Effects of Glyphosate and its Formulations on DNA Damage in HepaRG and HaCaT Cell Lines

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Abstract

Glyphosate (GLY) is an active ingredient found in herbicide formulations around the world. It affects the shikimate pathway in plants, blocking the activity of enolpyruvlshikimate-3-phosphate synthase. GLY has been around since the 1970s, but intensive use of GLY began with the introduction of GLY-resistant crops in the late 1990s. Conflicting reports exist as to whether GLY poses a cancer risk for humans, though it has a low toxicity profile for humans and mammals. Both European regulatory agencies and the USEPA have described GLY as unlikely to pose a carcinogenic hazard to humans, but the International Agency for Research on Cancer (IARC) and the California EPA have classified GLY as "probably carcinogenic to humans" and "known to the State of California to cause cancer," respectively.

It has been proposed that GLY induces oxidative stress potentially leading to cancer. To address this hypothesis, we tested GLY in two human cell lines, HepaRG (metabolically competent hepatocytes) and HaCaT (human keratinocytes) using the γ-H2AX assay which detects DNA double strand damage. Thirteen formulations with high concentrations ranging from 3 mM to 103mM and 5 actives including GLY, GLY salts, and other active ingredients in the formulations were tested at 1h and 24h at 10 concentrations. The results were then compared the effects of glyphosate and its formulations on cell viability (CellTiter-Glo assay) to determine whether GLY induces oxidative stress and DNA damage. While positive controls diquat and etoposide elicited a marked increase in γ-H2AX phosphorylation at concentrations lower than those that reduced cell viability, glyphosate and its formulations showed no or minimal changes in γ-H2AX phosphorylation and then only at concentrations that induced significant loss of cell viability. In fact, the only formulation to show some DNA damage also contains diquat as an active ingredient. This data suggests that GLY does not induce double strand DNA damage in HaCaT and HepaRG cells. It should be noted that the formulations marginally increased oxidative stress only after significant loss of cell viability. The results were very similar in both the HepaRG and HaCaT cells, suggesting that xenobiotic metabolism has negligible impact on toxicity.

Objectives

- Determine whether GLY and GLY formulations cause double strand DNA (dsDNA) damage using the γ-H2AX assay which detects DNA double strand damage.
- Evaluate the ability of GLY and GLY formulations to increase H_2O_2 and decrease cell viability and decrease glutathione concentrations.

Methods

The cells were seeded into 384 well plates, with HaCaT incubating for 24h and HepaRG for 5 days. The dosing scheme consisted of 10 concentrations in duplicate at third log intervals. Plates were run in triplicate. After 24h the CellTiterGlo™, Glutathione Glo (GSHGlo™) and ROSGlo ™ (Promega, Madison WI) plates were read for luminescence on a Clariostar plate reader.

The plates used for the H2AX1 assay were fixed using 4% formalin, followed by exposure to primary mouse antibody (EMD Millipore # 05-636) and secondary goat anti-mouse antibody (Invitrogen# A11-029), stained with Hoechst dye, and imaged using the IXM imaging system.

Test Articles

13 Glyphosate formulations
- Glystar Plus
- Cornerstone Plus
- Roundup Custom
- Roundup Super Concentrate
- Roundup Concentrate Plus
- Durango DMA
- Kill-Zall
- Remuda Full Strength
- Roundup PowerMax
- Touchdown Total
- Buccaneer Plus
- Halex GT
- Roundup WeatherMax

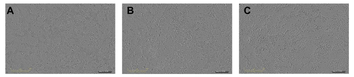
5 Actives
- Aminomethylphosphonic Acid (AMPA)
- Glyphosate
- Glyphosate Isopropyl ammonium salt (IPA)
- Metolachlor
- Mesotrione

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

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.

Image 1: Phase contrast

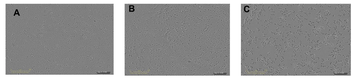
Phase Contrast Imaging of control and glyphosate formulation treated wells



A - Control HepaRG cells: Cells are a mixture of hepatocyte-like cells that form a cord like architecture with a cobblestone appearance and cholangiocytes that are flat and diffuse cells with poorly defined nuclei.

B - Mid dose level of Glystar Plus: Some areas of the culture appear to be pulling apart from one another while other areas appear normal. Little evidence of dead cells. Cellular ATP is approximately 50% of controls.

C - High dose Of Glystar Plus. Hepatocytes display a condensed phenotype, while the cholangiocytes appear to have detached from the plate. ATP is < 10% of control.



A - Control HaCaT cells: Cells human keratinocytes that form a monolayer of small cells.

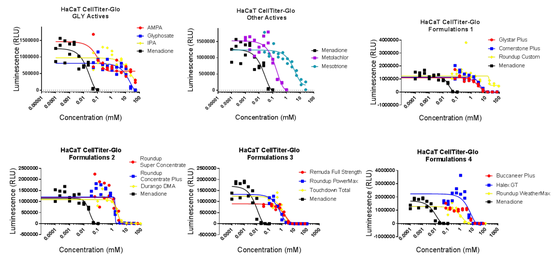
B - Mid dose level of Glystar Plus: Focal areas of the culture appear to be pulling apart from one another. Other areas of the culture appear normal. Some evidence of dead cells. Cellular ATP is approximately 50% of controls.

C - High dose of Glystar Plus. HaCaT cells display a condensed phenotype, and a large number of cells appear to have detached from the plate. ATP is < 10% of control.

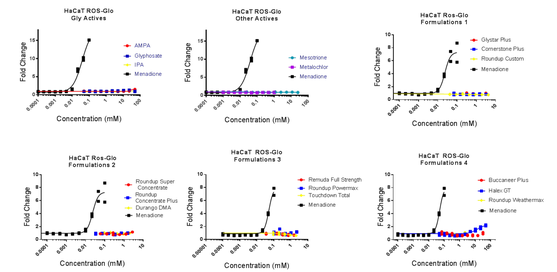
These images are examples of the relationship between cell morphology and the corresponding cell viability, as measured by ATP-depletion. In cultures where cellular ATP levels were less than 10% of the controls, there is considerable evidence of cell death based on morphology (B). In cultures where cellular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

Figure 1. HaCaT 24h Results

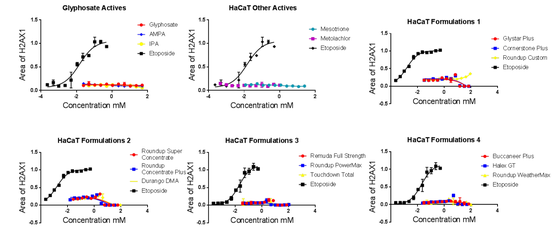
CTG HaCaT 24h



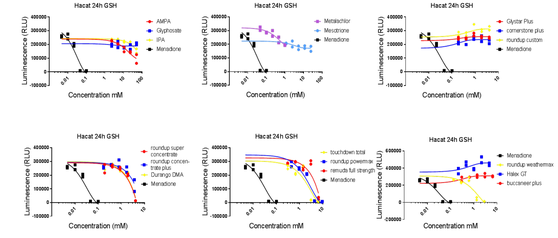
HaCaT ROS-Glo



H2AX HaCaT 24h



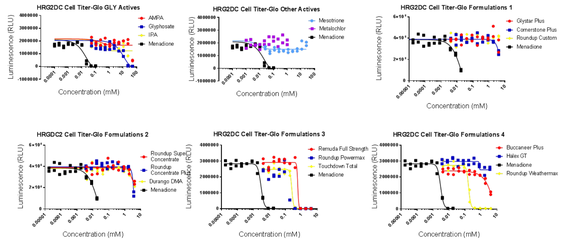
GSH Glo HaCat 24h



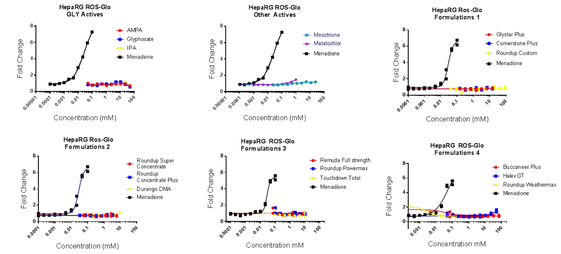
Note: Concentrations of Formulations are based on free glyphosate

Figure 2. HRG2DC 24h Results

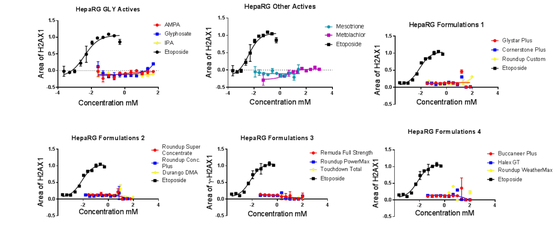
HRG2DC Cell Titer-Glo 24h



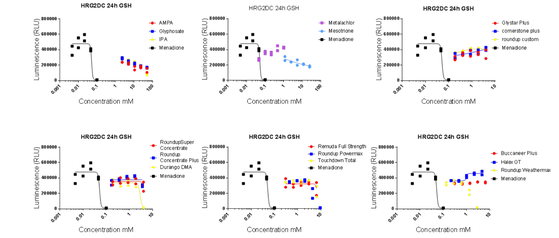
HRG2DC ROS-Glo 24h



HRG2DC H2AX 24h

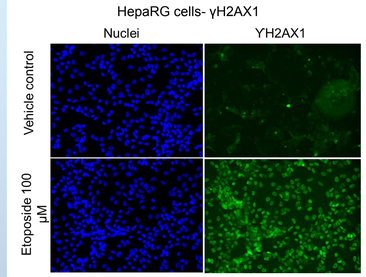


HRG2DC GSH glo 24h



Note: Concentrations of Formulations are based on free glyphosate

Image 2: Example of γ-H2AX1



- Early cellular response to Double-strand-breaks
- Discrete nuclear foci are formed as a result of H2AX phosphorylation

Conclusions

Gly and its formulations decreased cell viability and induced cell death at concentrations of 10mM or above.

Our positive controls for oxidative stress and DNA damage induced changes in these pathways at concentrations below those that altered cell viability

Gly and its formulations did not induce DNA damage and oxidative stress

References

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Acknowledgements

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