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In-depth comparative toxicogenomics of glyphosate and Roundup herbicides: histopathology, transcriptome and epigenome signatures, and DNA damage

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Abstract

Background. Health effects from exposure to glyphosate-based herbicides is an intense matter of debate. Toxicity including genotoxicity of glyphosate alone has been repeatedly tested over the last 40 years. Contrastingly, few studies have conducted comparative investigations between glyphosate and its commercial herbicide formulations, such as Roundup. We thus performed the first in-depth comparative toxicogenomic evaluation of glyphosate and a typical European Union Roundup formulation by determining alterations in transcriptome and epigenome profiles.

Methods. Glyphosate and the European Union reference commercial formulation Roundup MON 52276 (both at 0.5, 50, 175 mg/kg bw/day glyphosate equivalent concentration) were administered to rats in a subchronic 90-day toxicity study. Standard clinical biochemistry and kidney and liver histopathology was performed. In addition, transcriptomics and DNA methylation profiling of liver and selective gene expression analysis of kidneys was conducted. Furthermore, a panel of six mouse embryonic reporter stem cell lines validated to identify carcinogenic outcomes (DNA damage, oxidative stress, and protein misfolding) were used to provide insight into the mechanisms underlying the toxicity of glyphosate and 3 Roundup formulations.

Results. Histopathology and serum biochemistry analysis showed that MON 52276 but not glyphosate treatment was associated with a statistically significant increase in hepatic steatosis and necrosis. Similar lesions were also present in the liver of glyphosate-treated groups but not in the control group. MON 52276 altered the expression of 96 genes in liver, with the most affected biological functions being TP53 activation by DNA damage and oxidative stress as well as the regulation of circadian rhythms. The most affected genes in liver also had their expression similarly altered in kidneys. DNA methylation profiling of liver revealed 5,727 and 4,496 differentially methylated CpG sites between the control group and the group of rats exposed to glyphosate and MON 52276, respectively. Direct DNA damage measurement by apurinic/apyrimidinic lesion formation in liver was increased with glyphosate exposure. Mechanistic evaluations showed that two Roundup herbicides but not glyphosate activated oxidative stress and misfolded protein responses.

Conclusions. Taken together, the results of our study show that Roundup herbicides are more toxic than glyphosate, activating mechanisms involved in cellular carcinogenesis and causing gene expression changes reflecting DNA damage. This further highlights the power of high-throughput 'omics' methods to detect metabolic changes, which would be missed by relying solely on conventional biochemical and histopathological measurements. Our study paves the way for future investigations by reporting a panel of gene expression changes and DNA methylation sites, which can serve as biomarkers and potential predictors of negative health outcomes resulting from exposure to glyphosate-based herbicides.

Introduction

Glyphosate is the most widely used herbicide worldwide. Although the use of glyphosate remained limited during the first two decades after its market introduction in 1974, worldwide use increased exponentially from the 1990s due to the rapid adoption of agricultural practices involving cultivation of transgenic soybeans, maize, cotton, sugar beet and other crops genetically engineered to tolerate applications of glyphosate formulated products such as Roundup (Benbrook 2016). This was facilitated by substantial reductions in the cost of glyphosate-based herbicides (GBHs) due to improvements in manufacturing processes and the opening of the market to competitors when Monsanto's patent on glyphosate expired in 2000 (Perry et al. 2019). In addition, GBHs have increasingly been sprayed shortly before harvest to cause crop desiccation allowing a more rapid harvest, and for weed control in amenity and industrial areas.

The toxicity of glyphosate on human health and the environment is a controversial topic (Mesnage and Zaller 2021). The intensity in the debate on glyphosate toxicity increased dramatically when the World Health Organization's International Agency for Research on Cancer (IARC) classified this compound as a probable (Class 2A) human carcinogen (Guyton et al. 2015). Although the existence of a direct link between glyphosate exposure and carcinogenesis in human populations at typical low exposure levels still remains to be established, IARC identified evidence in the peer reviewed scientific literature that glyphosate and commercial GBHs cause oxidative stress and DNA damage in animal and *in vitro* model systems (Guyton et al. 2015). However, findings from genotoxicity studies were often contradictory. Among the assays reviewed by IARC to evaluate possible genotoxic mechanisms, 77% reported positive results (Benbrook 2019). Whether glyphosate increases cancer incidence in animal studies is still not certain. A recent collation and re-evaluation of data from laboratory animal model systems, has highlighted evidence of the carcinogenic potential of glyphosate and GBHs (Portier 2020). However, tumor findings in two-year rodent studies were not consistent with poor reproducibility; that is, the same type of cancer was not consistently found in different investigations. Issues concerning the reproducibility of chemical-induced carcinogenicity was first comprehensively documented 20 years ago (Gottmann et al. 2001). This study compared 121 pairs of replicate rodent assays conducted either by the National Cancer Institute/National Toxicology Program or documented in the general scientific literature. It was found that the results of only 57% of these paired studies were concordant with respect to carcinogenic outcome (Gottmann et al. 2001).

The mechanism or mechanisms by which GBHs may be carcinogenic are not fully known. Recent studies have provided mechanistic details suggesting a link between mitochondrial dysfunction caused by glyphosate and an increase in production of reactive oxygen species leading to DNA damage. Glyphosate has been known to affect the function of mitochondria since the end of the 1970s by interference with the respiratory chain (Olorunsogo 1990; Olorunsogo and Bababunmi 1980; Olorunsogo et al. 1979). More recently, in a study of *Caenorhabditis elegans* (Bailey et al. 2018), it was found that the glyphosate formulation TouchDown® Hitech inhibited mitochondrial respiration, resulting in a decreased proton gradient integrity and an inhibition of ATP production. This was concomitant with changes in hydrogen peroxide production that correlated with up-regulation of glutathione-S-transferase

expression. In addition to inhibiting the shikimate pathway in plants, glyphosate generates oxidative stress in plant cells through hydrogen peroxide formation by interfering with the mitochondrial respiratory electron transport chain (Gomes and Juneau 2016). Mitochondrial dysfunction induced by glyphosate was further confirmed by a study in zebrafish, *Danio rerio*, in which behavioural impairments induced by a GBH were linked to the effects of glyphosate on mitochondrial complex enzymatic activities and its resulting increase in reactive oxygen species production (Pereira et al. 2018). Studies on human peripheral blood mononuclear cells also suggest that glyphosate and its formulated products could generate DNA strand-breaks, and purine and pyrimidine oxidation via oxidative stress (Wozniak et al. 2018).

In the study presented here, we focused on the liver, which is a major target of injury by oxidative stress and inflammation (Cichoz-Lach and Michalak 2014). An increasing number of studies have revealed non-target effects of glyphosate on liver function (Benedetti et al. 2004; Beuret et al. 2005; Chan and Mahler 1992; Mesnage et al. 2017; Milić et al. 2018; Mills et al. 2020; Pandey et al. 2019). Some of these studies describe molecular phenotypes overlapping those found in cases of fatty liver disease, and which were associated with oxidative damage (Mesnage et al. 2017; Mills et al. 2020; Pandey et al. 2019). The acceptable daily intake of glyphosate in the European Union (EU) between 2002 and 2017 was based on liver damage detected after long-term administration of 60 mg/kg bw/day of glyphosate in rats (Commission 2002).

Another important knowledge gap, which confuses the debate on glyphosate carcinogenicity, is whether co-formulants included in GBHs could amplify glyphosate adverse effects. A meta-analysis has shown a correlation between the frequency of GBH use and exposure and incidence of non-Hodgkin's lymphoma (NHL) (Zhang et al. 2019). It was estimated that more than 750 different commercial herbicide formulations contained glyphosate in the US in 2015 (Guyton et al. 2015). These GBHs contain ingredients other than glyphosate, which also have toxic properties in humans (Sawada et al. 1988; Seok et al. 2011). The exposure to MON 0818 (a mixture of co-formulants) was identified as a worker safety issue during the 1980s because it was linked to occupational eye and skin injuries (Blondell 1986). Generally, although co-formulants are also toxic, long-term testing is not as a rule required prior to their commercial use and their composition remains company proprietary, confidential information. Thus, co-formulant toxicology and human exposure health effects remain a major gap in the regulation of these compounds (Mesnage and Antoniou 2018). The main surfactant present in GBHs, ethoxylated tallowamines, also known as POEA, was banned in the EU in 2016. However, it is not known if the most recent generation of GBHs containing co-formulant surfactants that are claimed to be less toxic, can still cause adverse health effects at doses which glyphosate alone is considered safe.

With the need to compare the toxicity of pesticide active principles with their commercial formulations, we performed a subchronic study for 90-days in rats administered with glyphosate (0.5, 50, 175 mg/kg bw/day), or its representative EU commercial herbicide formulation Roundup MON 52276 at the same glyphosate equivalent doses. In a first report of results from this toxicity study, we described how glyphosate and MON 52776 altered the function of the rat gut microbiome and brought about changes in the serum metabolome suggestive of oxidative stress damage (Mesnage et al. 2021). Alterations of the gut microbiome glyphosate and MON 52776 were also reported in male and female rats of the same strain treated from pregnancy (Mao et al. 2018). In the follow-up investigation presented here, we

combined deep phenotyping to highlight any alterations in the epigenome (DNA methylation) and transcriptome profile in liver (Figure 1). Since carcinogen hazard identification and risk assessment is increasingly relying on mechanistic data (Smith et al. 2016), we also used a mammalian stem cell-based genotoxicity experimental system (ToxTracker Assay) designed to discriminate a chemical's ability to induce DNA damage, oxidative stress, and protein misfolding to provide insight into the mechanisms underlying the toxicity of glyphosate and 3 commercial GBHs (Hendriks et al. 2016).

Our findings reinforce the urgent need to incorporate the assessment of toxicity of commercial pesticide formulations as well as their active principles as part of regulatory procedures to establish true no observed adverse effect level (NOAEL) and acceptable daily intake (ADI) values (Landrigan and Belpoggi 2018; Vandenberg et al. 2017). Overall, our results highlight the ability of in-depth molecular profiling afforded by epigenome and transcriptome analysis to evaluate molecular mechanisms underlying pesticide toxicity in animal model systems.

Material and methods

Experimental animals

The experiment was conducted with young adult female Sprague-Dawley rats at the Cesare Maltoni Cancer Research Center, Ramazzini Institute, (Bentivoglio, Italy) in accordance with Italian law regulating the use and humane treatment of animals for scientific purposes (Decreto legislativo N. 26, 2014. Attuazione della direttiva n. 2010/63/UE in materia di protezione degli animali utilizzati a fini scientifici. - G.U. Serie Generale, n. 61 del 14 Marzo 2014). The experiment was authorised by the ad hoc commission of the Italian Ministry of Health (authorization N. 447/2018-PR).

Treatment of animals was as previously described (Mesnage et al. 2021). In brief, groups of 12 female Sprague-Dawley rats of 8 weeks of age were administered for 90 days with glyphosate and MON 52276 as Roundup BioFlow (Italy) at the same glyphosate equivalent dose via drinking water of 0.5 mg, 50 mg and 175 mg/kg body weight per day (mg/kg bw/day), which respectively represent the EU acceptable daily intake (ADI), the EU no-observed adverse effect level (NOAEL) and the US NOAEL (European Food Safety 2015). In the group treated with the highest dose of Roundup, the concentration of MON 52276 had to be reduced starting from 6th week of treatment because of the lower solution consumption of the animals. The mean daily intake of glyphosate in animals of this group, for the whole period of treatment, was therefore 130 mg/kg bw/day instead of the 175 mg/kg bw/day.

Histopathology

Animals were checked for general status three times a day, seven days a week, except for non-working days when they were checked twice. All sacrificed animals were subjected to complete necropsy. Liver and kidneys were alcohol-fixed, trimmed, processed and embedded in paraffin wax. Sections of 3-6 µm were cut for each specimen of liver and kidneys and stained with haematoxylin and eosin. All slides were evaluated by a pathologist and all lesions of interest were re-evaluated by a second pathologist. The histopathological nomenclature of lesions adopted were classified according to the international nomenclature INHAND

(International Harmonization of Nomenclature and Diagnostic Criteria) and RITA (Registry of Industrial Toxicology Animal Data).

Biochemistry

Prior to sacrifice, animals were anesthetised by inhalation of a 70% CO_2/30% O_2 mixture, and approximately 7.5 ml of blood collected from the *vena cava*. Serum biochemistry was performed at IDEXX BioAnalytics (Stuttgart, Germany), a laboratory accredited to ISO 17025 standards. Sodium and potassium levels were measured by indirect potentiometry. Albumin was measured by a photometric Bromocresol green test. Alkaline phosphatase ALP was measured by IFCC with the AMP-buffer method, glucose by Enzymatic UV-Test (Hexokinase method), cholesterol by Enzymatic colour test (CHOD-PAP), blood urea nitrogen by enzymatic UV-Test, gamma-glutamyl-transferase by Kinetic colour test International Federation of Clinical Chemistry (IFCC), aspartate and alanine aminotransferase by kinetic UV-test (IFCC+ pyridoxal-5-phosphate), creatinine by kinetic colour test (Jaffe's method), lactate dehydrogenase (LDH) by the IFCC method, and triglycerides using an enzymatic colour test (GPO-PAP) on a Beckman Coulter AU 480 instrument.

DNA and RNA extraction

DNA and RNA were extracted from tissues taken at the time of sacrifice and which had been stored at -80°C. RNA extraction, library preparation, and cDNA sequencing was performed as described previously (Mesnage et al. 2020). Liver and kidneys from animals exposed to a dose of 50 mg/kg bw/day of glyphosate or to MON 52276 at the same glyphosate equivalent dose were used. In brief, genomic samples extracted from rat liver displayed a high molecular weight with DINs (DNA integrity score) ranging from 7.5 to 10, and average concentrations from 370 ng/µL. RNA was extracted from ~30 mg rat liver or kidney (from the cortical area) using the All Prep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. All liver samples had RNA integrity numbers (RIN) ≥ 7 as verified with the Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany).

Transcriptomics

Library preparation was performed using 100 ng total RNA using the NEBNext® Poly(A) mRNA Magnetic Isolation Module, the NEBNext® Ultra™ II Directional RNA Library Prep Kit, and indexed with NEBNext® Multiplex Oligos for Illumina® (96 Index Primers) (New England Biolabs, Ipswich, Massachusetts, USA). Sample libraries combined in equimolar amounts into a single pool at 1.1 pM were sequenced twice on the NextSeq500, and 75 bp paired-end reads were generated for each library using the Illumina NextSeq®500 v2.5 High-output 150 cycle kit (75bp paired-end reads, Illumina Inc., Cambridge, UK). A total of 455,308,427 reads (average of 12,647,456 ± 2,461,518 reads per sample) were generated for the 36 liver samples. The raw data is available at the GEO accession number GSE157426.

Reduced representation bisulfite sequencing

A total of 100 ng of total DNA was diluted and processed using the Premium Reduced Representation Bisulfite Sequencing (RRBS) Kit (Diagenode, Denville, NJ, USA) as per the manufacturer's instructions. Pooled libraries were sequenced to 75 base pair single end on a NextSeq 500 (Illumina, CA, USA). Data was aligned to the rat reference genome Rn6 with Bismark (Krueger and Andrews 2011). A total of 650,358,053 reads (average of 18,065,501 ±

4,846,025 reads per sample) were generated. The raw data from the RRBS analysis is available at the GEO accession number GSE157551.

DNA damage assay

DNA damage was measured in the liver of rats exposed to a dose of 50 mg/kg bw/day of glyphosate or to MON 52276 at the same glyphosate equivalent dose. We used a quantitative method to measure the formation of apurinic/apyrimidinic (AP) sites, which are common DNA lesions caused by oxidative damage. This was done using the DNA Damage Assay Kit (AP sites, Colorimetric) (Abcam, ab211154) (Abcam plc, Cambridge, UK) according to manufacturer's instructions.

RT-qPCR

We selected 5 genes indicative of oxidative damage to DNA from the transcriptomics of liver and measured their expression in kidney samples from rats exposed to a dose of 50 mg/kg bw/day of glyphosate or to MON 52276 at the same glyphosate equivalent dose. Extracted RNA was retrotranscribed to cDNA using the SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher scientific, Loughborough, UK). A total of 100 ng cDNA was then amplified using TaqMan™ assays for *Nr1d2* (Rn00596011_m1), *Gadd45g* (Rn01352550_g1), *Nr1d1* (Rn01460662_m1), *Ier3* (Rn03993554_g1), *Clec2g* (Rn01462968_m1), using *Actb* (Rn00667869_m1) as an internal reference standard. Reactions were conducted as technical duplicates, with a TaqMan™ Fast Advanced Master Mix (ThermoFisher scientific, UK) on the Applied Biosystems QuantStudio 6 Flex Real-Time quantitative PCR System. The delta-delta Ct method (Livak and Schmittgen 2001) was used to calculate the relative gene expression of the target genes in kidney samples.

ToxTracker Assay

The ToxTracker assay is a panel of six validated green fluorescent protein (GFP) gene-based mouse embryonic stem (mES) reporter cell lines that can be used to identify the biological reactivity and potential carcinogenic properties of newly developed compounds in a single test. The ToxTracker mES cell lines contain a GFP reporter gene inserted into genes whose expression is known to increase in response to various carcinogenic stimuli (oxidative stress, DNA damage, protein unfolding/misfolding). This assay was performed as previously described (Hendriks et al. 2016). We compared the genotoxic properties of glyphosate and MON 52276, but we also tested two other formulated GBH products: MON 76207 (Roundup PROMAX on the US market) and MON 76473 (Roundup ProBio on the UK market).

In brief, glyphosate, Roundup BioFlow (MON 52276), MON 76207 and MON 76473 were diluted to a glyphosate equivalent concentration of 50 mM in water and the pH adjusted to 7.2. First, wild type mES cells (strain B4418) were exposed to 20 different concentrations of the test substances to determine cytotoxic effects and select 5 concentrations to be tested in the six independent mES reporter cell lines. Induction of GFP reporter gene expression was determined after 24 h exposure using flow cytometry. Potential metabolic activation was included in the ToxTracker assay by addition of S9 liver extract from aroclor1254-induced rats (Moltox). Cells were exposed to five concentrations of the test samples in the presence of 0.25% S9 extract and required co-factors (RegenSysA+B, Moltox) for 24 h. Positive reference treatments with cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded/misfolded protein response) and aflatoxin B1 (metabolic activation of progenotoxins

by S9) were included in all experiments. Solvent concentration was the same in all wells and never exceeded 1% DMSO.

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. Only GFP induction at concentrations that do not cause more than 75% cytotoxicity are used for the ToxTracker analysis.

Statistical analysis

Incidence of non-neoplastic lesions was evaluated with a Fisher's exact test (one and two-tailed; one-sided results were also considered, since it is well established that only an increase in incidence can be expected from the exposure, and incidence in the control group are almost always 0). The analysis of linear trend, for incidence of pathological lesions, was obtained using the Cochran-Armitage trend test (OECD 2011; Shockley and Kissling 2018). Statistical significance for the serum biochemistry data was evaluated using a One-way ANOVA with post-hoc Tukey HSD (honestly significant difference).

RNA-seq data was analysed with Salmon (Patro et al. 2017). This tool was used to quantify transcript abundance by mapping the reads against a reference transcriptome (Ensembl Release Rattus Norvegicus 6.0 cDNA fasta). The Salmon output was then imported into R version 4.0 (Team 2019) using the Bioconductor package tximport. We created a transcript database containing transcript counts which was used to perform a differential gene expression analysis using DESeq2 (Love et al. 2014). We finally used goseq to perform a gene ontology and a KEGG pathway enrichment analysis accounting for transcript length biases (Young et al. 2010). The relationship between the different significant GO terms was visualised using REVIGO (Supek et al. 2011)

DNA methylation calls from RRBS data were extracted with Bismark (Krueger and Andrews 2011). The output from Bismark was then imported into R and analysed with Methylkit (Akalin et al. 2012). DNA methylation calls were annotated using RefSeq gene predictions for rats (rn6 release) with the package genomation (Akalin et al. 2014). Other annotations were retrieved using the genome wide annotation for rat tool org.Rn.eg.dbR package version 3.8.2. Statistical analysis was performed with logistic regression models fitted per CpG using Methylkit functions. P-values were adjusted to Q-values using the SLIM method (Wang et al. 2011).

Multi-omics integration was performed with the Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) tool using the R package mixOmics (v 6.13.3) (Singh et al. 2019). DIABLO is a supervised classification method that has been developed to integrate large 'omics' datasets and determine which genes/metabolites are discriminating experimental groups and acting in concert. The optimal number of variables in each dataset was determined using the tune.block.splsda function after 100 repeats. The measurement of the error rate was done using a leave-one-out approach.

Results

Our preceding analysis of the same animals investigated in this study indicated that glyphosate and to a greater degree Roundup MON 52276 gave rise to oxidative stress (Mesnage et al., 2021). The primary aim of this follow-up investigation was to determine if this oxidative stress resulted in toxic effects in the liver. Rats were exposed to glyphosate and its representative EU Roundup commercial formulation (MON 52276). The doses used were chosen as they represent the EU ADI (0.5 mg/kg bw/day), the EU NOAEL (50 mg/kg bw/day) and the US NOAEL (175 mg/kg bw/day). No significant differences were observed in feed consumption, nor in mean body weight, and only the group administered with the highest dose of MON 52276 (175 mg/kg bw/day glyphosate equivalent dose) had a reduced water consumption (Mesnage et al. 2021).

Roundup MON 52276 caused more serious liver lesions than glyphosate

All treated and control animals were subjected to a histopathological evaluation of the kidneys and liver. No lesions were detected in liver of control animals whereas a low frequency (2/8 animals) of pelvic mineralisation, inflammation and epithelial pelvic necrosis was observed in their kidneys (Table 1). Contrastingly, a dose-dependent and statistically significant increase in the incidence of liver lesions (fatty liver changes, necrosis) was observed in rats treated with MON 52276 (Table 1; Figure 2). An increase in liver and kidney lesions was also detected in animals treated with glyphosate, although this did not reach statistical significance (Table 1). Blood clinical biochemistry only revealed increased creatinine levels in the groups treated with glyphosate but not with MON 52276 (Figure 3). Considering the limited time of exposure (90 days), and the relatively small number of animals tested, it is possible that these non-statistically significant alterations in liver and kidney structure could be due to the glyphosate or MON 52276 exposure.

Liver transcriptomics reveals pathways of oxidative damage to DNA

In order to obtain insight into the possible effects of glyphosate and MON 52276 on liver function, we then used high-throughput molecular profiling techniques to search for molecular changes, which could act as predictors of the development of liver disease. We focused our analysis on liver samples from the cohort of rat exposed to the EU NOAEL (50 mg/kg bw/day).

Whilst a total of 20 genes had their expression altered (one gene was downregulated and 19 upregulated) by glyphosate (FDR = 5%) (Figure 4A), the exposure to MON 52276 at the same glyphosate equivalent dose altered the expression of 98 genes (50 genes downregulated and 48 upregulated) (Figure 4B). This correlated well with the findings from the histopathology analysis (Table 1) showing that MON 52276 was more toxic than glyphosate. Functional analysis failed to provide significant insights about the effects of glyphosate because of the low number of genes having their expression altered by this compound. However, important functional insights about the effects of MON 52276 were obtained. Using Gene Ontology (GO) annotations, we found that the exposure to MON 52276 affected the expression of genes associated with 173 biological processes (Figure 4D), with the most significant being the response to steroid hormones (adj.p = 0.0001) and radiation (adj.p = 0.002). A pathway analysis suggested that TP53 signaling (adj.p = 0.0008) and the regulation of circadian rhythms (adj.p = 0.01) were over-represented among the genes having their expression altered by MON 52276. Genes having their expression changed in relation to the TP53 signaling pathway were *Cdkn1a* (FC = -2.5), *Gadd45a* (FC = 1.7), *Chek2* (FC = 1.9), *Wesn2* (FC = -1.5) and *Gadd45g* (FC =

2.5). The two most affected genes were *Nr1d1* (FC = 8.8) and *Nr1d2* (FC = 3.1). Fold changes in gene expression of the genes having their expression altered by MON 52276 were well correlated to those affected by glyphosate alone (Figure 4C, Pearson's r = 0.83, p < 2.2e-16), suggesting that glyphosate affected the same pathways as MON 52276, even if only effects of MON 52276 reached statistical significance. Based on these results we were able to derive a short-list of genes whose expression was similarly altered by both glyphosate and MON 52276 and which could constitute a transcriptome signature for exposure to this class of herbicide (Table 2).

Gene expression was altered by glyphosate or MON 52276 in kidneys

Transcriptome alterations caused by both glyphosate and MON 52276 in liver were marked by statistically significant changes in the expression of 9 genes (Table 2) with consistent fold changes (Figure 4A and 4B). We hypothesized that these genes could be considered as a marker of oxidative DNA damage from glyphosate exposure (Figure 4D). In order to test this hypothesis, we measured expression of 5 genes from this list of 9 transcriptome biomarker candidates namely, *Nr1d1, Nr1d2*, *Clec2g, Ier3,* and *Gadd45g* in kidney, which showed the most significant alterations in liver samples. Only *Nr1d2*, *Clec2g* and *Gadd45g* transcripts were detectable in kidney samples with differential expression between these genes found to be comparable to that observed in the liver samples (Figure 5). Overall, this suggested that kidneys and thus potentially other tissues may have been damaged by the exposure to MON 52276 and glyphosate.

DNA methylation profiling reflected changes in the epigenome landscape

There was no difference in the percentage of methylated cytosines in CpG islands in either glyphosate or MON 52276 exposed rats (42.0 ± 1.6% for controls vs 42.6 ± 3.0% and 41.7 ± 1.7% for glyphosate and MON 52276 respectively). The distribution of the percentage DNA methylation per base was bimodal, as expected since a given C-residue is generally either methylated or not in a given cell (Figure 6A). CpG di-nucleotides near transcription start sites tend to be unmethylated (Figure 6B). Overall, we identified 5,727 and 4,496 differentially methylated CpG sites (DMS) (FDR < 0.05) with a modest methylation difference (> 10%) between the control group and the group of rats exposed to glyphosate and MON 52276, respectively (Figure 6C). When both treatments were compared, we found that 1100 CpG sites were found to be differentially methylated to the same degree in both glyphosate and MON 52276 treatment groups. The amplitude of these changes ranged from -35 to 34%. Percentage methylation changes were very well correlated between glyphosate and MON 52276 treatment groups (Pearson's r = 0.95, p < 2.2e-16), which suggests that they are not due to random variation (Figure 6D). Among these 1100 loci, DMS were in intergenic regions (50.7%) and introns (35.5%), and less within exons (8.4%) and at gene promoters (5.4%). DMS were located at an average distance of 59.8kb from transcriptional start sites (TSS).

We evaluated if some DMS were present in the genomic regions surrounding the differentially expressed genes (DEG) identified by RNA sequencing (Figure 4). No DMS were found in the genomic regions within 50 kb around the TSS of these genes. In addition, no gene had its expression changed among the differentially methylated CpG sites, which were attributed to promoters (Table 3). There was no correlation between the fold changes in gene expression and percentage methylation changes (Figure S1). Although the biological

consequences of these methylation changes could not be ascertained, they constitute a first step towards the creation of an epigenetic biomarker of glyphosate effects.

Alterations in liver transcriptome and caecum metabolome were correlated

DIABLO was then used to understand if changes in the transcriptome could be correlated with changes in the levels of serum and caecum metabolites already described in our previous study (Mesnage et al. 2021). The first components, which are projections of the different omics datasets separating the control from the treatment group were highly correlated to each other (Figure 7A). This confirms that the treatment with glyphosate is driving the clustering of these groups of samples. The overall classification error rate with majority vote was 10% and 6% with the first and second component, respectively. The disruption of the caecum microbiome by glyphosate or MON 52276 correlated well with changes in gene expression in liver (Figure 7B). Models established by considering the methylation profiles were of poor quality and could not be used to draw any conclusions. Collectively, although integration of the different omics datasets did not provide a causal relationship, it shows that effects on the caecum microbiome and the liver gene expression profiles are concomitant and confirmed that they can be associated with the effects of glyphosate.

Oxidative damage to DNA from increased rates of apurinic/apyrimidinic sites

Since alterations in gene expression reflected triggering of a DNA damage response, which could include oxidative stress, we measured DNA damage in the same liver samples. The rate of apurinic/apyrimidinic was increased by the exposure to glyphosate (Figure 8). This confirms that oxidative stress caused by glyphosate exposure resulted in damage to DNA.

Oxidative stress and unfolded protein responses

The ToxTracker assay was used to test if glyphosate and 3 GBHs activated mechanisms, which are known to be key characteristics of carcinogens. Cytotoxicity of the different GBHs was highly variable (Figure S2). The formulation MON 76207 was highly cytotoxic (LC50 ~ 107 µM), compared to MON 52276 (LC50 ~ 2.7 mM) and MON 76473 (LC50 ~ 1.8 mM). Glyphosate caused no cytotoxic effects at a concentration of 5 mM in the absence or presence of the metabolising S9 liver extract.

The correct functioning and accuracy of the ToxTracker assay was demonstrated by exposure to various reference substances and monitoring expression of the GFP reporter gene, which are known to increase in expression in response to various carcinogenic stimuli. The genotoxic compound cisplatin showed induction of a DNA damage response (*Bscl2* and *Rtkn* induction) and p53-mediated cellular stress (*Btg2* induction). Diethyl maleate induced primarily the oxidative stress related reporters *Srxn1* and *Blvrb*, tunicamycin induced the unfolded/misfolded protein stress response (*Ddit3* induction). The positive control compound aflatoxin B1, which requires metabolic activation to become genotoxic, selectively induced the *Bscl2* and *Rtkn* reporters when tested in the presence of S9 liver extract (Figure S3).

Figure 9 summarises the results of the ToxTracker assay for glyphosate and the three different GBHs (MON 52276, MON 76473, MON 76207). Induction of the reporter genes is shown in absence (Figure 9A to 9D) or presence of S9 liver extracts (Figure 9I to 9L). We also reported cell survival in absence (Figure 9E to 9H) or presence of S9 liver extract (Figure 9M to 9P). The two reporters reflective of oxidative stress are Srxn1-GFP and Blvrb-GFP. Induction of the Srxn1-GFP reporter is associated with activation of the Nrf2 antioxidant response and

activation of the Blvrb-GFP reporter is associated with the Hmox1 antioxidant response. Activation of the oxidative stress reporter Srxn1-GFP was observed with MON 76473 (Figure 9B, 9J) and MON 52276 (Figure 9D, 9L). MON 76207 weakly activated (>1.5-fold) the Srxn1-GFP reporter only in the presence of S9 metabolic liver extract. However, it should be noted that MON 76207 was highly cytotoxic (Figure S2), which could have masked the toxic effect of the surfactants present in this GBH formulation and thus interfere with the detection of its oxidative stress properties. The Ddit3-GFP reporter, associated with protein damage and an unfolded/misfolded protein response, was activated upon exposure to MON 76473 (Figure 9B, 9J) and MON 52276 (Figure 9D, 9L) in the absence and presence of S9 liver extract. Surprisingly, no activation of a DNA damage response (via *Bscl2* and *Rtkn* induction) and p53-mediated cellular stress (via *Btg2* induction) was observed.

Collectively, these ToxTracker assay results show that Roundup herbicides activated oxidative stress and unfolded/misfolded protein responses but glyphosate did not, corroborating our comparative toxicogenomic evaluation in the rats, which indicated that MON 52276 was more toxic than glyphosate (Figures 2).

Discussion

Many controversies exist around toxic effects of glyphosate and its commercial formulated herbicide products. In an effort to address some of the outstanding issues we compared the toxicity of glyphosate and its EU representative formulation Roundup MON 52276. The results presented in this study confirm the increased incidence of lesions (steatosis, necrosis) in the liver of animals treated with MON 52276 (Figure 2; Table 1), which was suggested from the analysis of the serum metabolite profile described in our previous publication (Mesnage et al. 2021). The finding of liver steatosis from exposure to MON 52276 is also in agreement with our observation that an ultra-low dose of Roundup Grand Travaux Plus administered to the same strain of Sprague-Dawley rats over a 2-year period resulted in non-alcoholic fatty liver disease (Mesnage et al. 2017). Although in this study glyphosate alone only caused limited changes in liver molecular and histological profiles, longer studies would be needed to understand if these non-statistically significant increases in signs of toxicity could lead to adverse health effects if exposure was prolonged.

Alterations in liver gene expression and methylation profiles observed in this study could provide insights into the mode-of-action of glyphosate as a carcinogen. We show that glyphosate caused oxidative DNA damage in liver by increasing the rate of formation of apurinic/apyrimidinic DNA sites. This could be linked with an alteration in the expression of genes associated with the induction/repair of DNA damage via an alteration in TP53 signaling. The genes which have their expression consistently altered by both glyphosate and Roundup MON 52276 (Table 2) can serve as a biomarker of early events leading to carcinogenesis. Molecular changes detected at early stages of carcinogenesis while no pathological effects are observable are increasingly used to predict long-term carcinogenesis (Locke et al. 2019). However, it is not clear whether glyphosate is a liver carcinogen even if some animal studies indicated that hepatocellular adenomas can develop in male Wistar rats after chronic exposure to glyphosate (Portier 2020).

Other studies have suggested that glyphosate causes oxidative damage to DNA, and that this can activate DNA repair mechanisms. A study on human peripheral blood mononuclear cells showed that glyphosate decreased the expression of the tumor suppressor gene *TP53,* and that this decreased expression was concomitant with an induction of 5-mC methylation of the *TP53* promoter (Woźniak et al. 2020). In addition, changes in the expression of the core-clock gene *NR1D1,* whose expression was most affected by the exposure to glyphosate in our study, are known to impact the circadian phenotype of *TP53*, ultimately affecting proliferation, apoptosis, and cell migration (Basti et al. 2020). Interestingly, disturbances of circadian genes are known to be involved in the etiology of NHL (Hoffman et al. 2009), providing a molecular mechanistic explanation for observations stemming from epidemiological studies showing that night-time work predisposes to NHL (Lahti et al. 2008). The circadian clock is known to impact cell proliferation and migration in lymphoma cells (Abreu et al. 2018).

DNA damage was observed with glyphosate but not with MON 52276 (Figure 7). One possible explanation for this apparent contradiction is that the damage caused by MON 52276 was sufficiently high to elicit DNA repair, while DNA damage for glyphosate was not detected by DNA lesion sensor mechanisms. This is corroborated by the observation of TP53 pathway activation with MON 52276 but not with glyphosate. It is thus possible that MON 52276 caused oxidative DNA damage, but this could not be detected because it had been repaired.

The findings from our comparative toxicogenomic evaluation in rats suggested that MON 52276 caused oxidative DNA damage. However, none of the GFP-reporters from the ToxTracker assay scored positive for activation of a DNA damage response or p53-mediated cellular stress (Figure 9), while the Srxn1-GFP reporter for oxidative stress was activated. This apparent discrepancy suggests that there are some uncontrolled factors, which can influence the detection of glyphosate genotoxicity. This is not necessarily surprising given the large number of studies with contradictory results and divergent interpretations (Benbrook 2019). It is plausible that DNA damage is only detectable *in vivo* because of a role of the gut microbiome as suggested in this study (Figure 7), or that the redox balance and the DNA damage repair capacity of the system influences the detection of DNA damage (Mikhed et al. 2015). It is also possible that glyphosate uptake *in vitro* is limited, as glyphosate is hydrophilic and enters cells via L-type amino acid transporters (Xu et al. 2016). The observation in an *in vitro* study, which showed that ~ 20% of ^{14}C-glyphosate entered HepG2 cells is in support of this possibility (Gasnier et al. 2011). It is also possible that DNA damage is the consequence of the activation of another type of toxicity process, which would not be detectable by the ToxTracker assay. For instance, DNA damage could be secondary to liver inflammation (Kawanishi et al. 2017).

Even if the biological significance of the observed changes in liver DNA methylation profiles (Figure 6) remain unclear, our findings constitute a first step towards the creation of a biomarker allowing the identification of glyphosate-associated pathologies in human populations. Although other epigenetics studies have been performed in different model systems (Duforestel et al. 2019; Kubsad et al. 2019; Woźniak et al. 2020), no *in vivo* biomarker of glyphosate exposure has been proposed or identified in laboratory animals. The increase in Tet3 activity, which was proposed to be a target of glyphosate in the mammary gland (Duforestel et al. 2019), was unchanged in our study. This could be due to tissue specific effects, or to the regulation of Tet3 activity at a protein rather than gene transcriptional level, which would not be detectable in our study. Since cancer risks are associated with genomic instability, early events in tumorigenesis can be reflected by epigenetic changes (Locke et al.

2019). For instance, the study of DNA methylation levels in Long Interspersed Nucleotide Element 1 (LINE-1) was found to be a surrogate for changes in global DNA methylation levels caused by exposure to pesticides in farmers (Alexander et al. 2017). Epigenetic biomarkers can also be more specific as in the case of the methylation levels of *WRAP53*, which is antisense to *TP53,* was found to be associated to pesticide exposure in a Mexican population (Paredes-Céspedes et al. 2019). Epigenetic signatures can also be compound-specific such as for smoking, and even inform on smoking habits and time since smoking cessation (Kaur et al. 2019).

While the co-formulants contained in the representative EU commercial GBH formulation MON 52276 are known to have one of the best safety profiles among agricultural surfactants, the results from the study presented here and in our previous investigation with the same animals (Mesnage et al. 2021) suggests that it is more toxic than previously assumed. Previous studies on human cells found that MON 52276 was no more toxic than glyphosate alone at equivalent concentrations (Mesnage et al. 2013), and that it was less toxic than glyphosate on rainbow trout and water fleas (Mesnage et al. 2019). However, our study shows that exposure to MON 52276 causes liver steatosis and necrosis and supports the need for long-term toxicity evaluations of the toxicity of formulated products. MON 76207 could even be more toxic as suggested by cytotoxicity profiles in mES cells. Since the residues of co-formulants are not monitored in human populations or in the environment, and the fact that there is no regulatory requirement to assess their toxicity in long-term studies, their health risks remain a major unknown quantity (Mesnage and Antoniou, 2018). Only a few studies have shown that the most used co-formulant POEA could persist in the environment after agricultural applications (Tush et al. 2018). No studies were performed to understand if post-harvest applications of GBH could expose consumers to co-formulant residues. Workers are always exposed to formulated products or their mixtures with additional pesticides, even if biomonitoring data is needed to understand internal exposures resulting from the application of agricultural sprays. However, since alterations in gene expression and DNA damage were consistently detected after the exposure to glyphosate alone *in vivo*, our study suggests that DNA damage caused by the exposure to glyphosate formulations could be attributed to the effects of glyphosate alone and not to a direct effect of its co-formulants.

Farmers frequently mix pesticides in order to overcome weed resistance to a single herbicide. Glyphosate is increasingly applied in combination with other active ingredients such as dicamba, mesotrione, metolachlor or 2,4-D in order to mitigate herbicide-resistance in large-scale monocultures of herbicide-tolerant GM crops (Bonny 2016; Vink et al. 2012). An increasing number of studies have found that pesticides can exert effects as mixtures at concentrations, which they do not show negative health outcomes in isolation (Martin et al. 2021). Future studies will have to evaluate the combined effects of glyphosate with other pesticides, since human populations are rarely exposed to single compounds.

Conclusions

Our study reports the first observations of the changes in the epigenome (DNA methylation profile) and transcriptome landscapes in the liver of rats exposed to glyphosate or its formulated product Roundup MON 52276. We also confirmed that glyphosate causes DNA damage and leads to activation of DNA repair mechanisms in a mammalian model system *in vivo*.

Mechanistic evaluation of carcinogenicity mode-of-action showed that Roundup herbicides can activate oxidative stress and an unfolded/misfolded protein response at concentrations at which glyphosate gave no effects. Our study further highlights the power of high-throughput 'omics' methods to detect metabolic changes, which are poorly reflected or completely missed within conventional biochemical and histopathological measurements upon which regulators currently rely. Thus, adoption by regulatory agencies of multi-omics analyses would result in more accurate evaluation of a chemical's toxicity and therefore better protection measures being enacted with major public health benefits. In addition, our study paves the way for future investigations by reporting a panel of genes and DNA methylation sites, which can serve as biomarkers of glyphosate exposure and negative health effects.

Acknowledgements

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Competing interests

RM has served as a consultant on glyphosate risk assessment issues as part of litigation in the US over glyphosate health effects. The other authors declare no competing interests.

Figures

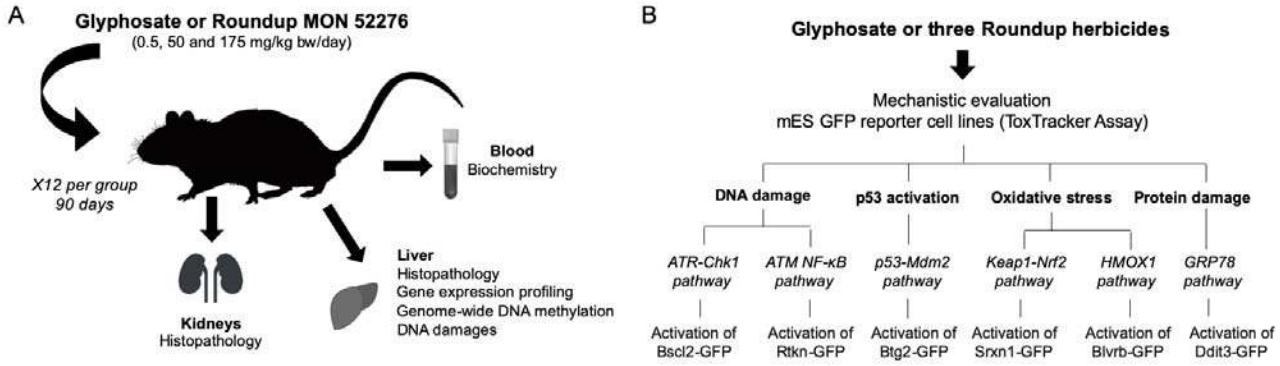

Figure 1. Experimental design. A. Sprague-Dawley rats were exposed to three concentrations of glyphosate or MON 52276 over 90-days. Molecular profiling techniques were applied in conjunction with standard histopathology and blood biochemistry analyses. **B.** The ToxTracker assay was used to identify the potential carcinogenic properties of glyphosate and three formulated glyphosate products (MON 52276, MON 76473 and MON 76207).

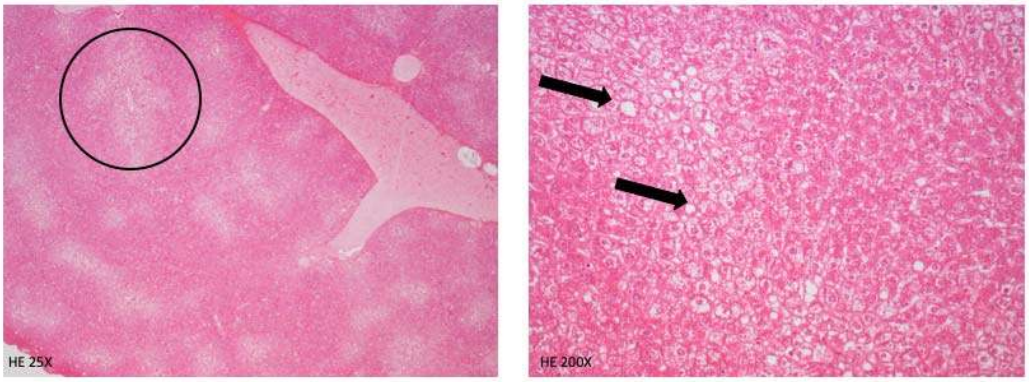

Figure 2. Exposure to Roundup MON 52276 causes liver damage. Female rats exposed to Roundup MON 52276 at a 175 mg/kg bw/day glyphosate equivalent dose cause a diffuse and severe fatty change with necrosis of hepatocytes (circle and arrows).

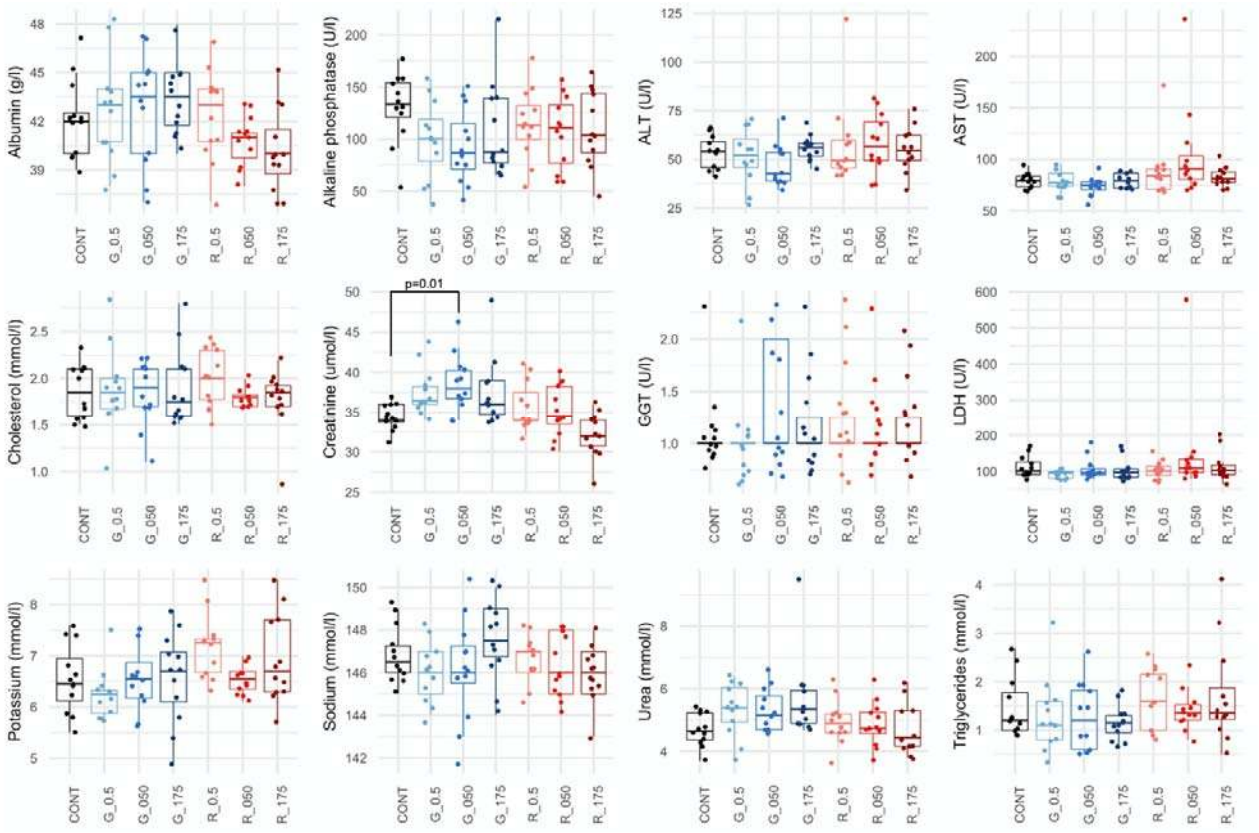

Figure 3. Serum biochemistry of rats following subchronic 90-day exposure to glyphosate and Roundup MON 52276. Clinical biochemistry evaluation in female rats administered with glyphosate (G_0.5: 0.5 mg/kg bw/day; G_050: 050 mg/kg bw/day; G_175: 175 mg/kg bw/day;) and MON 52276 (R_0.5: 0.5 mg/kg bw/day; R_050: 050 mg/kg bw/day; R_175: 175 mg/kg bw/day). Results at the end of the treatment period revealed minor changes with only creatinine showing a statistically significant increase at the highest dose of glyphosate (One-way ANOVA with post-hoc Tukey HSD).

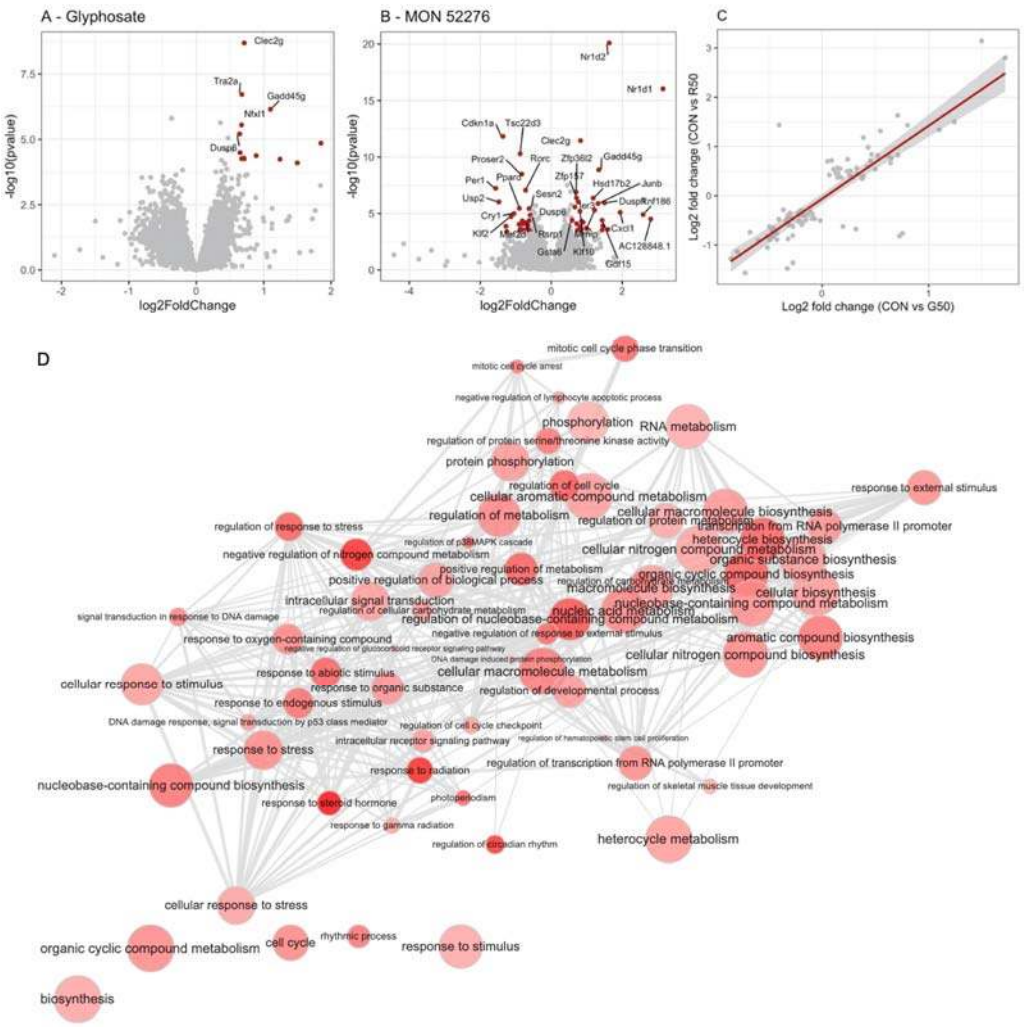

Figure 4. Transcriptome of liver from Sprague-Dawley rats exposed for 90-days to glyphosate or Roundup MON 52276. A volcano plot showing the fold changes and statistical significance in the expression of genes affected by exposure to glyphosate **(A)** or MON 52276 **(B)** at a dose of 50 mg/kg bw/day. Genes were considered as differentially expressed (red dots) if their count was found to be statistically significant after an analysis with DeSeq2. Fold changes in gene expression caused by glyphosate and MON 52276 were well correlated **(C)**. GO terms enriched for the group of rats exposed to MON 52276 (nodes) and their relation (edges) are shown **(D)**. Bubble color indicates the significant adjusted p-value ranging from 0.05 to 0.001.

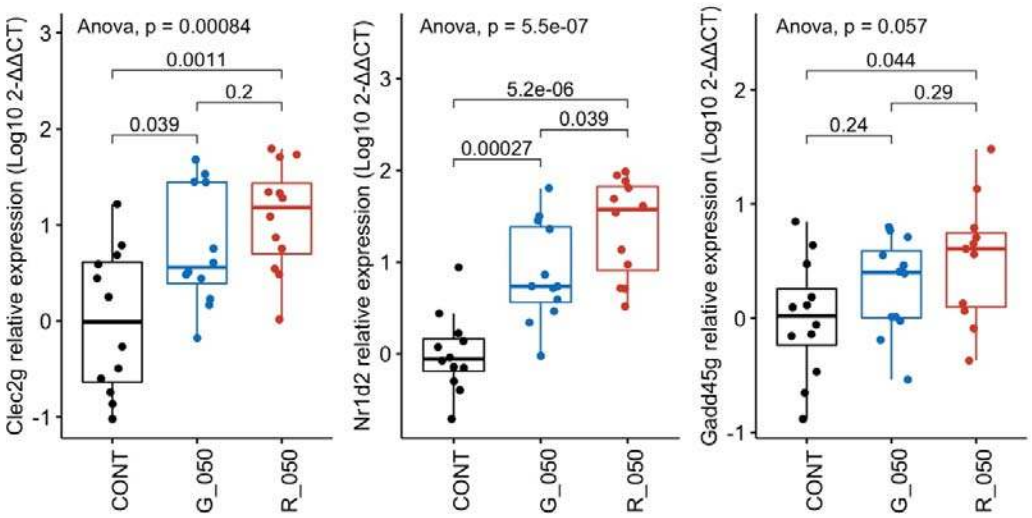

Figure 5. Alteration in expression of genes in kidney which had the most significant alterations in liver. We hypothesized that genes having statistically significant changes in their expression in liver could also be altered in kidneys. Expression of *Nr1d2*, *Clec2g* and *Gadd45g* transcripts were detectable in kidney samples of rats exposed to glyphosate (G_050) or MON 52276 (R_050) at a dose of 50 mg/kg bw/day, or to regular tap water (CONT), and reported here as delta-delta Ct values.

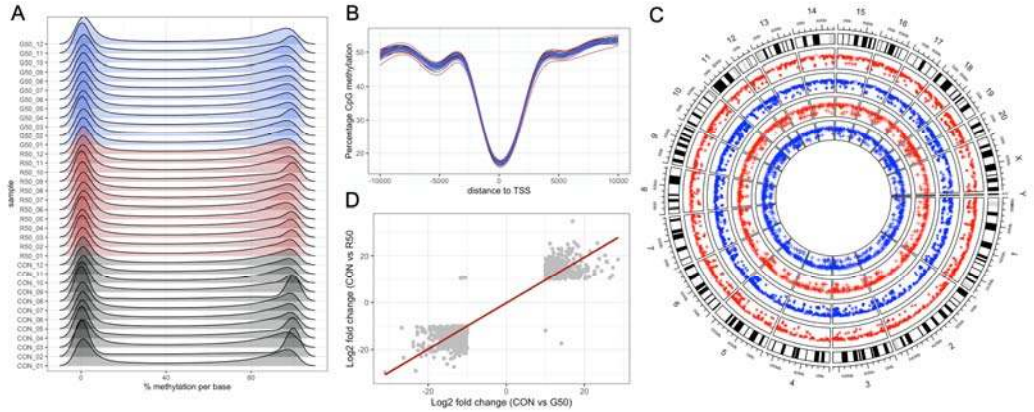

Figure 6. Reduced Representation Bisulfite Sequencing (RRBS) of liver samples from Sprague-Dawley rats exposed to glyphosate or its formulated product Roundup MON 52276. A. Distribution of percentage methylation per base **B.** CpG methylation around transcriptional start sites (TSS). **C.** Circos plot shows the location of differentially methylated CpG sites (blue track, hypomethylated; red track, hypermethylated; round symbols in outer tracks, glyphosate; cross symbols in inner tracks, MON 52276). **D**. Correlation of methylation changes for MON 52276 and glyphosate.

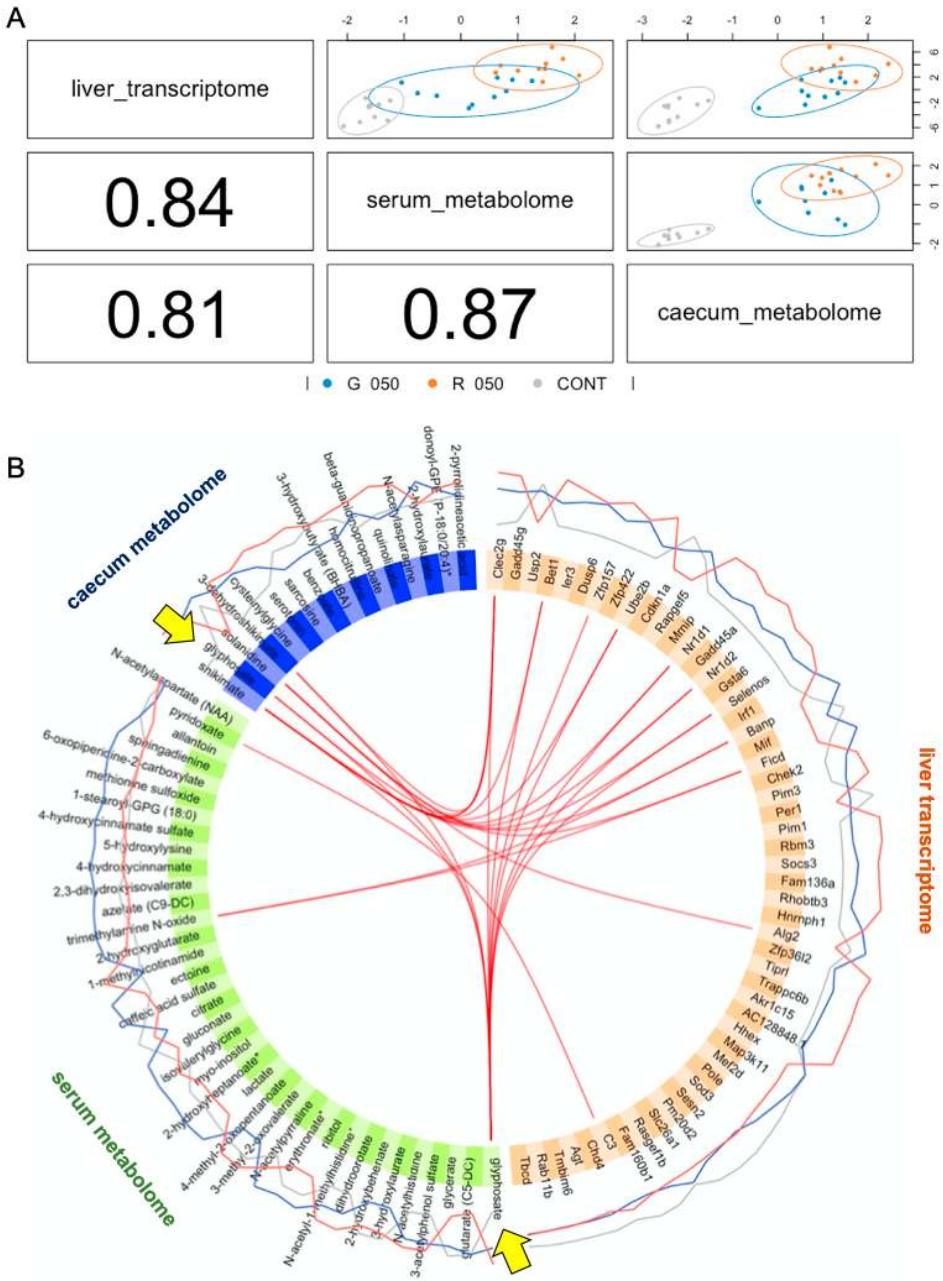

Figure 7. Changes in liver gene expression profiles in rats exposed to glyphosate or Roundup MON 52276 correlates with alterations in the serum-gut microbiota axis. The sample plot (**A**) shows the correlation between the first component from each data set. The Circos plot (**B**) shows correlations between the most discriminant metabolites/transcripts (r = cutoff = 0.7) of the serum metabolome (green), the caecum metabolome (blue), and the liver transcriptome (brown) samples. The links between each metabolite/transcript indicated the positive (red) or negative (blue) correlations. For the sake of clarity, a yellow arrow indicates the location of glyphosate.

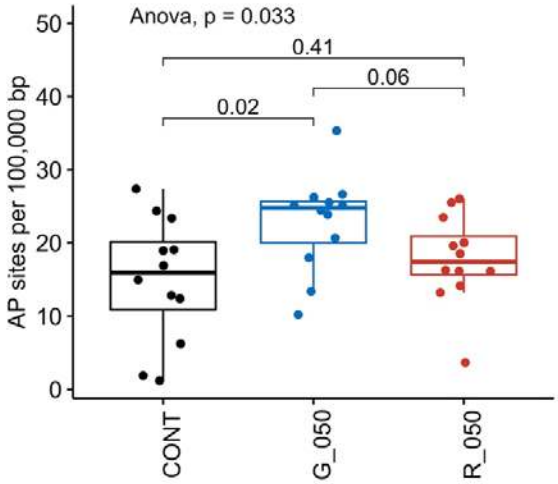

Figure 8. The exposure to glyphosate causes oxidative damage to DNA in rat liver. DNA from the liver of rats exposed to either 50 mg/kg bw/day glyphosate or the with an equivalent glyphosate dose of the commercial formulated product Roundup MON 52276 for 90-days, was analysed for oxidative damage by measuring the level of apurinic/apyrimidinic sites. A statistically significant increase in the rate of apurinic/apyrimidinic sites was detected in the glyphosate (G) treated animals but not in those exposed to MON 52276 (R) compared to untreated animals (CONT).

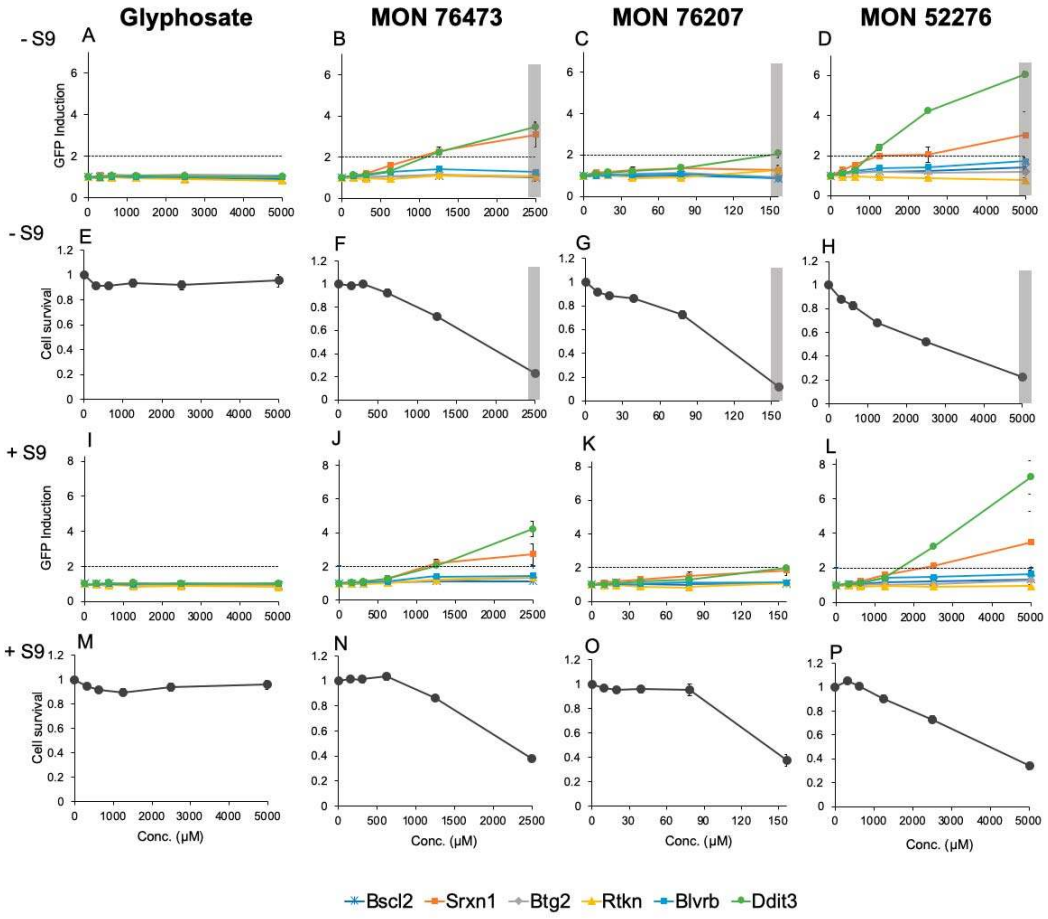

Figure 9. Three formulated GBHs activated oxidative stress and unfolded/misfolded protein responses in GFP-based mouse embryonic stem (mES) reporter cell lines. A total of six mES reporter cell lines were used to detect oxidative stress (Srxn1 and Blvrb), protein damage and an unfolded/misfolded protein response (Ddit3), the activation of a DNA damage response (Bscl2 and Rtkn) and p53-mediated cellular stress (Btg2). **Panels A to D:** induction of reporter gene expression in absence of S9 liver extracts and associated changes in cell survival **(panels E to H)**. **Panels I to L:** induction of the reporter gene expression in the presence of S9 liver extract and cell survival **(panels M to P)**. Grey shaded area denotes a concentration at which cell survival is less than 25% and thus no reliable interpretation regarding reporter gene induction from the treatment can be ascertained from this dose.

Tables

Table 1. Histopathological evaluation of liver and kidneys in adult female Sprague-Dawley rats administered for 90-days with glyphosate and Roundup MON 52276. The table shows incidence of lesions in the liver and kidneys of all animals. (**a**) Fatty change includes from mild to severe lesions or associated to necrosis. (**b**) One animal could bear more than one lesion*. Statistically significance (P≤0.05) was assessed using the Fisher Exact test (one-tailed test); # p-values (P≤0.01) associated with the Cochran-Armitage test for trend.

Lesions / Groups	**Control**		**Glyphosate**						**MON 52276**					
Dose (mg/kg)	**0**		**0.5**		**50**		**175**		**0.5**		**50**		**175**	
Liver:	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Fatty change (a)	0	0.0	2	16.7	2	16.7	0	0.0	0#	0.0	1#	8.3	3#	25.0
Necrosis (all types)	0	0.0	2	16.7	3	25.0	2	16.7	0#	0.0	1#	8.3	4*#	33.3
Inflammation, focal	0	0.0	0	0.0	1	8.3	2	16.7	0	0.0	0	0.0	0	0.0
Animal with liver lesions (b)	0	0.0	2	16.7	3	25.0	2	16.7	0#	0.0	1#	8.3	4*#	33.3
Kidneys:	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Hyaline droplets	0	0.0	5*	41.7	0	0.0	0	0.0	1	8.3	1	8.3	2	16.7
Proteinosis	0	0.0	2	16.7	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Degeneration renal tubules	0	0.0	0	0.0	1	8.3	0	0.0	0	0.0	0	0.0	0	0.0
Fibrosis renal tubules	0	0.0	1	8.3	0	0.0	1	8.3	0	0.0	0	0.0	0	0.0
Mineralization pelvis	1	8.3	2	16.7	1	8.3	0	0.0	3	25.0	5	41.7	0	0.0
Inflammation	1	8.3	2	16.7	1	8.3	5	41.7	4	33.3	2	16.7	4	33.3
Necrosis, epithelium pelvis	1	8.3	1	8.3	1	8.3	0	0.0	3	25.0	5	41.7	0	0.0
Cyst renal tubules	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Animal with kidney lesion (b)	2	16.7	8*	66.7	3	25.0	5	41.7	6	50.0	5	41.7	5	41.7

Table 2. Transcriptome signature in liver of Sprague-Dawley rats exposed to both glyphosate and Roundup MON 52276. Genes whose expression was statistically significantly changed (adj-p < 0.05) after exposure to both glyphosate and Roundup MON 52276 were selected. FC, fold changes for glyphosate (gly) or MON 52276 (mon).

Gene	Name	FC gly	FC mon
Clec2g	C-type lectin domain family 2	1.64	1.77
Gadd45g	Growth Arrest and DNA Damage Inducible Gamma	2.14	2.53
C3	Complement component 3	-1.29	-1.22
Dusp6	Dual specificity phosphatase 6	1.56	1.59
Ier3	Radiation-Inducible Immediate-Early Gene IEX-1	1.57	1.70
Nr1d2	Nuclear receptor subfamily 1 group D member 2	1.63	3.10
Dusp1	Dual-specificity phosphatase 1	2.36	2.83
Zfp422	Zinc finger protein 422	1.30	1.27
Nr1d1	Nuclear Receptor Subfamily 1 Group D Member 1	2.82	8.84

Table 3. Differentially methylated CpG sites located at gene promoters in liver of Sprague-Dawley rats exposed for 90-days to glyphosate or Roundup MON 52276. Liver samples were used to perform reduced representation bisulfite sequencing. Differentially methylated CpG sites in gene promoter regions are summarized. Also shown is the consequence on gene expression. (NA, not available; NE, not expressed; NS, not significant).

DMS for MON 52276	qvalue	meth.diff	gene.name	RNA
chr10:97895949 (-)	1.00E-55	13.5	WD repeat domain, phosphoinositide interacting 1	NS
chr2:88365913 (-)	2.00E-47	15.7	E2F transcription factor 5	NS
chr3:165740932 (-)	3.00E-43	10.4	zinc finger protein 64	NS
chr1:198181533 (-)	3.00E-41	10.6	uncharacterized LOC102551806	NA
chr20:6112412 (+)	1.00E-38	10.7	patatin-like phospholipase domain containing 1	NE
chr8:111686426 (-)	4.00E-31	12.6	SRP receptor subunit beta	NE
chr1:220466269 (-)	8.00E-29	13.8	transmembrane protein 151A	NS
chr3:172382496 (-)	4.00E-26	12.1	uncharacterized LOC102548488	NA
chr1:142220288 (+)	2.00E-20	15.1	uncharacterized LOC102553864	NA
chr6:6323934 (-)	4.00E-18	14.5	uncharacterized LOC103690379	NA
chr19:54317462 (+)	2.00E-15	11	interferon regulatory factor 8	NS
chr13:78999367 (+)	5.00E-13	-11.3	solute carrier family 9, member C2 (putative)	NE
chr13:90115074 (-)	8.00E-12	-10.1	Cd48 molecule	NS
chr15:18918298 (+)	1.00E-08	10.5	POTE ankyrin domain family member H	NS
chr5:22028484 (+)	2.00E-08	-18.1	uncharacterized LOC103692299	NA
chr17:80793952 (+)	2.00E-08	10.8	uncharacterized LOC102550536	NA
chr5:167091490 (+)	6.00E-07	11.3	microRNA 34a	NA
chr8:49709333 (+)	5.00E-06	10.5	FXYD domain-containing ion transport regulator 2	NS
chr5:169520975 (+)	3.00E-04	-10.7	chromodomain helicase DNA binding protein 5	NS
chr13:69606045 (+)	5.00E-04	12	uncharacterized LOC108352553	NA
chr4:157099430 (-)	9.00E-03	11.7	uncharacterized LOC102553636	NA
DMS for glyphosate	**qvalue**	**meth.diff**	**gene.name**	
chr3:57717495 (+)	2.00E-137	-16.9	cytochrome b reductase 1	NS
chr3:14455105 (+)	1.00E-104	-12.6	gelsolin	NS
chr3:14455057 (-)	6.00E-104	-12.5	gelsolin	NS
chr1:219852329 (-)	5.00E-74	17.6	leucine rich repeat and fibronectin type III domain containing 4	NE
chr13:90115074 (-)	3.00E-36	-17.5	Cd48 molecule	NS
chr15:33120272 (+)	6.00E-34	-13.7	RRAD and GEM like GTPase 2	NE
chr12:16912311 (+)	3.00E-30	12.9	transmembrane protein 184A	NS
chr11:72612213 (-)	1.00E-27	-10.8	3-hydroxybutyrate dehydrogenase 1	NS
chr5:159427478 (+)	3.00E-27	-10	peptidyl arginine deiminase 2	NE
chr14:83218464 (-)	1.00E-25	-12	DEP domain containing 5, GATOR1 subcomplex subunit	NS
chr5:22028484 (+)	4.00E-20	-26.8	uncharacterized LOC103692299	NA
chr13:78999367 (+)	2.00E-15	-11.1	solute carrier family 9, member C2 (putative)	NE
chr20:3350268 (+)	1.00E-13	13.7	alpha tubulin acetyltransferase 1	NE
chr1:226291649 (-)	2.00E-10	-11	myelin regulatory factor	NS
chr17:80793952 (+)	9.00E-08	10.3	uncharacterized LOC102550536	NA
chr1:87044466 (+)	1.00E-06	10.9	galectin 7	NE
chr1:166470212 (+)	3.00E-05	-14	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1	NS
chr14:80401981 (+)	4.00E-05	-12.1	carboxypeptidase Z	NS
chr13:69606045 (+)	2.00E-04	12.4	uncharacterized LOC108352553	NA
chr14:80401945 (+)	3.00E-04	-11.9	carboxypeptidase Z	NS
chr3:148103483 (-)	9.00E-03	10.5	defensin beta 25	NA

Supplemental Figures

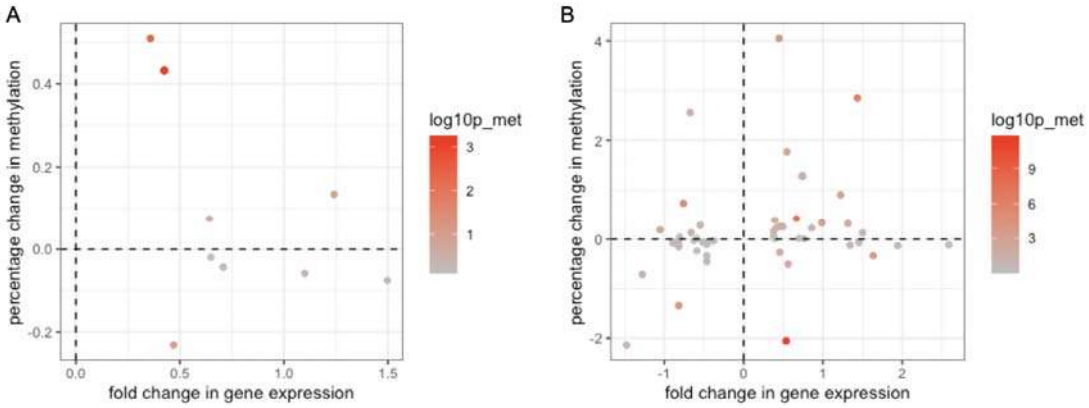

Figure S1. No correlation between the fold changes in gene expression and percentage methylation changes. RRBS of the liver samples was performed to assess if alterations in epigenetic (DNA methylation) status may be responsible for the glyphosate (**A**) and MON 52276 (**B**)-related changes in gene expression patterns. The fold changes in gene expression (RNA-seq) are compared to the percentage of change in methylation (RRBS) for the differentially expressed genes.

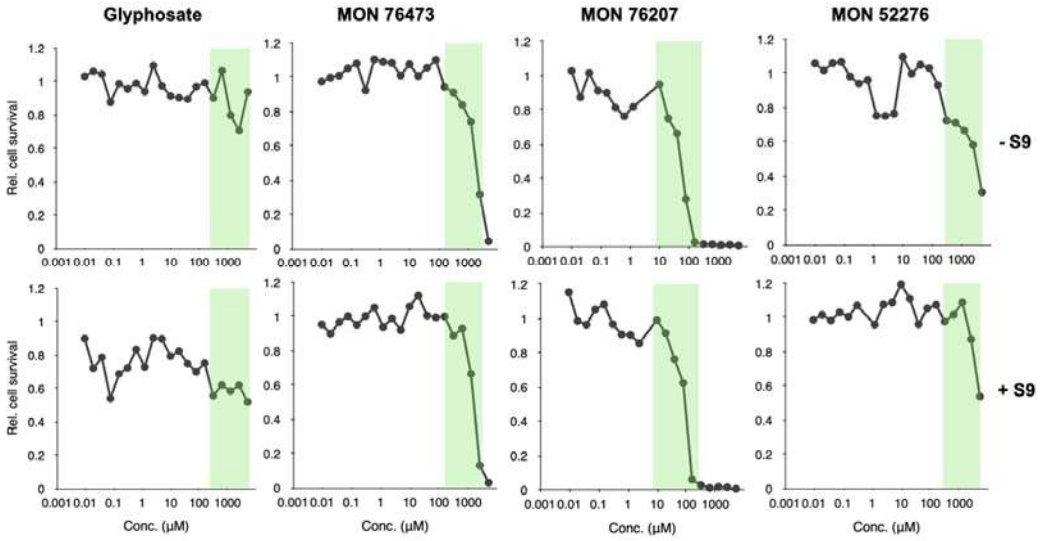

Figure S2. Cytotoxicity dose response evaluation of glyphosate and 3 Roundup herbicides in mouse embryonic stem cells. A total of 20 concentrations were tested for each compound to determine cytotoxicity thresholds. The green shaded area denotes the selected concentrations used in the ToxTracker assay.

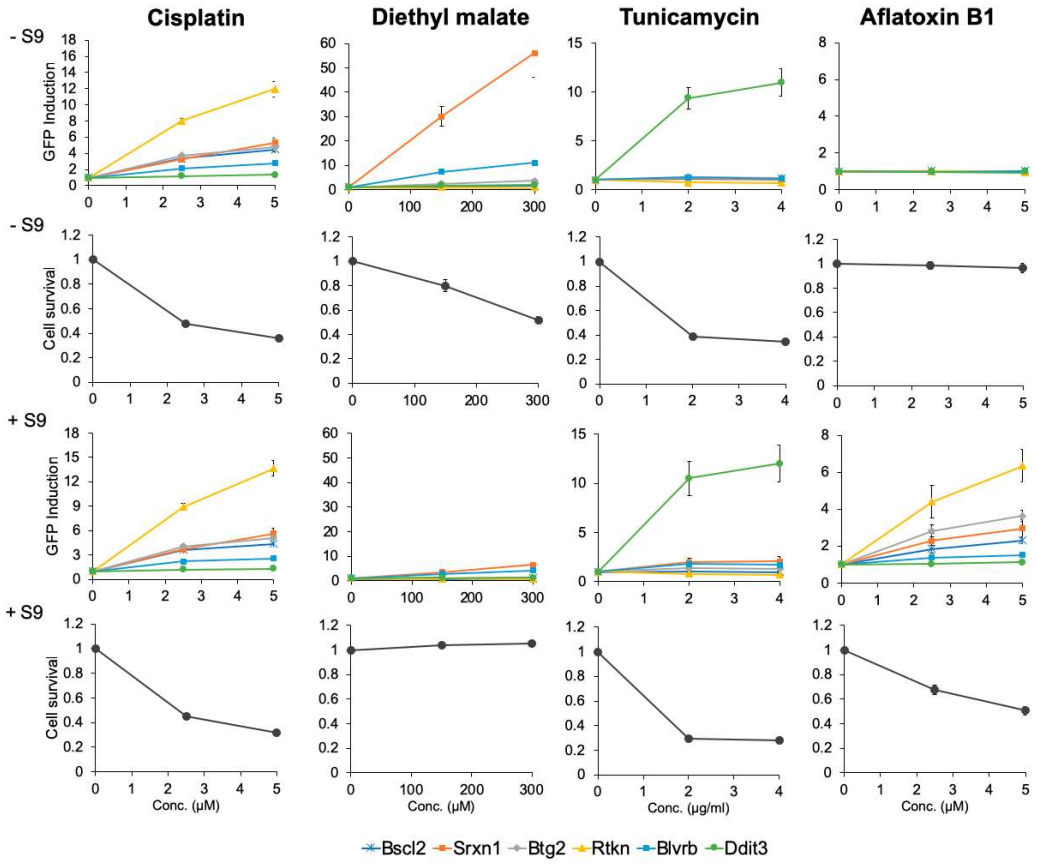

Figure S3. Positive reference treatments with cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded/misfolded protein response) and aflatoxin B1 (metabolic activation of progenotoxins by S9) were included in all the ToxtTracker experiments.

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