo EC_{50}s	
Compound	MW	mg/ml	uM	mg/ml	uM
Gly	169	2.06400	12.21302	not conv.	
Antimycin	548.63	0.00005602	0.00010	0.01465	0.02670
Diquat	362.06	0.00172	0.00476	0.007477	0.02065
Etoposide	588.56	0.32791	0.55713	0.1578	0.26811
Menadione	172.18	0.00524	0.03043	0.0146965	0.08536
TBHP	90.12	0.12046	1.33666	0.08624825	0.95704

Conclusions

- Glyphosate formulations decreased cell viability by >90% at glyphosate equivalents approximately 20-50 fold lower than glyphosate.
- In both the HaCaT and HepaRG cells, glyphosate, glyphosate isopropyl amine and AMPA, a glyphosate metabolite, did not increase hydrogen peroxide production.
- In both HaCaT and HepaRG cells, some, but not all glyphosate formulations caused a slight increase (≈ 2-3 fold) in hydrogen peroxide, but only at concentrations that produced a > 90% decrease in cellular ATP and increases in cell stress and death, as evaluated with phase contrast imaging.
- In contrast, the positive controls increased hydrogen peroxide (up to 15 fold) at concentrations that did not induce cell death.
- The positive controls were 10-1000 times more potent in the cell viability assay and in production of ROS. In addition, menadione induced ROS by approximately 4-5 fold more than the most active glyphosate formulation.
- The effects of glyphosate formulations appear to be due to the formulants and not to the glyphosate or other active ingredients.
- These data suggest that in the present study ROS induction by glyphosate formulations is most likely a result of significant cell death and not the cause of the cell death.

References

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Acknowledgements

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