Food and Chemical Toxicology 108 (2017) 30–42

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

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

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ARTICLE INFO

*Article history:*
Received 20 March 2017
Received in revised form
20 June 2017
Accepted 11 July 2017
Available online 12 July 2017

*Keywords:*
Endocrine disrupting effects
Pesticides
Glyphosate
Breast cancer
Estrogen receptor

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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http://dx.doi.org/10.1016/j.fct.2017.07.025

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,