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Evaluation of estrogen receptor alpha activation by glyphosate-based herbicide constituents

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ABSTRACT

The safety, including the endocrine disruptive capability, of glyphosate-based herbicides (GBHs) is a matter of intense debate. We evaluated the estrogenic potential of glyphosate, commercial GBHs and polyethoxylated tallowamine adjuvants present as co-formulants in GBHs. Glyphosate (≥10,000 μg/L or 59 μM) promoted proliferation of estrogen-dependent MCF-7 human breast cancer cells. Glyphosate also increased the expression of an estrogen response element-luciferase reporter gene (ERE-luc) in T47D-KBluc cells, which was blocked by the estrogen antagonist ICI 182,780. Commercial GBH formulations or their adjuvants alone did not exhibit estrogenic effects in either assay. Transcriptomics analysis of MCF-7 cells treated with glyphosate revealed changes in gene expression reflective of hormone-induced cell proliferation but did not overlap with an ERα gene expression biomarker. Calculation of glyphosate binding energy to ERα predicts a weak and unstable interaction (−4.10 kcal mol^{-1}) compared to estradiol (−25.79 kcal mol^{-1}), which suggests that activation of this receptor by glyphosate is via a ligand-independent mechanism. Induction of ERE-luc expression by the PKA signalling activator IBMX shows that ERE-luc is responsive to ligand-independent activation, suggesting a possible mechanism of glyphosate-mediated activation. Our study reveals that glyphosate, but not other components present in GBHs, can activate ERα *in vitro*, albeit at relatively high concentrations.

1. Introduction

Glyphosate-based herbicides (GBHs) are the most widely used pesticides worldwide. Glyphosate acts as a herbicide by inhibiting the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), causing a shortage of aromatic amino acids (Boocock and Coggins, 1983). Glyphosate occupies the binding site of the second substrate of EPSPS (phosphoenol pyruvate), mimicking an intermediate state of the ternary enzyme substrate complex (Schonbrunn et al., 2001). GBH are commercialized in the form of mixtures consisting of glyphosate and various co-formulants, which are required to stabilise and allow penetration of glyphosate into plants. The major class of co-formulants is represented by surfactants. They are generally included in commercial GBH formulations, but are also sold and used separately as adjuvants and added during the preparation of the agriculturally applied pesticide mixture. In 2014, the amount of GBH sprayed by farmers was equivalent to glyphosate being applied at 0.53 kg/hectare on all cropland worldwide (Benbrook, 2016). Glyphosate is routinely detected in foodstuffs (EFSA, 2014), air and rain (Majewski et al., 2014). The half-life of glyphosate is variable depending on environmental conditions. For example, it ranged from 47 to 315 days depending on light and temperature in a study using coastal seawater from the Great Barrier Reef (Mercurio et al., 2014). Epidemiological data on the human body burden of GBH residues is very limited but evidence suggests that glyphosate and its metabolites are wide-spread (Niemann et al., 2015). Although the contamination of human biological fluids by compounds used as co-formulants in commercial pesticides is poorly investigated, their

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presence in food (Ferrer et al., 2011) and in the environment (Berge et al., 2012; Tush and Meyer, 2016) is well documented.

A number of *in vivo* toxicity studies have suggested effects of glyphosate and its commercial formulations on reproductive organs (Mesnage et al., 2015a). This includes studies on the uterus (Guerrero Schimpf et al., 2017), the hypothalamic–pituitary–gonadal axis (Romano et al., 2012), testis (Cassault-Meyer et al., 2014) and ovaries (Armiliato et al., 2014). However, it is not clear whether toxic effects observed in these studies are due to endocrine disrupting mechanisms or result from a more general cytotoxicity mechanism. Glyphosate was suggested to have endocrine interference properties by inhibiting aromatase enzyme activity (Richard et al., 2005) and activating estrogen receptors (ER) (Thongprakaisang et al., 2013). In contrast, no evidence of potential interaction of glyphosate with the estrogen pathway has been detected in the Endocrine Disruptor Screening Program (EDSP) conducted by the US Environmental Protection Agency (EPA) (US EPA, 2015). Thus the endocrine disruptive capability of glyphosate remains uncertain.

Other modes of action can also be postulated for glyphosate-induced toxicity in a number of diverse species. Glyphosate inhibition of EPSPS in plants is by competitive inhibition of its substrate phosphoenolpyruvate (PEP) binding at the enzyme active site (Schonbrunn et al., 2001). Enzymes binding PEP are regulators of energy metabolism, in particular through the TCA cycle. Glyphosate off-target effects may include the disruption of these enzymes. Glyphosate can also interact at the substrate binding site and potentially inhibit mitochondrial succinate dehydrogenase (Ugarte, 2014). Overall, glyphosate has multiple non-specific biological activities and has been patented for a number of purposes, including use as a weedkiller (U.S. Patent No 3,799,758), a metal chelator (U.S. Patent No. 3,160,632), an anti-cancer and anti-viral compound (U.S. Patent No 5665713 A), and an anti-parasitic agent (U.S. Patent No 7771736 B2).

Surfactants contained in GBH can also be a source of toxicity (Mesnage et al., 2013). Populations of farmers exposed to adjuvants such as solvents or petroleum distillates presented a higher risk of developing hypospadias (Carmichael et al., 2013) and more allergic and non-allergic wheeze (Hoppin et al., 2017). However, the specific role of the pesticide-derived surfactants versus other sources of similar compounds found on farms is currently unknown. Since previous toxicity studies have revealed that the polyethoxylated tallowamine (POEA) class of adjuvants are potent toxicants *in vitro* (Mesnage et al., 2013), the investigation of the toxicological profile of POEA has been defined as a priority by the European Food Safety Authority (EFSA) (EFSA, 2015).

In order to address some of the gaps in the evidence pertaining to the endocrine disrupting capability of glyphosate and GBHs in general, we examined the estrogenic potential of these compounds in three human breast cancer cell lines. The effects of glyphosate, two forms of POEA co-formulant (technical grade and agricultural spray adjuvant) and 4 commercial GBH formulations containing different ratios of co-formulants were compared to estradiol and the known estrogen mimic bisphenol A (BPA). The E-screen assay employing cell proliferation of MCF-7 estrogen-dependent cells and an estrogen responsive element (ERE)-luciferase reporter gene assay in T47D-KBluc cells were used. In addition, effects on the transcriptome in hormone dependent MCF-7 cells using a microarray platform and by RNA-seq were also determined to ascertain if alterations in the gene expression profiles correlate with an established gene expression profile signature used to accurately predict estrogen receptor α (ERα) modulation (Ryan et al., 2016). Moreover, it is known that the activation of ER can be caused by the modulation of the phosphorylation of certain protein kinases due to the interplay between cellular signalling pathways (Driggers and Segars, 2002). These different modes of action can be revealed by transcriptomics analysis as they have distinct gene expression signatures (Dudek and Picard, 2008). The quantum mechanical behaviour of glyphosate ions within the ligand binding domain (LBD) of ERα was assessed using molecular dynamic simulation. Own N-layered Integrated Molecular Orbital and Molecular mechanics (ONIOM) calculations were undertaken from the lowest energy structures of molecular dynamic simulations in order to evaluate the binding energy of complexes.

Our results reveal that glyphosate (>10,000 μg/L) but none of the other compounds tested activate ERα. The predicted weak interaction between ERα and glyphosate, suggests that gene activation by this compound is through other mechanisms (for example, a non-ligand binding mechanism) than direct binding to the receptor.

2. Material and methods

2.1. Reagents

All reagents and chemicals, unless otherwise specified, were of analytical grade and were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Glyphosate (CAS Number: 1071-83-6) used was the PESTANAL® analytical standard (≥98.0%) obtained from Sigma-Aldrich (UK). The batch of glyphosate (≥98.0%) purchased from AccuStandard (New Haven, CT, USA) was tested exclusively in the ERE transcription luciferase reporter gene assay in an attempt to replicate previously published conditions and results suggesting estrogenic effects (Thongprakaisang et al., 2013). GBH formulations available on the market were Glyphogan (France, 39–43% isopropylamine salt of glyphosate, 13–18% of POEA (CAS 61791-26-2), homologation 9100537), Roundup Grand Travaux Plus (France, 450 g/L of glyphosate, 90 g/L of ethoxylated etheralkylamine, homologation 2020448), Roundup Original DI (Brazil, 445 g/L of glyphosate diammonium salt, 751 g/L of other ingredients, homologation no 00513) and Roundup Probio (UK, 441 g/L of the potassium salt of glyphosate, other ingredients, Registration Number 15539). These formulations were selected as they contain different types of surfactants (Mesnage et al., 2013), which could have different toxic effects. POEA was purchased from ChemService (West Chester, PA, USA). The agricultural spray adjuvant was Genamin T200 (France, 60–80% of POE-15, homologation 8500170).

2.2. Cell culture

The MCF-7, MDA-MB-231 and T47D cell lines were a kind gift from Prof Joy Burchell (Research Oncology Department, King's College London). T47D-KBluc cells were purchased from the American Type Culture Collection (ATCC, Teddington, UK) and harbour a stably integrated copy of a luciferase reporter gene under control of a promoter containing ERE (Wilson et al., 2004).

All cells were grown at 37 °C (5% CO2) in 75 cm^2 flasks (Corning, Tewksbury, USA) in a maintenance medium composed of phenol red free DMEM (Life Technologies, Warrington, UK), 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Buckinghamshire, UK), 2 mM glutamine (GE Healthcare Life Sciences) and 10 μg/ml penicillin/streptomycin (Life Technologies). Stock solutions of glyphosate, glyphosate-based herbicides, POEA and Genamin T200 surfactant formulation were prepared in serum-free medium and adjusted to pH 7.2. All assays described below have been performed at this pH. Estradiol, 3-isobutyl-1-methylxanthine (IBMX), and BPA were diluted in 100% ethanol to prepare stock solutions. Solvent concentrations for these two compounds were always below 0.5% (cell assays) and 0.0005% (transcriptome profiling). All treatments were diluted in a test medium containing phenol red free DMEM,

5% charcoal stripped FBS (Life Technologies, UK), 2 mM glutamine (GE Healthcare Life Sciences) and 10 μg/ml penicillin/streptomycin (Life Technologies, UK). Cells were released from the flask substrate using 0.05% trypsin EDTA (Life Technologies, UK) and counted using a haemocytometer prior to seeding. A 24 h recovery period was allowed for cell adherence in DMEM maintenance medium before cultures were subjected to the desired treatments.

2.3. E-screen assay

The E-screen allows the determination of estrogenic effects by determining ER-mediated cell proliferation in hormone responsive cells. This assay was performed as originally described (Soto et al., 1995) except that it was terminated using a MTT assay (Mosmann, 1983). Additionally, this test allows the measurement of cytotoxic effects since activity of mitochondrial dehydrogenase enzymes indirectly reflects cellular mitochondrial respiration (Mosmann, 1983). Briefly, cells were seeded into 48 well plates (Dutscher Scientific, Brentwood, Essex, UK) at a density of 20,000 cells per well in 250 μl maintenance medium. Following a 24 h incubation to allow cell attachment, the medium was changed to the medium containing the compounds. The test medium was refreshed after 3 days. Following another 3-day period of incubation, an MTT assay was performed as follows. Cells were incubated with 250 μl of MTT solution (1 mg/ml) for 2 h. The test was terminated by lysing the cells with dimethyl sulfoxide (DMSO) and optical density measured at 570 nm using the SPECTROstar Nano plate reader (BMG Labtech, Aylesbury, Bucks, UK). The proliferative effect was expressed as a percentage of the control cell culture receiving no treatment.

2.4. ERE-luciferase reporter gene assay

The ERE-mediated transcription of a luciferase reporter gene was determined in the T47D-KBluc cells using the Steady-Glo® luciferase assay system following the manufacturer's instructions (Promega, Southampton, UK). T47D-KBluc cells were seeded in white 96 well plates (Greiner Bio-One, Germany) at a density of 20,000 cells per well in 50 μl of maintenance medium and allowed to attach overnight. An initial 24 h incubation was performed in the absence of test substances in order to improve residual estrogen clearance and thus assay sensitivity. Test substances were added and plates were incubated for another 24 h before the addition of 50 μl Steady-Glo® luciferase reagent. The plates were left to stand for 10 min in the dark at room temperature to allow cell lysis. Bioluminescence was measured using the Orion II microplate luminometer (Berthold Detection Systems, Bad Wildbad, Germany). ER-mediated gene activation by test substances was confirmed by ascertaining if observed effects were subject to inhibition by the addition of 100 nM of the estrogen antagonist ICI 182,780 (CAS 129453-61-8) on induced luciferase activity.

2.5. Microarray gene expression profiling

MCF-7 cells were seeded into 96-well plates with maintenance medium at a density of 20,000 cells per well. After 24 h of steroid deprivation in hormone free medium, the cells were stimulated with test substances for 48 h in triplicate in three independent experiments. RNA extraction was performed using the Agencourt RNAdvance Cell V2 kit according to the manufacturer's instructions (Beckman Coulter Ltd, High Wycombe, UK). The samples were checked for RNA quality using the Agilent 2100 Bioanalyzer and quantified using a Nanodrop instrument (ND-1000 Spectrophotometer; Thermo Fisher Scientific, Wilmington, DE, USA). The RNA integrity number (RIN) was 9.7 ± 0.3. Subsequently, technical replicates of samples, which passed quality control (QC), were pooled appropriately such that the final input amount of each biological replicate was 3 ng. Transcriptome gene expression profiles were determined using the Affymetrix Human Transcriptome 2.0 Array (Affymetrix, High Wycombe, UK) as follows. Single Primer Isothermal Amplified (SPIA) cDNA was generated using the Ovation Pico WTA System V2 kit (Nugen, AC Leek, The Netherlands), following the manufacturer's instructions. Furthermore, the SPIA cDNA was subjected to a QC check to assess quality (Agilent 2100 Bioanalyzer) and quantity (Nanodrop ND-1000) for the next stage. The SPIA cDNA was fragmented and Biotin-labelled using the Encore Biotin Module (Nugen) according to the manufacturer's instructions. The fragmented and Biotin-labelled cDNA was subjected to a further round of QC checks to assess fragmentation size (Agilent 2100 Bioanalyzer). Hybridisation cocktails of the fragmented labelled-cDNA were then prepared (Nugen) and hybridised at 45 °C overnight. The arrays were washed and stained using wash protocol FS450_0001 as recommended for Affymetrix Human Transcriptome 2.0 Arrays on the GeneChip Fluidics 450 station. Ultimately, the arrays were scanned using the Affymetrix GeneChip Scanner. CEL files were quality control assessed in the Expression Console software package (Affymetrix) by using standard metrics and guidelines for the Affymetrix microarray system. Data were imported and normalised together in Omics Explorer 3.0 (Qlucore, New York, NY, USA), using the Robust Multi-array Average (RMA) sketch algorithm. These microarray data have been submitted to Gene Expression Omnibus (NCBI) and are accessible through accession number GSE86472.

2.6. RNA-sequencing gene expression profiling

RNA sequencing was performed by applying Illumina sequencing by synthesis technology as follows. Amount of RNAs for each library (100 ng) was a pool made up of 33 ng of RNA from each of the replicate wells for each sample. The preparation of the library was conducted by NEBNext Ultra Directional RNA (New England Biolabs, Hitchin, UK) following manufacturer's protocol. The amplified library was assessed using the Agilent 2100 Bioanalyzer for size and presence of adapter/primers/dimers, sized at ~400bp (including ~130bp adapter). The rRNA component was removed using the rRNA depletion module (New England Biolabs) following manufacturer's protocol. Libraries were pooled and sequenced on the Illumina HiSeq2500 platform (Illumina UK Chesterford, Essex, UK) using a Rapid Run v2 flowcell with on-board clustering in a 2 × 100 paired-end (PE) configuration. BCL files were processed and deconvoluted using standard techniques. The sequencing output FASTQ files contain the sequences for each read and also a quality score. We analyzed the quality scores and other metrics using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Contamination from rRNA was measured using an alignment script (http://genomespot.blogspot.co.uk/2015/08/screen-for-rrna-contamination-in-rna.html). Adapter sequences (standard TruSeq LT adapter seq) were removed/trimmed using cutadapt (Martin, 2011). Sequences were then aligned to the human genome (hg38 database) using the hierarchical indexing for spliced alignment of transcripts program HISAT2 (Kim et al., 2015). BAM files were imported into the Qlucore omics explorer, along with the GTF file for known genes in hg38 (downloaded from the UCSC Genome Database). Qlucore normalises data using a method similar to the trimmed mean of M-values normalization method (TMM), which corrects for transcript length and applies a log transformation (Robinson and Oshlack, 2010). This approach gives values similar to the quantification of transcript levels in reads per kilobase of exon model per million mapped reads (RPKM) (Robinson and Oshlack, 2010), and in addition, also incorporates the TMM normalization factor for an improved between-sample

normalization.

A total of 376.6 million raw reads were obtained (25.1 ± 5.4 million reads per sample). The average value of Q30, representing the probability of an incorrect base call 1 in 1000 times, was above 96%. The GC content (%GC) of the reads was on average 49%. A total of 90.92 ± 6.54% of the clean reads were mapped onto the human reference genome hg38. Among them, an average of 71.01 ± 5.82% and 18.25 ± 3.79% reads align concordantly exactly one time and more than one time, respectively. These RNA-seq data have been submitted to Gene Expression Omnibus (NCBI) and are accessible through accession number GSE87701.

2.7. Statistical analysis

The ERE transcription luciferase reporter gene assay (n = 4) and the E-Screen assay (n = 6) were performed 4 and 6 times in triplicate, respectively. Concentrations required to elicit a 50% response (AC50) were determined using a nonlinear regression fit using a sigmoid (5-parameters) equation calculated with GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA). For the transcriptome analysis, pair-wise comparisons of each tested substance to the negative control were performed using a *t*-test controlling for batch effects in Omics Explorer 3.0 (Qlucore, New York, NY, USA). Affymetrix microarray and RNA-seq gene expression profiling were performed 3 times in triplicate.

Data used for the functional analysis were selected at cut off p-values of <0.05 with fold change >1.2 to evaluate the ER activation signature as previously described (Ryan et al., 2016). Gene and disease ontology were analyzed using the Thomson Reuters MetaCore Analytical Suite recognizing network objects (proteins, protein complexes or groups, peptides, RNA species, compounds among others). The p-values are determined by hypergeometric calculation and adjusted using the method of Benjamini & Hochberg.

2.8. Molecular dynamic simulations and ONIOM calculations

The atomic coordinates of the complex formed by estradiol (ES) and the human ERα ligand binding domain were taken from the Protein Data Bank (entry code 1A52). For the construction of the model, only chain A of the homodimer was included, and the second set of duplicate coordinates was deleted. The protein contains 239 amino acids in the modelled monomer, starting from Leu 306 and ending with Leu 544. Coordinates for missing amino acid residues (297–305, 545–554), missing heavy atoms and hydrogen atoms, were reconstructed with the LEaP module of AmberTools 15 package (Case et al., 2015) and by using the AMBER FF14SB force field. The final ER contained 258 amino acid residues. All Arg, Lys, Asp, and Glu residues were modelled as charged, all tyrosines as neutral, and histidine residues were modelled according to published models (Celik et al., 2007). Of the 13 residues of histidine, three residues were protonated (ion imidazolium) in order to preserve the electroneutrality of the system (Table 1). Histidine 524 was chosen because it serves as a communication point between the ligand and the receptor by establishing a hydrogen bond network necessary to activate the ER (Celik et al., 2007). The termini were modelled as charged. Glyphosate was docked into the ER by using the ArgusLab software (M. A. Thompson, 2004). Since the charge of glyphosate (G) is −2, the ligand-receptor system was kept neutral by changing the charge of histidine residues of the environment (medium that surrounds the active site; that is, the remaining protein plus water) or of the active site itself (Table 1). The amino acid residues that form the active site are: LEU 346, LEU 349, ALA 350, GLU 353, LEU 384, LEU 387, MET 388, LEU 391, ARG 394, PHE 404, MET 421, ILE 424, LEU 428, GLY 521, Histidine 524, LEU 525, MET 528. The Estradiol-Estrogen Receptor (ES-ER), Glyphosate-Estrogen Receptor (G-ER), and ER systems were solvated in a pre-equilibrated water box using the TIP3P solvent model and extending 10 Å beyond the protein. Electrostatic potentials of estradiol ($C_{18}H_{24}O_2$) and glyphosate ($C_3H_6NO_5P^{2-}$) were calculated with the Gaussian 09 program using the HF/6-31G(d) level of theory (Frisch et al., 2009). Partial charges were fitted with the Restrained Electrostatic Potential (RESP) method (Bayly et al., 1993) using the Antechamber module of AmberTools 15. General AMBER force field (GAFF) parameters for glyphosate were assigned by LeaP (Wang et al., 2004).

2.8.1. Molecular dynamics simulations

All simulations were carried out with the SANDER module of AmberTools 15 in periodic boundary conditions. All bonds involving hydrogen atoms were constrained by using the SHAKE algorithm (Ryckaert et al., 1977). The particle mesh Ewald (PME) method was used to treat long-range electrostatic interactions (Petersen, 1995). Temperature regulation was done using a Langevin thermostat with collision frequency of 2 ps^{-1}. Prior to the molecular dynamic simulations we performed an energy minimization in order to remove the largest strains in the systems, then each system was gradually heated in the NVT ensemble from 0 K to 310 K over a period of 70 ps. The production runs of 5 ns were performed in an NPT ensemble at 310 K and a pressure of 1 atm employing a Berendsen barostat; the time step of the simulations was 2 fs with a non-bonded cut-off of 10 Å. Snapshots were extracted every 0.5 ps. The potential energy was automatically analyzed for each of the 10000 snapshots from each model. The result obtained from this analysis allows finding the structure or conformation of lowest energy. Visualizations of glyphosate and estradiol interactions with ER were produced using the Chimera visualization system (Pettersen et al., 2004).

2.8.2. QM/MM (quantum mechanics/molecular mechanics) calculations

The conformations of lowest energy corresponding to each model are submitted to geometry optimization. The AMBER optimized structures were used as a starting point for the QM/MM calculations, except the different ER versions, which are derived from its respective complexes by elimination of the ligand. These structures obey the conformational selection hypothesis (McGowan Lauren and Hamelberg, 2013). Water molecules that were within ~10 Å of the protein surface were kept for the QM/MM calculations. All other water molecules were deleted to facilitate the computation (Humphrey et al., 1996). Moreover, in the QM/MM calculations, all residues within 7 Å of the core active site residues were allowed to move without any constraints, while all other residues were frozen (Tao et al., 2009; Tao and Schlegel, 2010).

In QM/MM hybrid methods, part of the system that includes the chemically relevant region is treated quantum mechanically (QM) while the remainder, often referred to as the environment, is treated at the classical level using molecular mechanics (MM) force fields. This multiscale approach reduces the computational cost significantly as compared to a QM treatment of the entire system and makes simulations possible that otherwise would not be feasible (Gotz et al., 2014). ONIOM, a powerful hybrid method implemented in the Gaussian 09 package (Vreven and Morokuma, 2006), was used to estimate the binding energy of the Ligand-ER model complexes (ES-ER and G-ER).

In this work, we used a three-layer ONIOM (B3LYP/6-31 + G(d):PM6:AMBER) scheme and a two-layer ONIOM(PM6:AMBER) scheme to optimize the Ligand-ER and ER models, respectively. This includes estradiol or glyphosate (B3LYP/6-31 + G(d)), the active site (PM6), and the environment (AMBER).

Table 1
Molecular dynamics simulations and ONIOM calculations of glyphosate and estradiol-estrogen receptor models. Since the charge of glyphosate (G) is −2, the ligand-receptor system was kept neutral by changing the charge of histidine residues of the environment (medium that surrounds the active site; that is, the remaining protein plus water) or of the active site itself. The ONIOM theory level of B3LYP:PM6:AMBER and PM6:AMBER were used for the Ligand-ER and ER models respectively. The final ONIOM energy (Hartrees) is presented. The modification of the charge of the active site induced a big structural distortion in ER, thus the model G-ER:11 was not reliable.

Models	Histidine		Charge		Total charge	ONIOM energy	ΔE (kcal mol^{-1})
	Envir.	Act. Site	Envir.	Act. Site			
ES-ER	HIE 356, HID 398	HIE 524	0	0	0	948.69	−25.79
G-ER:11	HIP 356, HID 398	HIP 524	1	1	0	984.14	16.15
G-ER:20	HIP 356, HIP 398	HIE 524	2	0	0	988.87	−4.10
ER:00	HIE 356, HID 398	HIE 524	0	0	0	97.79	Nr
ER:11	HIP 356, HID 398	HIP 524	1	1	2	93.57	Nr
ER:20	HIP 356, HIP 398	HIE 524	2	0	2	98.27	Nr
ES-W	nr	nr	nr	nr	0	876.61	Nr
G-W	nr	nr	nr	nr	−2	916.35	Nr
W	nr	nr	nr	nr	0	25.75	Nr

ES, estradiol; G, glyphosate; ER, Estrogen Receptor; W, Water; HID, histidine residue protonated in the δ ("delta") position; HIE, histidine residue protonated in the ε ("epsilon") position; HIP, histidine residue protonated at both positions; Nr, not relevant.

Likewise, for the ES-W and G-W models, we used a three-layer ONIOM(B3LYP/6-31 + G(d):PM6:AMBER). This included estradiol or glyphosate (B3LYP/6-31 + G(d)), a water shell of 29 residues surrounding the ligand (PM6) and the remaining water (AMBER). Finally, for the W model we used a two-layer ONIOM(PM6:AMBER) comprising a water shell of 29 residues (PM6) and the remaining water (AMBER). The results of final energy of ONIOM calculations (hartree) are reported in Table 1. The main property to be considered is the binding energy of the G-ER complex, which could be defined with the following equation: G-W + ER → G-ER + W, in other words $\Delta E_{G\text{-}ER} = \{E_{G\text{-}ER} + E_W\} - \{E_{G\text{-}W} + E_{ER}\}$. Finally, the binding energy of the G-ER complex ($\Delta E_{G\text{-}ER}$) is compared to the binding energy of the ES-ER complex ($\Delta E_{ES\text{-}ER}$) to conclude on the binding of glyphosate to the ER.

3. Results

In this investigation we compared the estrogenic capability *in vitro* of glyphosate, two forms of the polyethoxylated tallowamine co-formulant (technical grade and agricultural spray adjuvant), and 4 commercial GBH formulations containing different ratios of co-formulants to that of estradiol and BPA (Fig. 1). An E-Screen assay was performed to determine if glyphosate was able to mimic estrogen-dependent cell proliferation (Fig. 1A). The positive control 17β-estradiol was very potent in inducing the proliferation of hormone-dependent MCF-7 human breast cancer cells, with the concentration required to elicit a 50% response (AC50) being 0.0013 μg/L. BPA was also able to promote cell growth (AC50 = 46 μg/L) at concentrations 10,000 to 100,000 higher than 17β-estradiol (Fig. 1A). Glyphosate promoted growth of the same cells starting at a concentration between 1000 and 10,000 μg/L and peaking at 100,000 μg/L (Fig. 1A, upper panel). Similar but less pronounced results were observed with the T47D cell line with cells retaining a response to glyphosate but not Roundup Probio (Fig. 1A, middle panel). This lower response of T47D cells may be accounted for the fact that they possess lower levels of ERα (Parl, 2000). Additionally, no proliferative effects were observed in the ER-negative, hormone-independent MDA-MB-231 cell line, suggesting that proliferative effects were mediated via the ER (Fig. 1A, lower panel). The commercial GBH formulation Roundup Probio, tested at an equivalent glyphosate concentration, induced a non-significant trend of proliferation in the MCF-7 cell line (Fig. 1A, top panel). Roundup Probio did not show ER activity at a glyphosate equivalent concentration, which induced a cell proliferative effect when glyphosate is tested alone. This may be explained by potentially higher toxicity of commercial GBH formulation (Fig. 1A), which could have resulted in cell death at the higher concentrations tested.

Glyphosate alone, but not Roundup Probio, was able to stimulate ERE-mediated transcription of the luciferase reporter gene starting at a concentration of 1000 μg/L (Fig. 1B). The test of two other commercial formulations, Roundup Original DI and Roundup Grand Travaux Plus, also gave negative results (data not shown). We also assayed a lower grade glyphosate, from AccuStandard, in an effort to confirm previously published results suggesting an estrogenic potential of this compound at a range of concentrations around 10^{-9} M (Thongprakaisang et al., 2013). The results we obtained were similar to those for PESTANAL® Analytical Standard glyphosate (Fig. 1B); that is, a luciferase reporter gene activation was elicited from 1000 μg/L (data not shown). In order to evaluate whether the cell proliferation and luciferase reporter gene stimulatory effects observed with glyphosate were mediated by through the ER, we next performed experiments with the addition of the potent antagonist ICI 182,780 (Fig. 1C). This antagonist was effective at suppressing ER activation induced by 0.001 and 0.01 μg/L but not at 0.1 μg/L of 17β-estradiol. The addition of ICI 182,780 effectively blocked the stimulatory effects of 2000–20,000 μg/L glyphosate, confirming agonist-like effects of this compound (Fig. 1C).

The investigation of the toxicological profile of the GBH surfactant POEA has been defined as a priority by EFSA (EFSA, 2015). We tested the estrogenic potential of technical grade POEA, of an agricultural surfactant mixture containing 60–80% POEA, and of a GBH commercial formulation (Glyphogan) containing 13–18% POEA. Our results clearly indicate that POEA is not estrogenic. POEA, alone or with glyphosate in a commercial formulation, did not induce the proliferation of MCF-7 cells (Fig. 2A) and failed to induce ERE-mediated luciferase reporter gene expression in T47D-KBluc cells (Fig. 2B). We obtained similar negative results with the agricultural POEA surfactant mixture with all cell lines and assays (data not shown).

MCF-7 cells treated for 48 h with glyphosate, the GBH formulation Roundup Probio, the adjuvant POEA, or bisphenol A, and the natural hormone 17β-estradiol were subjected to a full transcriptome profiling. Table 2 displays the 10 genes having the highest fold-change in expression, either down- or up-regulated. These data were then compared to a gene expression biomarker consisting of 46 genes with consistent expression changes after exposure to 7 agonists of ER (Ryan et al., 2016). The biomarker has a balanced accuracy for prediction of ERα activation of 94%. Genes having their levels altered after treatment with glyphosate were compared to the ER gene expression biomarker using the Running Fisher algorithm as previously described (Ryan et al., 2016). The cut-off for statistical significance was p-value ≤ 0.0001 after a Benjamini-Hochberg correction of $\alpha = 0.001$. Transcriptome profile

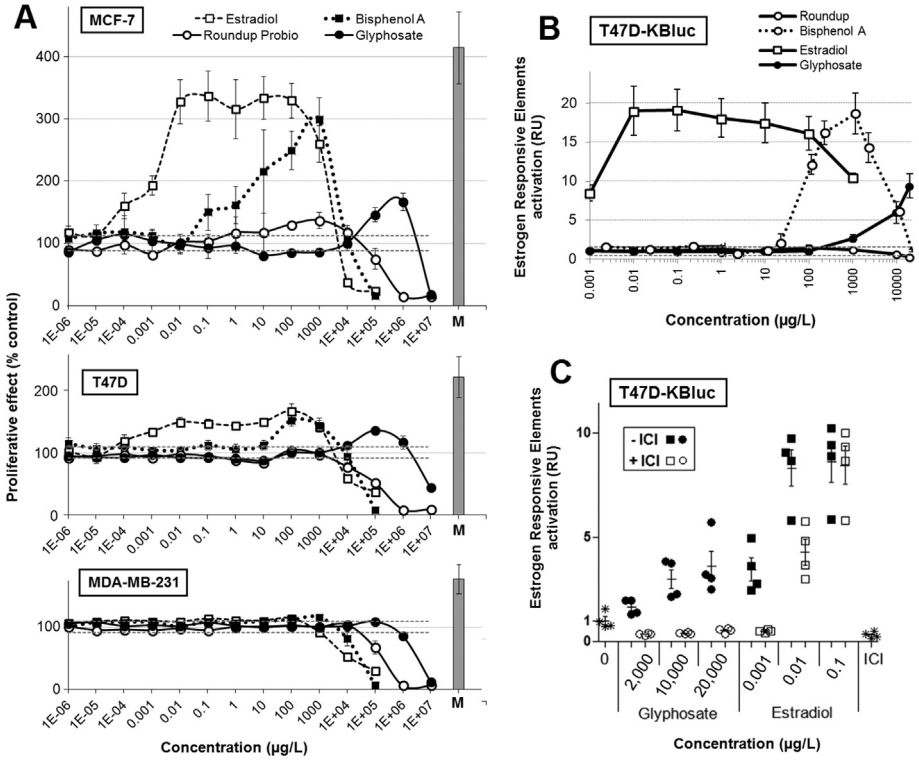

Fig. 1. Glyphosate at high concentrations can substitute for estradiol in promoting cell growth through estrogen receptors in human breast cancer cells. (A) Proliferative effect of glyphosate and Roundup Probio in an E-Screen bioassay. Hormone-dependent (MCF-7, T47D) and hormone independent (MDA-MB-231) human breast cancer cells were cultured under hormone-free conditions (n = 6). Proliferative effects by MTT assay following 6 days exposure to either glyphosate or Roundup Probio with estradiol and bisphenol A treatments acting as positive controls at the indicated concentrations. Proliferation is expressed as a percentage increase in cell number compared to culture in the absence of the natural hormone or test substance. Upper bound and lower bound of the biological variability (SEM) of the non-treated cells (negative control) are represented by dotted lines. M, proliferative effect of medium containing non-steroid hormone stripped FBS. **(B)** Estrogen receptor activation in T47D-Kluc cells harbouring a ERE-luciferase reporter gene. Cells were treated for 24 h with glyphosate alone, a Roundup formulation (Roundup Probio), BPA or estradiol and activation of expression measured by a bioluminescence assay (n = 4). **(C)** Luciferase assays of T47D-Kluc cell cultures treated with glyphosate or estrodiol in the absence (black squares, circles) or presence (white squares, circles) of the estrogen antagonist ICI 182,780 (n = 4). Note decrease in luciferase reporter gene expression indicating action of test substances via the ER.

alterations resulting from exposure to BPA and estradiol (Fig. 3A) were highly statistically significant due to the similar expression of biomarker genes in terms of fold-changes and direction to that of the biomarker. Glyphosate treatment at the highest concentration (10,000 µg/L; Fig. 3A) produced changes in the expression of only 3 genes present in the ER biomarker (p-value = 0.0037, Fig. 3C), which did not achieve the threshold for activation. The analysis of gene ontology confirms that genes having their expression altered by treatment of MCF-7 cells with 10,000 µg/L glyphosate were involved in cell cycle regulation, as well as in stimulation by steroid hormones (Fig. 3B). The transcriptome of glyphosate-treated cells was also reflective of cell death through apoptosis. The GO terms "regulation of apoptotic process" and "cell death" were significantly enriched (FDR adjusted p-values of 2.3e-5 and 2.0e-5, respectively) in MCF-7 cells treated with 10,000 µg/L glyphosate, but not at the two lower concentrations tested.

We also measured the effects of the commercial GBH formulation Roundup ProBio, and of the adjuvant POEA, on the transcriptome profiles of hormone-dependent MCF-7 human breast cancer cells. POEA was tested at two sub-cytotoxic concentrations (10 µg/L, 100 µg/L). Roundup ProBio was assessed at the glyphosate equivalent concentrations of 1 µg/L (environmental level), 100 µg/L and 1000 µg/L (showing a cell proliferative trend). The statistical analysis of differential expression showed that genes having their function altered by Roundup or POEA had low fold changes (Table 2). No genes whose expression was increased or repressed by POEA treatment had fold changes higher than 2. Although we consider that low fold changes in expression of a sufficient number of genes can have a toxicological relevance (Mesnage et al., 2015a), the study of transcriptome profiles shows that POEA alone is unlikely to have estrogenic effects. This was confirmed in that none of the treatments exhibited statistically significant correlations to the ER biomarker (Fig. 3A,C).

In order to confirm endocrine disturbances provoked by glyphosate, RNA extracted from MCF-7 cells treated with glyphosate (10,000 µg/L), estradiol (0.27 µg/L) or bisphenol A (80 µg/L) for 48 h were subjected to a full RNA-Seq analysis using the Illumina sequencing platform. The expression of genes found commonly altered in both the microarray and the RNA-seq analysis were well correlated (Fig. 4A) for 17β-estradiol (Pearson r = 0.81), BPA (r = 0.86) and glyphosate (r = 0.71). Overall, the RNA-seq method was more sensitive and identified 2–3 times more genes whose expression was significantly altered compared to the microarray approach (Fig. 4B). A total of 5102, 2939 and 1083 genes had their expression significantly disturbed by estradiol, BPA and glyphosate, respectively. Although their gene expression profiles were different, MCF-7 cells treated by these 3 chemicals presented alterations reflecting a response to steroid hormones and a modulation of cell proliferation, although the significance of the overlapping genes and those in these pathways was lower for glyphosate than the other compounds (Fig. 4C). We next applied the ER gene expression biomarker (Ryan et al., 2016) to see if we could detect ERα agonist effects after transcriptome profiling using RNA-Seq (Fig. 4D and E) platforms. The results were similar to those

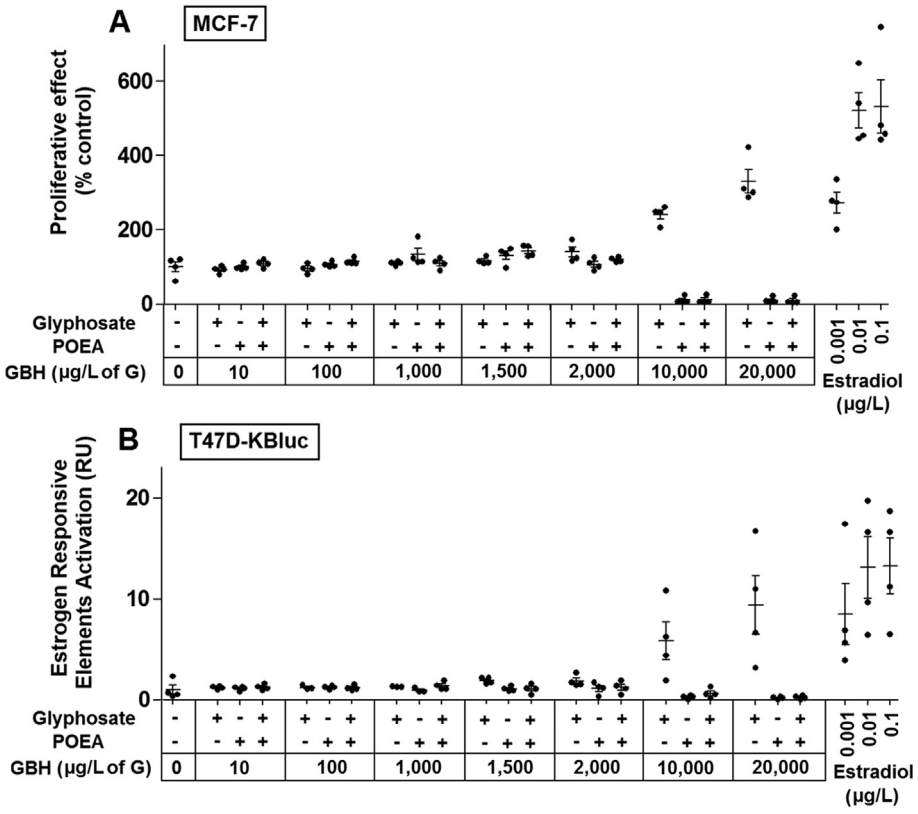

Fig. 2. Comparison of cell growth and estrogen receptor activation in human breast cancer cells treated with glyphosate, a GBH (Glyphogan), or POEA: a glyphosate-based herbicide adjuvant (polyethoxylated tallow amine; POEA) is not estrogenic in human mammary cells. (A) Proliferative effect of glyphosate alone, a glyphosate-based herbicide containing the adjuvant POEA (Glyphogan), or POEA alone in the E-Screen bioassay. Hormone-dependent MCF-7 human breast cancer cells were cultured under hormone-free conditions (n = 4) and proliferative effects measured by MTT assay following 6 days exposure to either the natural hormone (estradiol) or test substance (Glyphogan, POEA). **(B)** Glyphosate but not Glyphogan Roundup formulation or POEA stimulate ERE-luciferase reporter gene expression in T47D-Kluc cells. T47D-Kluc cells were treated for 24 h with the test substances and then subjected to a bioluminescence luciferase reporter gene assay (n = 4). +/−, indicates the presence or absence of the indicated chemical.

obtained using the microarray data; glyphosate failed to pass the threshold of significance for ERα activation. Although the RNA-seq platform was able to identify more statistically significant genes than microarrays, the genes altered differed between the two methods. Among those that are part of the ERα activation biomarker, the genes that were altered by glyphosate in the microarray analysis (CD44, PGR, and MYB) were different from those identified by RNA-seq (CXCl12, ABHD2, and EFNA1).

The ability of glyphosate to bind to ERα was evaluated by molecular dynamics simulations and ONIOM calculations. Since the charge of glyphosate (G) is −2, the ligand-receptor system was kept neutral by changing the charge of histidine residues of the environment (medium that surrounds the active site; that is, the remaining protein plus water) or of the active site itself (Table 1). An analysis of root mean square deviations (RMSD) of the lowest energy structures originating from the ER molecular dynamics simulations was performed on 230 residues (6 → 235). Our results indicate that ER:11 (RMSD = 2.014) presents a larger structural deviation than ER:20 (RMSD = 1.474). The charge on histidine 524 at the active site induces some structural distortion in ER and the ER:11 model was thus discarded. In relation to the number of active site water molecules (Table 1), the behaviour of ER:20 → G-ER:20 is similar to the reference ER:00 → ES-ER. A number of water molecules are replaced by the ligand, and by virtue of the above, the addition of the charges in the environment does not produce too large a distortion in receptor protein structure.

The three crucially important residues for estradiol binding to ER via the creation of a hydrogen bond network are HIS 524, GLU 353 and ARG 394 (Fig. 5C). In addition, there are 3 water molecules within 4 Å of the estradiol molecule within the active site. The binding of the hydroxyl group at the C17 position to HIS 524 maintains ER in the active conformation. The interaction with the opposite side of the estradiol molecule (with the hydroxyl group at the C3 position), is stabilized by creating a hydrogen bound network with ARG 394, GLU 353 and a water molecule. Aside from these hydrogen bonds, the active site is mostly hydrophobic and is thus appropriate for binding of estradiol. Results from the molecular dynamics simulations of glyphosate-ER interactions reveal that glyphosate enters the active site with a large number of water molecules (Fig. 5B). The glyphosate phosphate group interacts with ARG 394 by creating hydrogen bonds. (Note: arginine residue is positively charged.) A total of 21 water molecules enter the active site within 4 Å of the glyphosate; these molecules are mostly located at one side of the glyphosate molecule. The positively charged LYS 529 residue (which does not belong to the active site), stabilizes the opposite end of the glyphosate molecule (at the carboxyl group) by creating a water hydrogen bond network. Furthermore, on the other side of the ER binding site, LEU 387 and LEU 391 maintain hydrophobic contacts with the methylene groups of glyphosate. We notice that glyphosate is unlikely to interact with HIS 524, a residue having a pivotal role in maintaining protein structure in the biologically active agonist conformation. The ONIOM binding energy of G-ER:20 (−4.10 kcal mol^{-1}) is approximately 6 times greater than ES-ER (−25.79 kcal mol^{-1}). These results strongly imply that the binding of glyphosate at the active site of the receptor is weak and unstable, suggesting that glyphosate is

Table 2
List of the 10 most up- or down-regulated genes after the treatment by glyphosate, Roundup, POEA, bisphenol A, or 17β-estradiol. MCF-7 cells treated by glyphosate (1 μg/L, 100 μg/L, 10,000 μg/L), Roundup (1 μg/L of glyphosate, 100 μg/L of glyphosate, 1000 μg/L of glyphosate), by the adjuvant POEA, by bisphenol A (80 μg/L) or by the positive control estradiol were subjected to a full transcriptome microarray analysis.

17β-estradiol (0.0027 μg/L)		17β-estradiol (0.027 μg/L)		17β-estradiol (0.27 μg/L)		Bisphenol A (80 μg/L)		POEA (100 μg/L)		POEA (10 μg/L)	
ALDH1A3	0.4	ALDH1A3	0.2	ALDH1A3	0.2	SEMA5A	0.3	NRF1	0.6	RNU7-163P	0.6
SEMA5A	0.4	PRICKLE2-AS3	0.2	PSCA	0.2	ALDH1A3	0.3	RNU6-1281P	0.6	SNORA54	0.6
PPARG	0.5	SEMA5A	0.3	TFPI	0.2	PSCA	0.3	RP3-486I3.5	0.6	IREB2	0.6
PSCA	0.5	PSCA	0.3	PRICKLE2-AS3	0.2	PRICKLE2-AS3	0.3	SNORA54	0.7	LINC00403	0.6
IGFBPL1	0.5	EPAS1	0.3	EPAS1	0.3	GABBR2	0.3	RNA5SP144	0.7	RP11-10N23.2	0.6
EPAS1	0.5	TFPI	0.3	LIPH	0.3	EPAS1	0.3	RP3-425P12.5	0.7	ANKRD10-IT1	0.6
PSCA	0.5	LIPH	0.3	SEMA5A	0.3	LIPH	0.4	RNU7-75P	0.7	COX17	0.6
S1PR3	0.5	SAT1	0.3	DAB2	0.3	PPARG	0.4	RNU6-1024P	0.7	RP11-473O4.4	0.6
MIR3911	0.5	PSCA	0.3	SAT1	0.3	PSCA	0.4	HIST2H2AB	0.7	RNU6-385P	0.6
RP11-398B16.2	0.5	RND1	0.3	PSCA	0.3	UPK2	0.4	AC099344.2	0.7	ACTL6A	0.6
RNU6-807P	2.3	EGR3	5.2	MYBL1	5.5	MYB-AS1	2.8	RP5-865N13.1	1.4	LOC339978	1.4
MYB-AS1	2.5	MYBL1	5.5	EGR3	5.6	MGP	2.9	LOC100506142	1.4	LOC100134937	1.4
STC1	2.5	MYB	6.0	AC106875.1	6.4	AC005534.8	3.1	FKSG29	1.4	FLJ45079	1.4
AC005534.8	2.6	AC106875.1	6.3	GREB1	6.5	AC106875.1	3.3	RNA5SP39	1.4	MIR99A	1.4
MYB	3.0	GREB1	6.6	MYBL1	6.5	GREB1	3.3	TBX3	1.4	RP11-61L14.6	1.5
AC106875.1	3.2	GREB1	7.1	GREB1	7.1	MYB	3.7	LOC101927783	1.5	DHRS3	1.5
GREB1	3.2	MGP	7.2	PGR	7.1	GREB1	3.8	LINC00396	1.5	RP11-148B18.4	1.5
PGR	3.4	PGR	7.8	MGP	9.0	PGR	4.2	FLJ45513	1.5	MIR103B2	1.6
GREB1	3.7	PGR	10.0	PGR	9.2	MGP	4.2	MIR99A	1.5	MIR103A2	1.7
PGR	4.6	MGP	12.4	MGP	15.0	PGR	6.2	RNU6-601P	1.6	MIR425	1.8
Glyphosate (1 μg/L)		**Glyphosate (100 μg/L)**		**Glyphosate (10,000 μg/L)**		**Roundup (1000 μg/L of G)**		**Roundup (100 μg/L of G)**		**Roundup (1 μg/L of G)**	
RNU6-385P	0.5	RP3-486I3.5	0.4	SEMA5A	0.5	SNORA54	0.5	MIR548A2	0.5	LRCH3	0.6
TNXB	0.6	RNU7-141P	0.5	DXO	0.5	RNU4ATAC	0.5	NFE2L3	0.5	MIR4328	0.6
RNA5SP303	0.6	MYB	0.6	ALDH1A3	0.6	TNXB	0.6	RNU6-177P	0.5	LOC101928000	0.6
RNU6-667P	0.6	RNU7-172P	0.6	PRICKLE2-AS3	0.6	RNU6-611P	0.7	PGR	0.5	RNU5B-4P	0.7
RP11-276G3.1	0.7	RNU7-113P	0.6	RNU7-134P	0.6	TICAM2	0.7	SNORD58A	0.6	ZNF404	0.7
RNU6-611P	0.7	RNA5SP303	0.6	XXbac-BPG254F23.7	0.6	AC005304.1	0.7	RNU6-998P	0.6	RNU6-322P	0.7
TGFB1	0.7	MIR3662	0.6	RP11-417B4.2	0.6	RNU6-893P	0.7	LINC00403	0.6	ADAM20P1	0.7
CHIC1	0.7	RNU6-1024P	0.6	AC073046.25	0.6	ZNF202	0.7	TMEM123	0.6	RP11-77M5.1	0.7
CCIN	0.7	SNORD20	0.6	AAK1	0.6	VPS35	0.7	LOC101928000	0.6	RAD54L2	0.7
RP11-473E2.2	0.7	RNU6-998P	0.7	LIPH	0.6	MIR4514	0.7	MIR3140	0.6	RP11-276G3.1	0.7
CRTAP	1.5	PDE11A	1.5	AC005534.8	1.6	CRTAP	1.4	AL592528.1	1.5	AP001469.5	1.4
PTPRS	1.5	MIR3074	1.6	SNORA70B	1.6	MAST4-AS1	1.4	RNU6ATAC18P	1.5	PLEKHA4	1.4
SNORA5A	1.5	AC010894.3	1.6	MYB-AS1	1.7	SLC25A30-AS1	1.4	PAIP2B	1.5	SNORA70B	1.5
RP11-575A19.2	1.5	RNU6-306P	1.7	STC1	1.8	RNU6-853P	1.4	RP11-319F12.2	1.5	PCAT1	1.5
RNU6-1258P	1.5	RNU4-38P	1.7	MIR99A	1.8	RNU4-40P	1.5	SART3	1.6	ATP11A-AS1	1.5
RN7SKP56	1.5	MIR99A	1.7	AC106875.1	2.0	SNORA5A	1.5	RNA5SP498	1.6	RP5-865N13.1	1.5
LOC729451	1.5	MIR532	1.8	GREB1	2.1	RP5-1022J11.2	1.5	RNA5SP498	1.6	MIR4733	1.5
MIR532	1.6	MIR501	1.8	MYB	2.2	RP11-218L14.4	1.5	RP11-300E4.2	1.6	LOC101927557	1.5
ALG9	1.6	RNU6-1004P	2.3	PGR	2.3	RNU4-38P	2.0	RNU6-388P	1.7	SNORD41	1.7
SNORD59B	1.6	RNU4-60P	3.1	GREB1	2.4	SNORD1A	2.2	MIR4256	2.3	RNU4-62P	1.9

unlikely to bind to ERα.

Based on the results of the molecular dynamics simulations and the results of ERα biomarker, we hypothesized that the increase in ERE-luciferase reporter gene expression caused by glyphosate (Fig. 1) is due to a ligand-independent mechanism. A possible route for this ligand-independant activation is via cellular pathways (such as the cAMP-dependent protein kinase (PKA) pathway) that modulate the balance between cell proliferation and apoptosis (Berger et al., 2013). In order to show that the ERE-luc reporter is responsive to ligand-independent activation, we treated the T47D-Kluc cells with 500 μM of 3-isobutyl-1-methylxanthine (IBMX), an activator of the PKA pathway which raises cAMP levels, and monitored activation of the ERE-luciferase reporter gene. Our result (Fig. 6) shows that 500 μM IBMX was able to stimulate ERE-mediated transcription of the luciferase reporter gene. Further studies would be needed to determine if glyphosate modulates the PKA pathway.

4. Discussion

A previous study reported observation that glyphosate exhibited estrogenic activity in promoting MCF-7 cell proliferation and inducing ERE-mediated-luciferase reporter gene expression in the T47D-KBluc cells at concentrations comparable to those of estradiol (Thongprakaisang et al., 2013). We were not able to replicate these results. However, it should be noted that our experimental conditions differed to a small degree from those of Thongprakaisang and colleagues and were not directly comparable with our estrogen withdrawal period during cell culture was shorter (1 day vs 5 days). It is possible that the estrogen withdrawal protocol used in the present study may not be long enough for clearance of residual estrogen and may thus affect the dose of glyphosate needed to activate the ERE luciferase reporter gene. However, we believe that this difference in the duration of the estrogen withdrawal period between our study and that of Thongprakaisang and colleagues is not a significant contributing factor to the observed differences in experimental outcome. First, we optimized our protocol to reach the required sensitivity to detect the activity of weak agonists such as bisphenol A (Mesnage et al., 2017a). Second, the finding of Thongprakaisang and colleagues that a dose of 10e-12 M of glyphosate had a greater estrogenic effect than estradiol raises major concerns and suggests the possible presence of contaminants. Third, it is surprising that Thongprakaisang and colleagues were not able to find a no observable effect level for glyphosate in

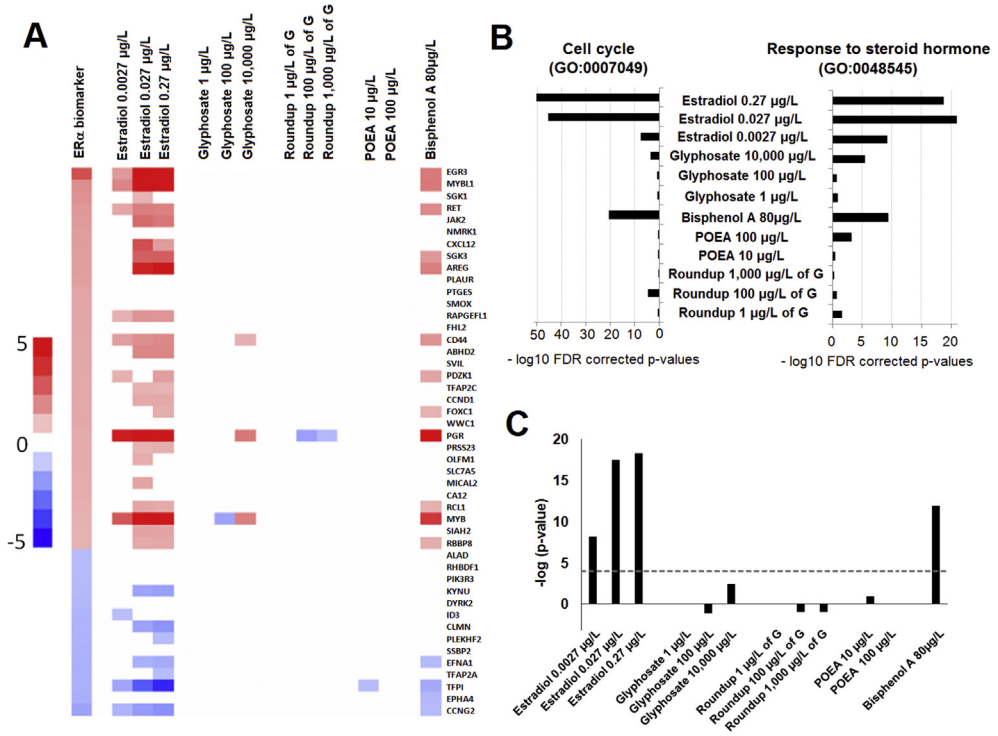

Fig. 3. Determination of estrogenic effects of glyphosate through a gene expression biomarker of ESR1 activity. (A) MCF-7 cells following 24 h adaptation to steroid hormone-free conditions were treated with the indicated concentrations of glyphosate, the commercial formulation Roundup Probio, POEA adjuvant or the natural hormone 17β-estradiol for 48 h. Transcriptome profiles of cell cultures following treatment was by microarray analysis. Lists of genes whose expression was statistically significantly altered was then scrutinized against an ER gene expression biomarker consisting of 46 genes (listed on right hand side). Results shown as a heat map showing the expression of genes in the ER biomarker following exposure to the indicated compound. The fold-change values for ER biomarker genes are the average across 7 agonist treatments. **(B)** Gene ontology analysis reflects hormone induced cell proliferative effects. The p-values are determined by a hyper-geometric calculation and corrected for multiple comparisons (FDR). **(C)** Bar plots showing the significance of the correlation by their −log10 (p-value)s. Classification of activation or suppression required $p < 0.0001$.

their ERE-luciferase assay. In contrast our results suggest that glyphosate is a weak activator of ERα in hormone-dependent human breast cancer cells and that it may exert this effect via a ligand-independent pathway. The glyphosate intake necessary to reach a systemic concentration representative of the estrogenic effects shown here would only be encountered in cases of extreme exposures (incidental ingestion, mishandling) (Niemann et al., 2015; Roberts et al., 2010). GBH exposures causing acute moderate to severe clinical outcomes are generally associated with glyphosate plasma concentrations ranging from 18.9 to 104.2 mg/L, while death occur at glyphosate concentrations larger than 734 mg/L (Roberts et al., 2010). However, problems potentially arising from chronic, environmentally relevant low dose exposure to GBHs as well as to glyphosate *per se* have not been thoroughly investigated although there is evidence to suggest that this avenue of investigation is worth pursuing (Guyton et al., 2015; Mesnage et al., 2015b, 2017b).

It has been reported elsewhere that glyphosate can induce proliferation of MCF-7 cells (Lin and Garry, 2000) at a concentration that corresponds to that showing estrogenic effects in our experiments (Fig. 1). Interestingly, in this previous study, glyphosate provoked similar proliferative effects in both the presence and absence of charcoal dextran-treated culture medium, suggesting that proliferative effects were not mediated by activation of ER (Lin and Garry, 2000). An evaluation of the glyphosate binding energy confirmed that this compound is unlikely to activate ER (Fig. 5). It is known that estrogenic effects can be caused by an activation of ER in a ligand-independent manner. The modulation of the phosphorylation of some protein kinases, as well as modification at the level of second messengers, can activate ER due to interplay between cellular signalling pathways. For example, the suppression of PI3K signalling results in induction of ER-dependent transcriptional activity (Bosch et al., 2015). Proliferative effects provoked by exposure to toxic agents changing the apoptosis/proliferation balance could be accompanied by an activation of estrogenic pathways (Berger et al., 2013). The activation of p53 during apoptosis is accompanied by an increased expression of ERα in MCF-7 cells (Angeloni et al., 2004). This is due to protein-protein interactions between ERα and p53 and p53 binding to the *ESR1* promoter (Berger et al., 2013). This provides one explanation for the observed activation of ERα by glyphosate (Fig. 1). Glyphosate has been shown to cause apoptosis, as measured by caspase 3/7 activation, at 10–20 mg/L (Benachour and Seralini, 2009; Koller et al., 2012). In addition, glyphosate can act as a protonophore, causing a disturbance in liver cell mitochondrial respiration from ~10,000 µg/L (Olorunsogo, 1990). This encompasses the concentrations at which we observed estrogenic effects of glyphosate (Fig. 1) and thus provides a possible mechanism of action.

The presence of glyphosate-associated cytotoxic effects could also explain discrepancies between the results we obtained with the ERα biomarker (Fig. 3) and those from the cellular assays (Fig. 1). As shown (Fig. 1), the efficacy of ERα activation by glyphosate through a receptor-independent mechanism is lower than for BPA. If the resulting alterations in gene expression patterns by glyphosate are weak, they may be below the threshold at which correlations with the ERα biomarker can be made. However, this lack of correlation between glyphosate cellular effects and the ERα biomarker may also be due to the thresholds used to identify statistically significant changes. Indeed, 42 out of the 46 genes that constitute the ERα biomarker had their expression altered (up- or

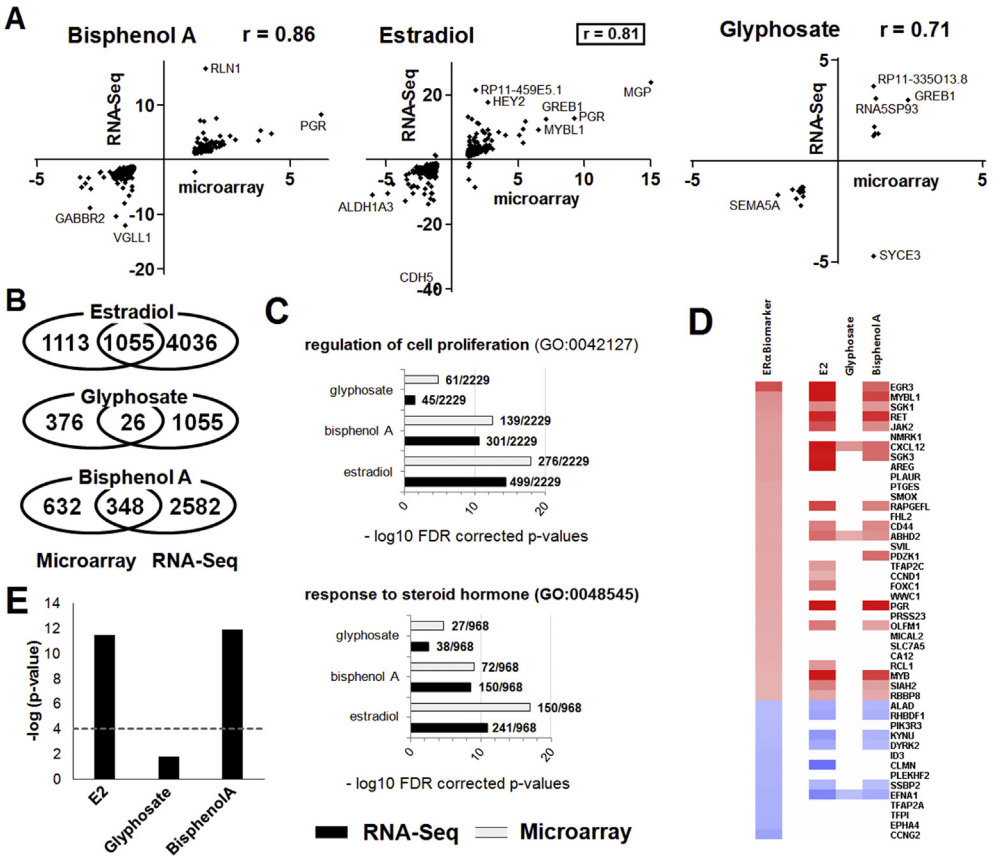

Fig. 4. Comparison of RNA-seq and microarray platforms in evaluating potential endocrine disrupting effects of glyphosate. RNA extracted from MCF-7 cells were subjected to a full transcriptome profiling using the Illumina RNA sequencing (RNA-Seq) or Affymetrix microarray technology platforms. **(A)** Pearson correlation coefficients between the RNA-Seq and microarray data. The fold changes of genes having an altered expression by the two methods are presented. **(B)** Venn diagrams showing the number of genes uniquely or commonly disturbed in the RNA-seq or the microarray platforms. **(C)** Gene ontology analysis of terms associated to MCF-7 hormone-induced proliferation in the transcriptome profiles obtained by RNA-seq or microarray experiment analyzed using the Thomson Reuters MetaCore Analytical Suite recognizing network objects (proteins, protein complexes or groups, peptides, RNA species, compounds among others). The p-values are determined by hypergeometric calculation and adjusted using the method of Benjamini & Hochberg. Ratios show the total number of network objects belonging to each term in comparison to those disturbed by the treatments. **(D)** Heat map of statistically significant genes examined against an ER gene expression biomarker (gene list on the right hand side, p-values of <0.05 with fold change > 1.2). **(E)** Bar plots showing the significance of the correlation between each bioset and the ER biomarker (−log10 (p-value)s. Classification of activation or suppression required $p < 0.0001$.

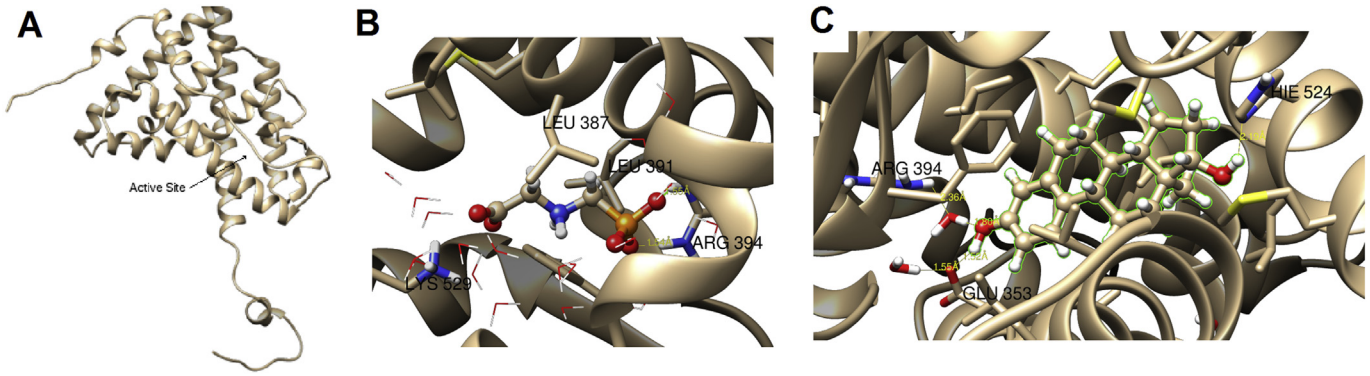

Fig. 5. Molecular dynamics simulation of glyphosate and estradiol binding to human estrogen receptor alpha (ERα). (A). ER Model showing the position of the active (ligand binding) site of **ERα**. **(B).** Glyphosate (ball and stick depiction) binding simulation at the **ER** active site is weak and less stable (G-ER:20 model, binding energy of −4.10 kcal mol^{-1}). Distances between interacting heteroatoms are listed in Ångstrøms. The environment water molecules are not shown. **(C).** Estradiol (ball and stick depiction) binds strongly to **ER** (ES-ER model, binding energy of −25.79 kcal mol^{-1}); this binding specificity and stability is conferred by a network of direct and water (stick) molecule-mediated interactions. Note: the water molecule constituting a bridge between ES and ARG 394.

down-regulated) in conformity with the biomarker but failed to pass the threshold for statistical significance (data not shown).

Our finding that PKA activation by IBMX can induce ERα-mediated reporter gene expression (Fig. 6) suggests that the ERE-luc reporter is responsive to ligand-independent activation. The stimulation of PKA signalling pathway, increasing cAMP levels, is known to activate the transcriptional activity of ESR1, even in the absence of ligand, by associating with cyclin D1 (Driggers and

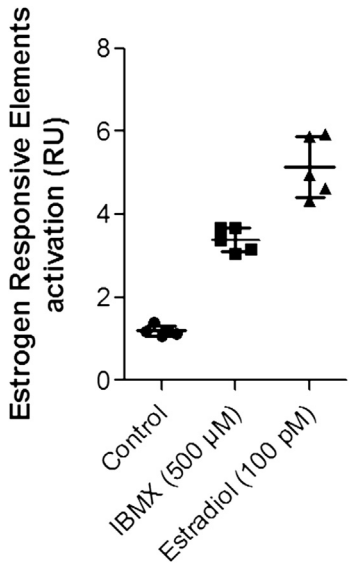

Fig. 6. IBMX, a ERE-luciferase reporter gene expression in T47D-Kluc cells. T47D-Kluc cells were treated with 500 μM of 3-isobutyl-1-methylxanthine (IBMX) for 24 h and ERE-luciferase reporter gene expression was measured. Cells treated with 100pm estradiol acted as a positive stimulatory control. Results from 5 technical replicates are presented with their standard deviation. Control, cells without estradiol or IBMX. RU, relative units.

Segars, 2002). It is thus plausible that glyphosate is activating ERα through a ligand-independent mechanism albeit at high concentrations. However, further studies would be needed to determine if glyphosate modulates the PKA pathway or another signalling pathway. Compounds that function through ligand-independent ERα activation may fail to be detected by the ERα biomarker, which was established to detect activity of agonist and antagonist functioning directly through ERα (Ryan et al., 2016). The observation that ligand-independent (growth factors and cAMP) and ligand-dependent (estradiol) activation of ER have distinct ERα-dependent transcriptional responses in MCF-7 cells (Dudek and Picard, 2008) supports such a mechanism. Additionally, liganded and unliganded activation of ER has been shown to give a different pattern of phosphorylation of this receptor (Maggi, 2011). Therefore, future studies determining the phosphorylation of ERα could provide new insights into the mechanism by which glyphosate activates various signalling pathways. This could also be studied by the investigation of the effects of signalling pathway inhibitors on glyphosate-induced ERE activation. A previous study in ERE-Luc reporter gene transfected MCF-7 cells shows that 1 μM of forskolin, an activator of the PKA pathway, could induce ERE activation but with a lower potency than 10 nM of E2. Furthermore, H89, a PKA inhibitor could not reverse E2-induced ERE activation (Yum et al., 2009).

The comparison of estradiol and glyphosate binding energies shows that glyphosate is unlikely to activate ERα in a similar manner to the natural hormone. Our results are consistent with those of previously published studies, which reported that glyphosate ER activity is negative in the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) (Mansouri et al., 2016). This is not altogether surprising since glyphosate and estradiol are structurally very distinct, with glyphosate not displaying the features of ER agonists. The structural features of glyphosate making it an unlikely ER agonist can clearly be seen when comparing it to known estrogen mimics such as BPA. The binding free energy of estradiol and BPA to ER has been shown to be −34.88 kcal/mol and −23.77 kcal/mol, respectively (Li et al., 2015). BPA binding to ERα is comparable to that of estradiol, with two phenol moieties pointing to the two ends of the ER hydrophobic pocket, one ring to residue Glu 353 and the other to His 524 (Li et al., 2015). As a consequence, BPA traps the twelfth helix (H12) of the binding pocket in an active conformation, allowing the binding of a coactivator in order to transduce downstream gene transcription signalling (Li et al., 2015). As glyphosate lacks features such as the two phenol moieties possessed by BPA this could evidently account for its ability to stably interact with the ER ligand binding site even in the presence of potential associated water molecules. However, our model did not take into account the capacity of glyphosate to bind cations and it is possible that the chelating properties of glyphosate may influence its biological activity at the level of binding to ERα.

Although the health risks of the stated active principle of a given pesticide are evaluated by regulatory agencies, in reality exposures to both animal and human populations also include the adjuvants that are invariably present in commercial products. In order to determine any estrogenic potential of adjuvant co-formulants, we tested a number of GBH formulations (Roundup ProBio, Glyphogan, Roundup Grand Travaux Plus, and Roundup Original DI). Our results (Fig. 1) did not show any estrogenic effects. However, we did observe cytotoxicity at glyphosate-equivalent concentrations lower than those required to elicit a proliferative response to glyphosate alone. We have demonstrated that commercial formulations can be up to 1000 times more toxic than glyphosate in human cell lines due to surfactant-mediated cell membrane disruption, with POEA being ~10,000 times more toxic than glyphosate alone (Mesnage et al., 2013). However, the composition of co-formulants of different formulations is variable. The characterisation of the potential for endocrine-mediated mode of action of POEA has been defined by EFSA as a data gap precluding a proper risk assessment (EFSA, 2015). POEA is considered as a major contributor to incidences of poisoning in humans. In particular, the recent review of the toxicological profile of POEA by EFSA highlights concerns about its genotoxic potential regarding DNA damage at concentrations that do not cause cytotoxicity, as well as potentially severe reproductive and developmental toxicities (EFSA, 2015). We report here for the first time the lack of endocrine-disrupting effects of POEA at the level of ER. Other studies have reported that POEA can inhibit aromatase (a key enzyme in the balance of sex hormone production) in human placental tissue culture cells (Defarge et al., 2016). This effect is explained by the known disturbance of the endoplasmic reticulum, as surfactants such as POEA are known to disrupt membrane structure (Cserháti, 1995).

In conclusion, our investigation reveals that glyphosate activates ERα in breast cancer cells but only at relatively high concentrations, and that this activation is through a ligand-independent pathway. This result is in contrast to a previous study, which reported that glyphosate could activate ER with a potency similar to estradiol. Our results suggest that humans exposed to glyphosate would not exhibit ER activation at typical exposure levels. However, the clinical relevance of human exposure to glyphosate, either alone or mixed with natural hormones, hormonal therapies, or other environmental pollutants, warrants further investigation.

Competing interests

The authors declare they have no competing interests.

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