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The surfactant co-formulant POEA in the glyphosate-based herbicide RangerPro but not glyphosate alone causes necrosis in Caco-2 and HepG2 human cell lines and ER stress in the ToxTracker assay

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ABSTRACT

The toxicity of co-formulants present in glyphosate-based herbicides (GBHs) has been widely discussed leading to the European Union banning the polyoxyethylene tallow amine (POEA). We identified the most commonly used POEA, known as POE-15 tallow amine (POE-15), in the widely used US GBH RangerPro. Cytotoxicity assays using human intestinal epithelial Caco-2 and hepatocyte HepG2 cell lines showed that RangerPro and POE-15 are far more cytotoxic than glyphosate alone. RangerPro and POE-15 but not glyphosate caused cell necrosis in both cell lines, and that glyphosate and RangerPro but not POE-15 caused oxidative stress in HepG2 cells. We further tested these pesticide ingredients in the ToxTracker assay, a system used to evaluate a compound's carcinogenic potential, to assess their capability for inducing DNA damage, oxidative stress and an unfolded protein response (endoplasmic reticulum, ER stress). RangerPro and POE-15 but not glyphosate gave rise to ER stress. We conclude that the toxicity resulting from RangerPro exposure is thus multifactorial involving ER stress caused by POE-15 along with oxidative stress caused by glyphosate. Our observations reinforce the need to test both co-formulants and active ingredients of commercial pesticides to inform the enactment of more appropriate regulation and thus better public and environmental protection.

1. Introduction

Glyphosate, an N-phosphonomethyl-derivative of glycine, is used as an active ingredient in herbicides, such as Roundup, to control weeds in agricultural fields and urban environments, and also to desiccate crops shortly before harvest. Commercial glyphosate-based herbicides (GBHs) are a complex mixture of glyphosate and other ingredients called co-formulants. The major GBH co-formulants are surfactants, which allow glyphosate penetration through plant cell walls (Anderson and Girling, 1983). Although co-formulants are listed as "inert" by GBH manufacturers as they are deemed not to have target herbicidal action, it has long been known that these complex mixtures of compounds are highly toxic in their own right (Mesnage et al., 2019) and ignoring their toxicity constitutes a regulatory oversight (Mesnage and Antoniou, 2018).

There are various routes through which humans can be exposed to GBH co-formulants. First, GBH applicators in agricultural, urban and domestic settings are subject to uptake via inhalation and dermal absorption. In addition, non-occupational exposure to glyphosate-based herbicide application can arise from aerial spraying in both an agricultural context (cultivation of glyphosate tolerant genetically modified crops) (Paz-y-Miño et al., 2007) and in an effort to control coca and poppy production in Colombia (Solomon et al., 2007). Second, for populations at large foodstuffs constitute the main source of co-formulant ingestion, especially those derived from crops sprayed with GBHs just prior to harvest (e.g., cereals, pulses). Given the recognised toxicity of GBH co-formulants (Mesnage and Antoniou, 2018; Mesnage et al., 2019), it is essential to assess the health implications of exposure to the complete GBH formulations and not just glyphosate alone. However, a major obstacle to such investigations is that co-formulants used in GBHs are generally held by manufacturers as

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proprietary confidential information. As a result, although the full composition of a GBH must be submitted to regulatory agencies, details of co-formulants in most cases are withheld and do not appear on the product specification sheets provided to applicators and consumers.

Understanding the human health implications of exposure to GBH co-formulants has become a priority within the European Union (EU), who has launched a biomonitoring programme to try and address this concern (HMB4EU, 2018). The HMB4EU initiative has highlighted the co-formulant polyoxyethylene tallow amine (POEA) as a priority substance to evaluate exposure to GBH (HMB4EU, 2018). In 2016, the EU Commission recommended to Member States that POEA-type co-formulants be banned from use in GBHs (Mesnage et al., 2019).

As a result of the phasing out of POEA use, manufacturers of GBHs have been replacing this co-formulant with other surfactants. Previously, we developed and validated a mass spectrometry method to measure the urinary excretion of surfactants present in Roundup MON 52276, the European Union GBH representative formulation (Mesnage et al., 2021b). In this study we identified that the listed surfactant co-formulant in Roundup MON 52276, Dogiden 4022, is a propoxylated quaternary ammonium compound (Mesnage et al., 2021). Furthermore, in this previous study we demonstrated that our method was highly accurate, precise, sensitive and reproducible to estimate the oral absorption of MON 52276 surfactants in rats exposed to this GBH via drinking water. Based on this prior success, the first objective of the investigation presented here was to use a similar mass spectrometry approach to evaluate the surfactant composition in the widely used US GBH formulation RangerPro marketed by Bayer Corporation, which would lay a foundation for the development of new environmental epidemiology studies.

The second objective of this study was to assess the cytotoxicity of RangerPro compared to glyphosate and POE-15 tallow amine. In a previous investigation, a comparison of 9 GBHs to glyphosate by combining mass spectrometry and cell culture to identify the contribution of POEA to the toxicity of these products, found that all the formulations were more cytotoxic than glyphosate alone (Mesnage et al., 2013). We therefore chose to determine the cytotoxicity profile of RangerPro using two different human tissue culture cell line model systems namely Caco-2 epithelial cells as a representative of the intestinal epithelium, which is the first tissue exposed to GBHs through the diet, and HepG2 hepatoma cells, which are a known reliable model to evaluate glyphosate toxicity (Gasnier et al., 2011). In addition, we also used a mammalian stem cell-based genotoxicity experimental system (ToxTracker Assay), which is designed to evaluate a chemical's ability to induce DNA damage, oxidative stress or activation of an unfolded protein response (Hendriks et al., 2016) and thus assess its carcinogenic capability.

2. Materials and methods

2.1. Chemicals

Standard reagents were of analytical grade and obtained from Sigma Aldrich (Gillingham, Dorset, UK), unless otherwise stated. Glyphosate was obtained from Sigma Aldrich N (CAS: 1071-83-6, purity ≥96%, catalog no: 337757). The formulation RangerPro was sourced from the US market. POE-15 tallow amine was purchased from ChemService (distributed by Greyhound Chromatography and Allied Chemicals, Birkenhead, UK). Stock solutions of glyphosate, RangerPro and POE-15 were prepared in foetal bovine serum (FBS)-free tissue culture cell medium. RangerPro was diluted accordingly in phenol red-free Dulbecco's Modified Eagle Medium (DMEM).

2.2. Mammalian cell tissue culture

The human colonic tumour-derived epithelial cell line Caco-2 was obtained from the American Type Culture Collection (ATCC) and used between passages 46 and 66. The human hepatoma HepG2 cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC) and was used between passages 53 and 65. Both cell lines were grown in phenol red-free DMEM supplemented with 10% FBS, 10 mg/ml 200 mM L-glutamine and 10 mg/ml penicillin/streptomycin. DMEM without phenol red, FBS, penicillin/streptomycin and trypsin (0.05 and 0.5%), L-glutamine, penicillin/streptomycin and DMSO were all obtained from ThermoFisher Scientific (ThermoFisher Scientific, Loughborough, UK). Cells were maintained in 75 cm^2 flasks (Corning, Tewksbury, USA) under standard culture conditions of 37 °C and 5% CO_2 air atmosphere. For experimentation, cells were seeded into 96 well plates from stock cultures that were no more than 70% confluent.

2.3. Cytotoxicity assays

Caco-2 and HepG2 cells were seeded at 20,000 cells/well in 100 μ l of medium in clear 96 well plates. Following a 24 h incubation, cells were treated with the test substances diluted accordingly to the desired concentrations in tissue culture maintenance medium. After a further 24 h incubation, an MTT assay was performed to assess cell proliferation and thus cytotoxicity according to the manufacturer's instructions. Cells were incubated for 2 h in MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution at 1 mg/ml in PBS. The resulting formazan precipitate was then dissolved by addition of 100 µl dimethyl sulfoxide (DMSO) and quantified spectrophotometrically at 560 nm using a GloMax Multi Microplate Multimode Reader (Promega, Madison, USA). Cell viability was expressed as a percentage relative to the negative control, untreated cell samples.

2.4. ToxiLight cell membrane damage assay

Caco-2 and HepG2 cells were seeded at 10,000 cells/well in 50 µl of medium in white-walled, clear-bottomed 96-well plates and incubated for 24 h. Subsequently, Caco-2 cells were treated with the concentrations of pesticides that produced the lethal concentration 50 (LC50; 50% cell viability), the cytotoxicity threshold (LC99) or with the LC99/5. HepG2 cells were also treated at concentrations corresponding to LC50, LC50/2 and (LC50/2)/2 values. Following incubation, the ToxiLight kit (Lonza, Slough, Berkshire, UK) was used according to the manufacturer's instructions to assess cell membrane rupture. Briefly, a 50 µl aliquot of the AK reagent was added to each well and after 5 min the plates were read using the GloMax Multi Microplate Multimode plate reader with excitation 485 nM and emission 520 nM settings. The background luminescence from wells with tissue culture medium alone was subtracted, and luminescence compared relative to the negative control, untreated cell samples. Triton X100 (0.05%) was used as a positive control, cell membrane damage-inducing agent.

2.5. Oxidative stress

Caco-2 and HepG2 cells were seeded at 10,000 cells/well in 80 μ l medium in 96 well white-walled plates, incubated for 24 h and then treated with the test substances at the desired concentrations. The positive control, reactive oxygen species (ROS)-inducing agent treatment was with 50 μ M menadione. Immediately after treatment, 20 µl H_2O_2 substrate was added to each well. At 6 h post treatment, 100 µl ROS-Glo detection reagent (Promega, Southampton, UK) was added per well, as per manufacturer's instructions. The plates were then incubated at room temperature in the dark for 20 min and the luminescence read using the GloMax Multi Microplate Multimode plate reader at excitation 485 nM and emission 520 nM settings.

2.6. Mass spectrometry analysis

The glyphosate-based herbicide RangerPro formulation samples were diluted 1:100 with acetonitrile-water (1:1) containing 0.1% formic

acid prior to their injection into the LC-MS/MS system. The UHPLC-MS/MS instrument employed was a Thermo Scientific Accela™ UHPLC coupled to a Thermo Scientific TSQ Quantum Access™ mass analyser with a heated electrospray ionization source (HESI-II). The mass spectrometer was operated in positive ion mode and data acquired with Xcalibur software. The injection volume was 5 μl and the chromatographic separation was achieved using a Thermo Scientific Accucore C18 column (2.6 μm, 100 × 2.1 mm) maintained at 40 °C. A binary gradient profile was developed using water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 200 μl/min (min). HPLC grade acetonitrile and HPLC grade water were from Fisher Scientific, and LC-MS grade formic acid was from Merck (Merck Life Science UK Limited, Gillingham, UK). Separations were conducted under the following chromatographic conditions: 95% solvent A for 0.2 min, decreased to 5% over 15 min, maintained for 5 min at 5% before being increased to 95% over 0.1 min. Column equilibration time was 4.2 min, with a total runtime of 25 min. Mass spectrometric conditions were as follows: spray voltage 3.5 kV, capillary temperature 350 °C, vaporizer temperature 300 °C, sheath gas 50 au, auxiliary gas 5 au, full scan mode 150–1200 m/z.

2.6.1. ToxTracker assay system

The ToxTracker assay system was used as previously described (Mesnage et al., 2021f). Briefly, first cytotoxicity profiles of the test substances was undertaken using the wild-type murine embryonic stem (mES) cell line B4418, with cells exposed to 20 different concentrations of the test substances at a maximum concentration of 10 mM or 1 mg/ml. Cytotoxicity was estimated by cell count after 24 h exposure by flow cytometry and was expressed as the percentage of viable cells compared to untreated control cells.

Based on the cytotoxicity analysis, 5 concentrations of the test substances were selected for testing in the ToxTracker system. ToxTracker consists of six mES cell lines (Hendriks et al., 2016). Each cell line was seeded in 96-well cell tissue culture plates at 50,000 cells per well. After 24 h, the test substances were added. The response to the test materials was evaluated at five concentrations in a 2-fold dilution series. GFP reporter gene expression was determined after 24 h by flow cytometry to assess induction compared to untreated control cultures taking into account cytotoxicity and cell viability corrective measures. Possible effects from metabolic activation of test substances was assessed by conducting the same assays in the presence of 0.25% S9 rat liver extract plus required co-factors (Moltox, Boone, NC, USA).

The following treatments were included as positive controls for the various ToxTracker measures: cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded protein response) and cyclophosphamide (metabolic activation of progenotoxins by S9 rat liver extract).

2.7. Statistical analysis

The dose response on Caco-2 and HepG2 cells was used to determine cytotoxicity thresholds using nonlinear dose-relationships. We determined the LC50, the concentration at which 50% of the cells are viable. Statistical analysis was performed by one-way ANOVA in GraphPad prism 5. Primary ToxTracker data were generated as flow cytometry (.fcs) files. Mean GFP expression and viable cell concentrations after treatment were exported as text files (.csv) that are imported into Microsoft excel for calculation of reporter gene induction and cytotoxicity. ToxTracker is considered to give a positive response when a compound induces at least a 2-fold increase in GFP reporter gene expression in any of the six cell assay systems. GFP induction at test substance concentrations that do not cause more than 75% cytotoxicity are used for the ToxTracker analysis.

3. Results and discussion

We determined the surfactant composition of RangerPro, a widely used GBH in the US, using a mass spectrometry approach. The comparison of RangerPro to the POE-15 tallow amine standard revealed that both products contain a complex mixture of surfactants (Fig. 1A). Peaks for polyoxyethylene tallow amines with several different polyoxyethylene chain lengths were observed (Fig. 1B). This is due to the successive addition of ethylene oxide molecules to form POE does not occur at the same speed for each POE in the melange. Thus, when the chemical reaction is stopped, the POE chains produced have different lengths. Although our results do not allow either the identification of every peak in the mass spectrometry spectra or the quantification of the different polyoxyethylene tallow amines, comparison of the m/z profiles of the two products confirmed that POE-15 tallow amine is a component of RangerPro.

Our results demonstrate that RangerPro contains POE-15 tallow amine, and thus offers a starting point for conducting human surveys of co-formulant exposure. Given the established toxicity of POEA, there is a need to conduct biomonitoring studies and assess for their presence in population groups, especially those which are spraying pesticides containing surfactants, and correlate this exposure to the presence of glyphosate. However, exposure to surfactants can be from multiple sources such as washing products and cosmetics as well as pesticides. Therefore, additional experiments would be required to ascertain that POE-15 tallow amines found in humans originate from pesticide exposure or from the use of other products.

We next investigated the cytotoxic potential of RangerPro compared to glyphosate alone to reveal the greater potential health risks from exposure co-formulants present in the herbicide formulation. A Caco-2 cell cytotoxicity assay was performed, which gave LC50 values of 125 μg/ml, 17,200 μg/ml, and 5.7 μg/ml, for RangerPro, glyphosate and POE-15, respectively (Fig. 2A to C). Thus, based on this assay RangerPro was ~22 times and POE-15 > 3000 times more cytotoxic than glyphosate. The products were also tested in HepG2 cells, which gave LC50 values of 96 μg/ml, and 2 μg/ml, for RangerPro, and POE-15, respectively (Fig. 2E to F). Glyphosate was not cytotoxic and even tended to increase the number of vial cells, which could reflect an hormetic effect by which the survival of the cells is increased by a protective response to induced oxidative stress as demonstrated in other studies (Calabrese and Baldwin, 2003). This phenomenon by which mild-induced stress can give rise to a positive physiological counter-response inducing maintenance and repair systems, has already been described by our group after an exposure to a low-dose pesticide mixture (Mesnage et al., 2021e), and for other pesticide exposures on both target (Tang et al., 2019) and non-target (Calabrese and Baldwin, 2003) species. Overall, our results demonstrate a marked enhanced toxicity from the presence of the POE-15 surfactant and possibly other related surfactants in the RangerPro formulation.

A large number of studies have shown that POEA surfactants contribute to the toxicity of GBHs. Studies showing that POE-15 tallow amine is more toxic than glyphosate stretch back to 1979 (Folmar et al., 1979). The formulation MON 2139 containing POE-15 tallow amine was 10–40 times more toxic than glyphosate in different fish species (Folmar et al., 1979; Wan et al., 1989). Further studies showed that the lethal concentration at which 50% of rainbow trout (*Oncorhynchus mykiss*) were killed was 86 mg/l for glyphosate, and 8.2 mg/l for MON 2139. The herbicide MON 2139 was also 10 to 50 times more toxic than glyphosate in four North American amphibian species (Howe et al., 2004; Mann and Bidwell, 1999) or Microtox bacterium, microalgae, protozoa and crustaceans (Tsui and Chu, 2003). In a previous investigation using three mammalian tissue culture cell lines (HepG2, HEK293, JEG3), we showed that 9 GBHs were up to 100 times more cytotoxic and POE-15 was 10,000 times more toxic than glyphosate alone (Mesnage et al., 2013). Thus, our results showing that RangerPro is 22 times and POE-15 > 3000 times more cytotoxic than glyphosate alone in Caco-2

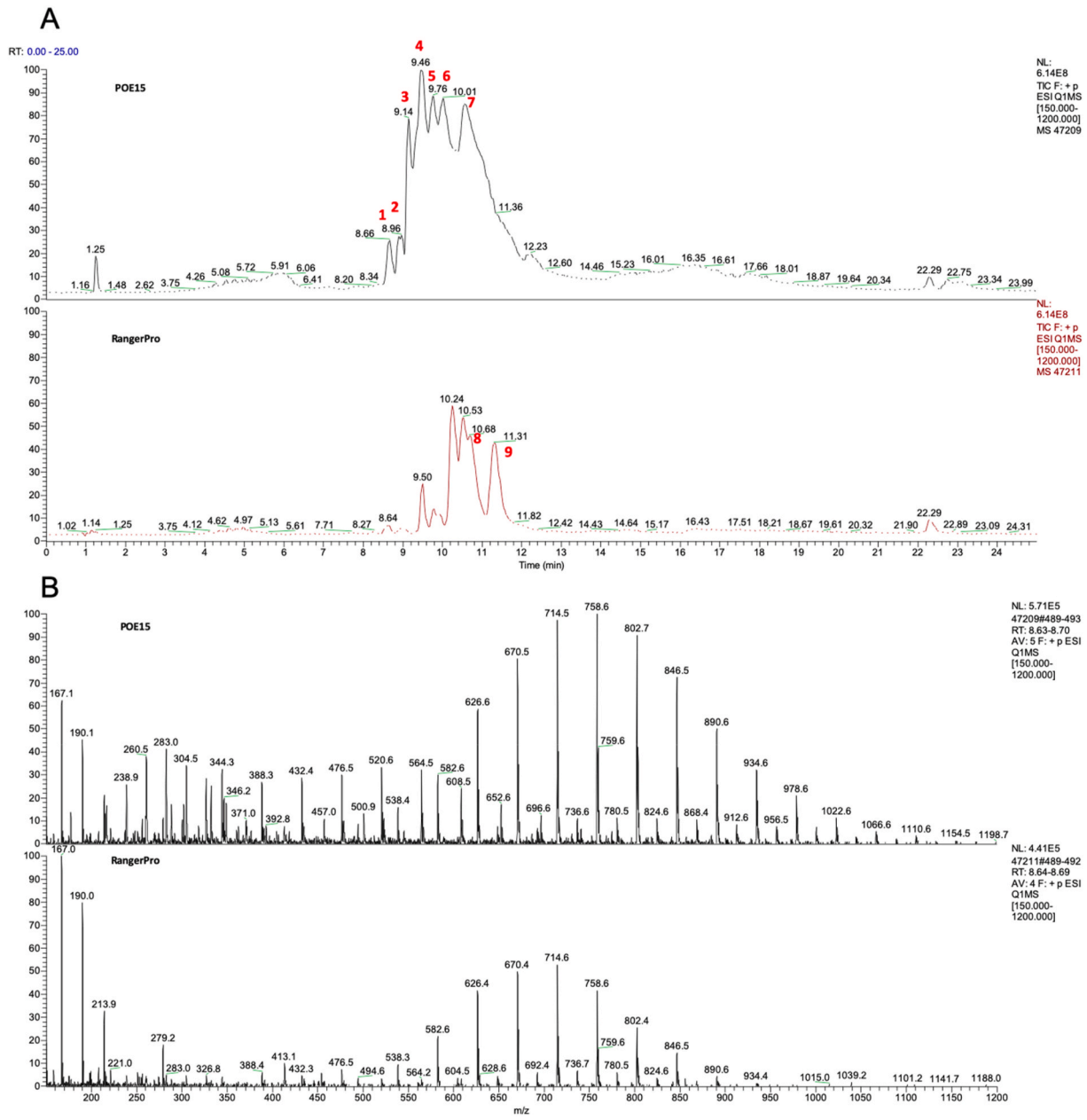

Fig. 1. Representative spectrum of the GBH RangerPro obtained by liquid chromatography-mass spectrometry reveals the presence of POE-15 tallow amine. (A) Chromatograms of RangerPro and POE-15 show the presence of a mixture of co-formulants in the herbicide, which elute at different retention times from the mass spectrometry column. **(B)** The mass spectra (bottom panel) are obtained by extracting the TIC (Total Ion Chromatogram) of peak 1 for POE-15, retention time: 8.7 min (TIC between 8.63 and 8.70 min) show that peaks are separated by 44 m/z increments, which is similar to what is seen with RangerPro suggesting that each peak corresponds to a different molecule belonging to a homologous series of surfactants with a different number of ethoxylation units. Mass spectra from the other peaks 2–9 are available as supplementary data.

cells (Fig. 2) is in accord with these earlier observations.

The mechanism of action by which POEA caused cytotoxicity in previous studies was by disrupting the structure of cell membranes (Mesnage et al., 2013). Therefore, we next undertook an analysis to see if RangerPro also caused cell membrane disruption (Fig. 3). We tested the LC50 (cytotoxic), LC99 and LC99/5 (non-cytotoxic) threshold concentrations. RangerPro and POE-15 treatment resulted in a statistically significant increase in adenylate kinase release indicative of induced cell membrane damage at all concentrations tested in both Caco-2 (Fig. 3A) and HepG2 (Fig. 3B) cells. Glyphosate did not cause any measurable membrane damage in either of the two cell lines, confirming that the cytotoxic capability of RangerPro can be attributed to the membrane disrupting potential of the surfactants included as co-formulants.

Since glyphosate has frequently been described as a disruptor of redox status in mammalian cells (Mesnage et al., 2015; Wang et al., 2022; Martínez et al., 2020), we tested to see if either glyphosate or RangerPro could increase production of hydrogen peroxide, an indicator of oxidative stress (Fig. 4). In Caco-2 cells, the positive control compound menadione provoked a significant (6-fold) increase in hydrogen peroxide production in comparison to the negative control untreated samples (Fig. 4A, NC and PC values). Although some of the test compounds caused an increase in oxidative stress, effects were limited with none reaching significance (Fig. 4). Glyphosate induced a dose-dependent oxidative stress response in HepG2 cells (Fig. 4B). The difference between the results of the Caco-2 and HepG2 oxidative stress assays could also suggest that glyphosate was metabolised by the

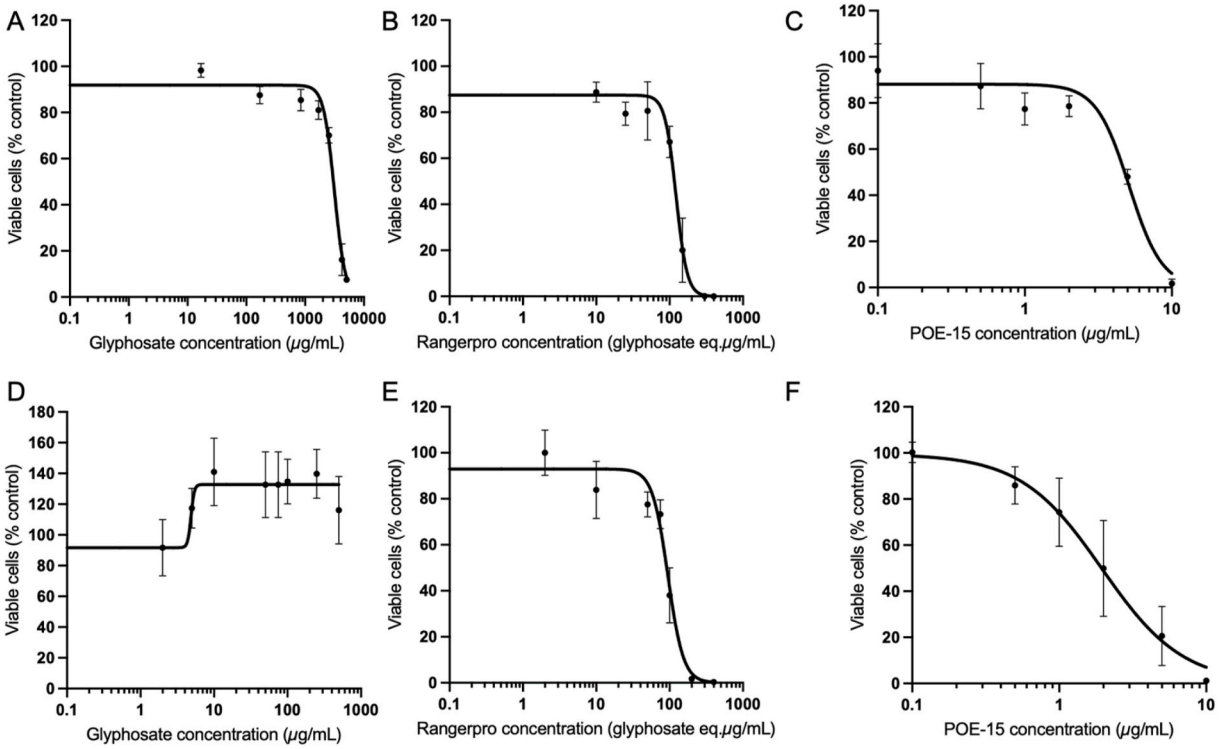

Fig. 2. RangerPro is markedly more cytotoxic than glyphosate alone in Caco-2 and HepG2 cells. Caco-2 cells (A to C) or HepG2 cells (D to F) were treated with the test substances for 24 h, and viability determined by an MTT assay. The concentration of RangerPro is expressed as glyphosate equivalent concentrations. Cell viability was expressed as a percentage relative to the negative control, untreated cell samples. The assay was performed in triplicate and data is expressed as mean ± SD (standard deviation) of 3 independent replicates.

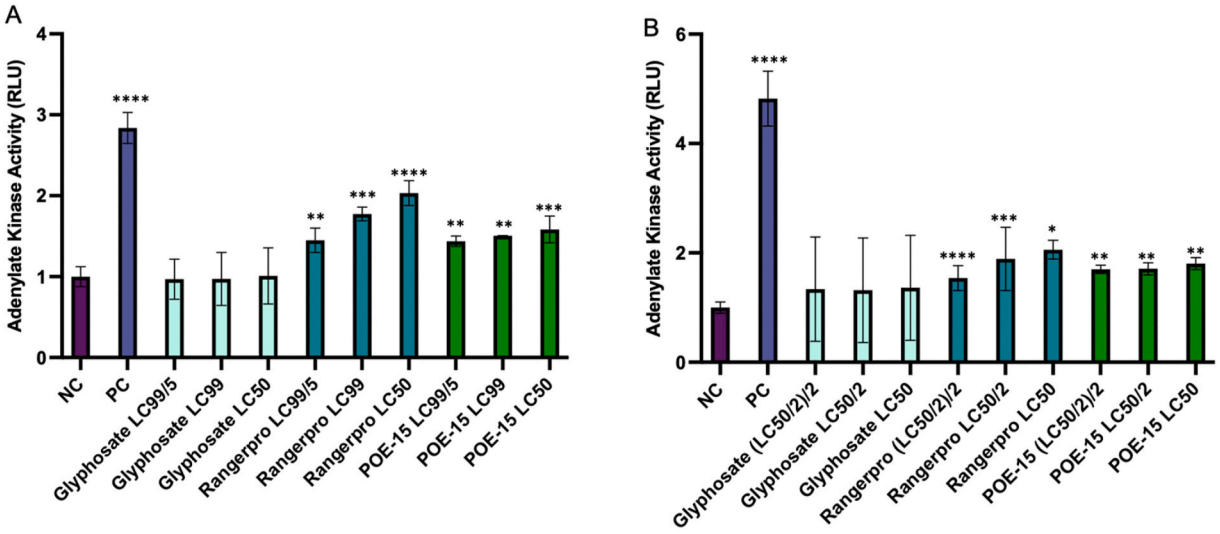

Fig. 3. Cytotoxicity of RangerPro and POE-15 in Caco-2 and HepG2 cells is via a plasma membrane disruption mechanism. Caco-2 (A) or HepG2 (B) cells were treated with 3 concentrations of each test substance, which corresponded to the lethal concentration (LC) thresholds determined by the MTT cytotoxicity assay. The positive control (PC) was 0.05% Triton-X100 non-ionic detergent, which is known to cause cell plasma membrane damage. Following treatment, cultures were assessed for plasma membrane damage and necrosis using the Toxilight assay system, which measures release of adenylate kinase from damaged cells. The assay was performed in triplicate and data is expressed as mean ± SD (standard deviation) of at least 3 independent replicates. Adenylate kinase activity shown is represented as fold change in relative light units (RLU) compared to the negative control (NC) untreated cell culture samples. Statistical analysis was performed by one-way ANOVA in GraphPad prism 5 (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

xenobiotic metabolising enzymes in hepatocytes into a more toxic compound. This hypothesis is supported by animal experiments suggesting that glyphosate is metabolised to glyoxylate in the liver (Ford et al., 2017). In general, toxicokinetic evaluations performed with glyphosate suggest that the main metabolite of glyphosate is aminomethyl phosphonic acid (Anadón et al., 2009). However, it should be noted that the generation of oxidative stress can be time-dependent and it is not clear if our experimental design fully captures glyphosate's capability for inducing this type of toxicity. It is plausible that changes in gut microbiota composition could promote oxidative stress

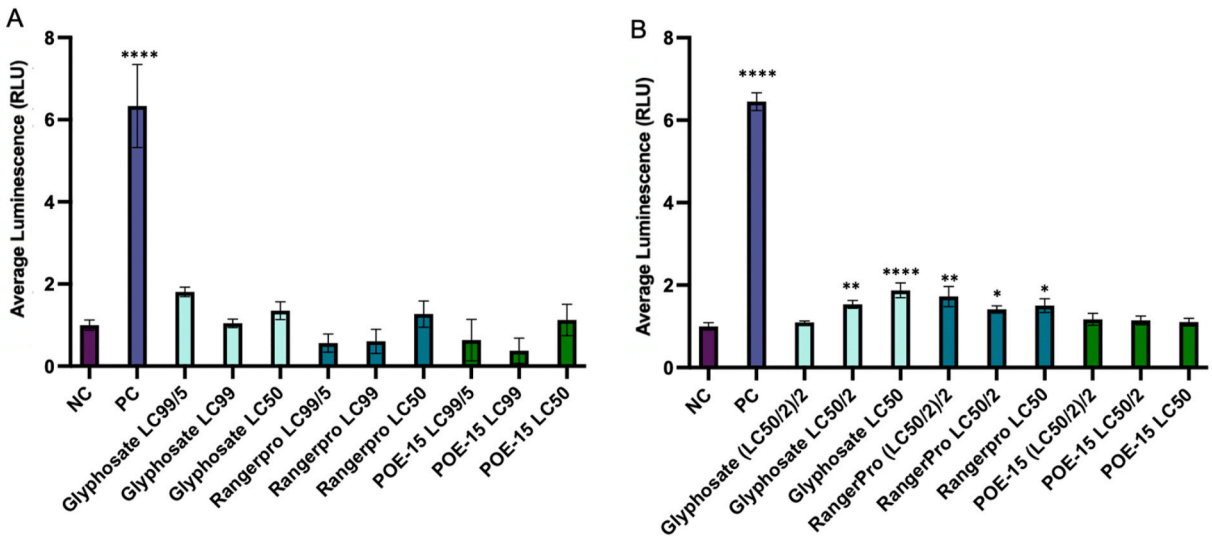

Fig. 4. Oxidative stress measured by hydrogen peroxide production. *Caco-2 (A) or HepG2 (B) cells were treated for* 6 h *at respective LC50, LC99 and LC99/5 thresholds with glyphosate, RangerPro and POE-15. Treatment with 50 μ M menadione acted as a positive control (PC) treatment. Production of hydrogen peroxide was the oxidative stress marker detected by the ROS-Glo luciferase reporter system. The assay was performed in triplicate and data is expressed as mean ± SD (standard deviation) of 3 independent replicates.*

since we recently showed that the gut microbiome metabolite profile in rats exposed to glyphosate reflected changes in the redox status of the microbial community (Mesnage et al., 2021d). In addition, our most recent study has shown that exposure to RangerPro, and to a lesser extent glyphosate, affected the growth of gut bacteria, which in turn reduced competition and allowed opportunistic fungi to proliferate (Mesnage et al., 2021c).

In order to gain deeper insight into mechanisms of toxicity, especially any carcinogenic potential, of glyphosate, RangerPro and POE-15 we conducted an evaluation of their effects in the ToxTracker assay in the presence or absence of S9 metabolising liver extract fractions. The ToxTracker assay system consists of six different mES cell lines expressing reporter gene constructs, which provide a readout of potential carcinogenic activity (Hendriks et al., 2016). The reporter loci are: Bscl2 (DNA damage-associated Berardinelli-Seip congenital lipodystrophy 2), which informs on the activation of the ATR-CHK1 DNA damage signalling pathway (Smith et al., 2010). A second biomarker to monitor activation of a DNA damage response is Rtkn (Rhotekin), which is a indicator of NF-kB signalling reflecting double-strand DNA breaks (Thumkeo et al., 2013). Btg2-GFP reflects activation of the p53 tumour suppressor response pathway (Winkler, 2010). Oxidative stress is evaluated with Srxn1 (Sulfiredoxin 1) reflecting an Nrf2 antioxidant response and Blvrb (Biliverdin Reductase B) reflecting an Nrf2 independent response. Protein damage or endoplasmic reticulum (ER) stress is evaluated as the unfolded protein response by measuring Ddit3 (DNA damage-inducible transcript 3) expression (Cao et al., 2019).

Cytotoxicity of RangerPro and its ingredients glyphosate and POE-15 in normal mouse embryonic stem cells varied markedly (Supplementary Data). POE-15 was the most cytotoxic substance. The formulation RangerPro was also cytotoxic while glyphosate caused no cytotoxicity even at a concentration tested of 5 mM in either the absence or presence of the metabolising S9 liver extract (Supplementary Data). We then tested POE-15 and RangerPro in the ToxTracker assay (Fig. 5). Cytotoxicity and genotoxicity profiles for glyphosate have already been reported (Mesnage et al., 2021a) and are reproduced here for comparative purposes in accordance with the publisher's (Elsevier's) data reuse policy. At the maximum tested concentration in the absence of a metabolising system more than 50% cytotoxicity was observed for RangerPro and POE-15. None the three compounds activated the two reporters for genotoxicity, nor the reporter for p53 activation. Activation of the oxidative stress reporter Srxn1-GFP was observed for RangerPro in the presence of S9 extract (Fig. 5H). In the presence of S9 extract, POE-15 activated the Srxn1-GFP reporter 1.98-fold (Fig. 5I). The Ddit3-GFP reporter for the unfolded protein response was activated upon exposure to RangerPro in the absence (Fig. 5B) and presence (Fig. 5H) of S9 extract. For POE-15, an activation of Ddit3-GFP in absence of S9 extract was observed (Fig. 5C) whilst the induction levels in the presence of S9 extract (Fig. 5I) did not pass the 2-fold threshold for a positive ToxTracker response.

In conclusion, our results demonstrate that RangerPro contains POE-15 tallow amine at a concentration at which it could exert necrotic effects and activate the unfolded protein response in mammalian cells. Since the unfolded protein response is a known consequence of alterations in the function of the ER (Hetz, 2012), and given the known membrane damaging potential of POE-15 (Mesnage et al., 2013), our results suggest that POE-15 caused ER stress. In addition, to these mechanisms, glyphosate and RangerPro but not POE-15 caused oxidative stress in HepG2 cells. Our results suggesting that glyphosate alone caused oxidative stress in HepG2 cells are supported by a large number of other studies in a variety of models systems (Mesnage et al., 2015) including a recent study on the small intestine (Tang et al., 2020). The toxicity resulting from RangerPro exposure is thus multifactorial involving ER stress caused by the surfactant with oxidative stress caused by glyphosate.

Future studies would also need to take into account that the effects of glyphosate are multifactorial, and that co-formulants included in GBH formulations could contribute to carcinogenesis. Altogether, our results reinforce the need to test co-formulants when comparing formulations and active ingredients in order to not only provide a mechanistic understanding of toxic effects but also to inform the enactment of regulation for more appropriate and thus better public and environmental protection from these substances.

CRediT author statement

Robin Mesnage: Conceptualization, Writing- Original draft preparation, Visualization, Supervision. Scarlett Ferguson: Investigation, Formal analysis, Writing- Reviewing and Editing. Inger Brandsma: Formal analysis, Writing- Reviewing and Editing. Nynke Moelijker: Resources, Investigation. Gaonan Zhang: Resources, Investigation. Francesca Mazzacuva: Investigation, Methodology, Writing- Reviewing and Editing. Anna Caldwell: Writing- Reviewing and Editing. John

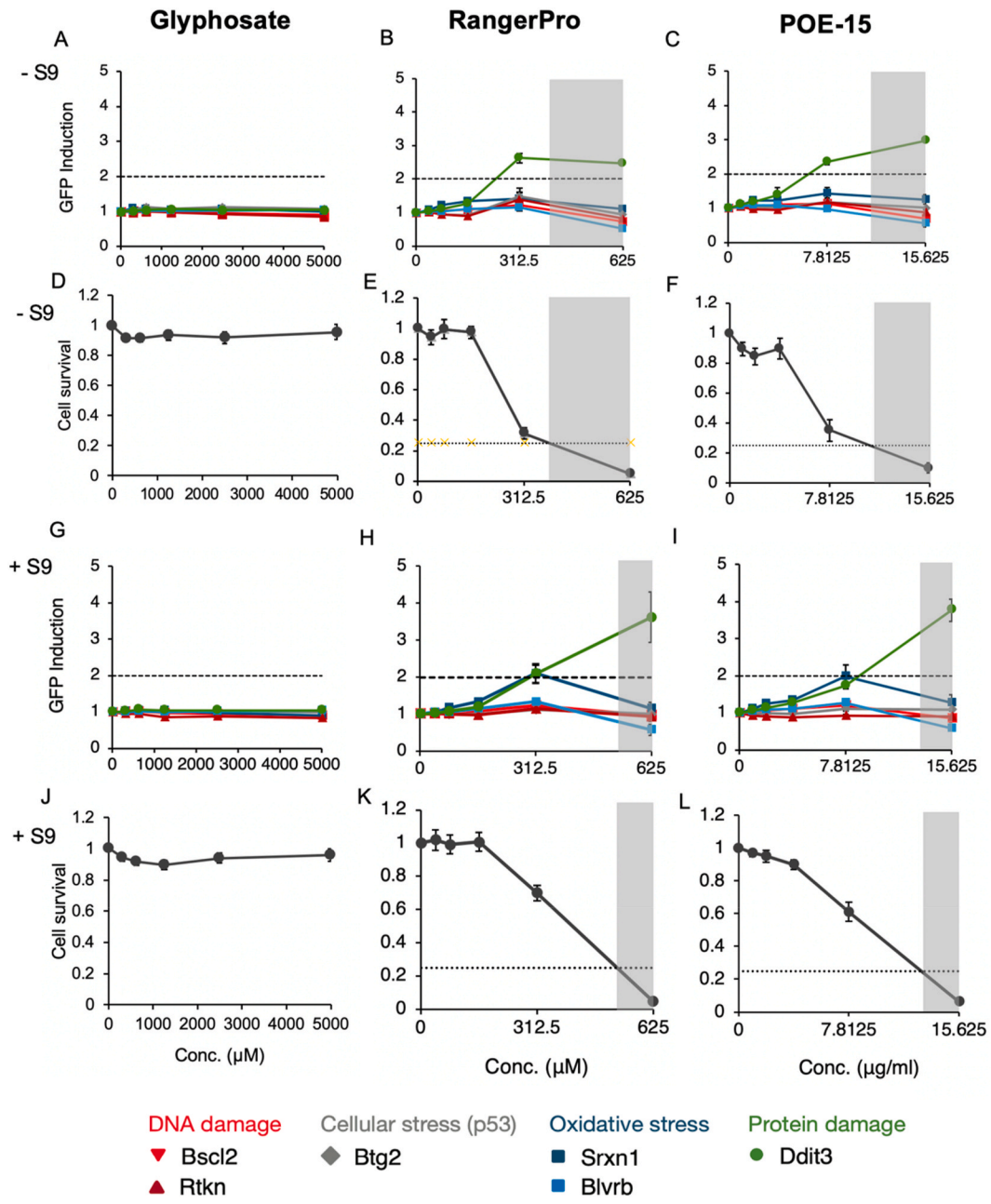

Fig. 5. Activation of mechanisms known to be key characteristics of carcinogens by glyphosate, RangerPro or POE-15 employing the ToxTracker assay system. The six mES reporter cell lines that constitute the ToxTracker assay system were used to detect readouts of oxidative stress (Srxn1 and Blvrb), protein damage and an unfolded protein response (Ddit3), the activation of a DNA damage response (Bscl2 and Rtkn) and p53-mediated cellular stress (Btg2). Panels A to C: induction of reporter gene expression in absence of S9 metabolising liver extract and associated changes in cell survival (panels D to F). Panels G to I: induction of reporter gene expression in the presence of S9 liver extract and cell survival (panels J to L). Grey box covers measurements with less than 25% cell survival and were not taken into consideration due to too high cytotoxicity. Note: the data pertaining to glyphosate exposure (panels A, D, G, J) have been reported previously (Mesnage et al., 2021a) and are reproduced here for comparative purposes in accordance with the publisher's (Elsevier's) data reuse policy.

Halket: Writing- Reviewing and Editing. Michael N Antoniou: Project administration, Supervision, Writing- Reviewing and Editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Robin Mesnage is supported by a grant to Michael Antoniou from the Sustainable Food Alliance (USA). Inger Brandsma, Nynke Moelijker and Gaonan Zhang are employees of Toxys. Robin Mesnage acts in an expert consulting or advisory capacity with a law firm involved in litigation in the US over glyphosate/Roundup health effects.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.113380.

References

Anadón, A., Martínez-Larrañaga, M.R., Martínez, M.A., Castellano, V.J., Martínez, M., Martin, M.T., Nozal, M.J., Bernal, J.L., 2009 Oct 8. Toxicokinetics of glyphosate and its metabolite aminomethyl phosphonic acid in rats. Toxicol. Lett. 190 (1), 91–95.

Anderson, N.H., Girling, J., 1983. The uptake of surfactants into wheat. Pestic. Sci. 35, 399–405.

Calabrese, E.J., Baldwin, L.A., 2003. Hormesis at the national toxicology program (NTP): evidence of hormetic dose responses in NTP dose-range studies. Nonlinearity Biol. Toxicol. Med. 1 (4), 455–467. https://doi.org/10.1080/15401420390271056.

Cao, Y., Trillo-Tinoco, J., Sierra, R.A., et al., 2019. ER stress-induced mediator C/EBP homologous protein thwarts effector T cell activity in tumors through T-bet repression. Nat. Commun. 10 (1), 1280. https://doi.org/10.1038/s41467-019-09263-1.

Folmar, L.C., Sanders, H.O., Julin, A.M., 1979. Toxicity of the herbicide glyphosphate and several of its formulations to fish and aquatic invertebrates. Arch. Environ. Contam. Toxicol. 8 (3), 269–278.

Ford, B., Bateman, L.A., Gutierrez-Palominos, L., Park, R., Nomura, D.K., 2017. Mapping proteome-wide targets of glyphosate in mice. Cell Chem. Biol. 24 (2), 133–140. https://doi.org/10.1016/j.chembiol.2016.12.013.

Gasnier, C., Laurant, C., Decroix-Laporte, C., et al., 2011. Defined plant extracts can protect human cells against combined xenobiotic effects. J. Occup. Med. Toxicol. 6 (1), 3. https://doi.org/10.1186/1745-6673-6-3.

Hendriks, G., Derr, R.S., Misovic, B., Morolli, B., Calléja, F.M.G.R., Vrieling, H., 2016. The extended ToxTracker assay discriminates between induction of DNA damage, oxidative stress, and protein misfolding. Toxicol. Sci. 150 (1), 190–203. https://doi.org/10.1093/toxsci/kfv323.

Hetz, C., 2012. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat. Rev. Mol. Cell Biol. 13 (2), 89–102. https://doi.org/10.1038/nrm3270.

HMB4EU, 2018. Second List of HBM4EU Priority Substances and Chemical Substance Group Leaders for 2019-2021. Document Ref Ares(2018)4423297 - 28/08/2018.

Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K., Veldhoen, N., 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ. Toxicol. Chem. 23 (8), 1928–1938.

Mann, R.M., Bidwell, J.R., 1999. The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. Arch. Environ. Contam. Toxicol. 36 (2), 193–199.

Martínez, M.A., Rodríguez, J.L., Lopez-Torres, B., Martínez, M., Martínez-Larrañaga, M. R., Maximiliano, J.E., Anadón, A., Ares, I., 2020 Feb. Use of human neuroblastoma SH-SY5Y cells to evaluate glyphosate-induced effects on oxidative stress, neuronal development and cell death signaling pathways. Environ. Int. 135, 105414.

Mesnage, R., Antoniou, M.N., 2018. Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Front. Public Health 5, 361.

Mesnage, R., Bernay, B., Seralini, G.E., 2013. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. Toxicology 313 (2–3), 122–128. https://doi.org/10.1016/j.tox.2012.09.006.

Mesnage, R., Defarge, N., Spiroux de Vendomois, J., Seralini, G.E., 2015. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. Food Chem. Toxicol. 84, 133–153. https://doi.org/10.1016/j.fct.2015.08.012.

Mesnage, R., Benbrook, C., Antoniou, M.N., 2019. Insight into the confusion over surfactant co-formulants in glyphosate-based herbicides. Food Chem. Toxicol. 128, 137–145. https://doi.org/10.1016/j.fct.2019.03.053.

Mesnage, R., Brandsma, I., Moelijker, N., Zhang, G., Antoniou, M.N., 2021 Nov. Genotoxicity evaluation of 2,4-D, dicamba and glyphosate alone or in combination with cell reporter assays for DNA damage, oxidative stress and unfolded protein response. Food Chem. Toxicol. 157, 112601.

Mesnage, R., Ibragim, M., Mandrioli, D., et al., 2021a. Comparative toxicogenomics of glyphosate and Roundup herbicides by mammalian stem cell-based genotoxicity assays and molecular profiling in Sprague-Dawley rats. Toxicol. Sci., kfab143 https://doi.org/10.1093/toxsci/kfab143.

Mesnage, R., Mazzacuva, F., Caldwell, A., Halket, J., Antoniou, M.N., 2021b. Urinary excretion of herbicide co-formulants after oral exposure to roundup Mon 52276 in rats. Environ. Res. 197, 111103.

Mesnage, R., Panzacchi, S., Bourne, E., et al., 2021c. Glyphosate and its formulations Roundup Bioflow and RangerPro alter bacterial and fungal community composition in the rat caecum microbiome, 2021 bioRxiv 11 (19), 468976. https://doi.org/10.1101/2021.11.19.468976.

Mesnage, R., Teixeira, M., Mandrioli, D., et al., 2021d. Use of shotgun metagenomics and metabolomics to evaluate the impact of glyphosate or Roundup Mon 52276 on the gut microbiota and serum metabolome of Sprague-Dawley rats. Jan Environ. Health Perspect. 129 (1), 17005. https://doi.org/10.1289/EHP6990.:870105, 10.1101/870105.

Mesnage, R., Teixeira, M., Mandrioli, D., et al., 2021e. Multi-omics phenotyping of the gut-liver axis reveals metabolic perturbations from a low-dose pesticide mixture in rats. Commun. Biol. 4 (1), 471. https://doi.org/10.1038/s42003-021-01990-w.

Paz-y-Miño, C., Sánchez, M.E., Arévalo, M., Muñoz, M.J., Witte, T., De-la-Carrera, G.O., Leone, P.E., 2007. Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. Genet. Mol. Biol. 30, 456–460.

Smith, J., Tho, L.M., Xu, N., Gillespie, D.A., 2010. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. Adv. Cancer Res. 108, 73–112. https://doi.org/10.1016/b978-0-12-380888-2.00003-0.

Solomon, K.R., Anadón, A., Carrasquilla, G., Cerdeira, A.L., Marshall, J., Sanin, L.H., 2007. Coca and poppy eradication in Colombia: environmental and human health assessment of aerially applied glyphosate. Rev. Environ. Contam. Toxicol. 190, 43–125.

Tang, S., Liang, J., Xiang, C., et al., 2019. A general model of hormesis in biological systems and its application to pest management. J. R. Soc. Interface 16 (157), 20190468. https://doi.org/10.1098/rsif.2019.0468.

Tang, Q., Tang, J., Ren, X., Li, C., 2020. Glyphosate exposure induces inflammatory responses in the small intestine and alters gut microbial composition in rats. Environ. Pollut. 261, 114129 https://doi.org/10.1016/j.envpol.2020.114129.

Thumkeo, D., Watanabe, S., Narumiya, S., 2013. Physiological roles of Rho and Rho effectors in mammals. Eur. J. Cell Biol. 92 (10–11), 303–315. https://doi.org/10.1016/j.ejcb.2013.09.002.

Tsui, M.T., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52 (7), 1189–1197. https://doi.org/10.1016/S0045-6535(03)00306-0.

Wan, M.T., Watts, R.G., Moul, D.J., 1989. Effects of different dilution water types on the acute toxicity to juvenile Pacific salmonids and rainbow trout of glyphosate and its formulated products. Bull. Environ. Contam. Toxicol. 43 (3), 378–385.

Wang, X., Lu, Q., Guo, J., Ares, I., Martínez, M., Martínez-Larrañaga, M.R., Wang, X., Anadón, A., Martínez, M.A., 2022 Jan 6. Oxidative stress and metabolism: a mechanistic insight for glyphosate toxicology. Annu. Rev. Pharmacol. Toxicol. 62, 617–639.

Winkler, G.S., 2010. The mammalian anti-proliferative BTG/Tob protein family. J. Cell. Physiol. 222 (1), 66–72. https://doi.org/10.1002/jcp.21919.