Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity

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A B S T R A C T

Pesticides are always used in formulations as mixtures of an active principle with adjuvants. Glyphosate, the active ingredient of the major pesticide in the world, is an herbicide supposed to be specific on plant metabolism. Its adjuvants are generally considered as inert diluents. Since side effects for all these compounds have been claimed, we studied potential active principles for toxicity on human cells for 9 glyphosate-based formulations. For this we detailed their compositions and toxicities, and as controls we used a major adjuvant (the polyethoxylated tallowamine POE-15), glyphosate alone, and a total formulation without glyphosate. This was performed after 24 h exposures on hepatic (HepG2), embryonic (HEK293) and placental (JEG3) cell lines. We measured mitochondrial activities, membrane degradations, and caspases 3/7 activities. The compositions in adjuvants were analyzed by mass spectrometry. Here we demonstrate that all formulations are more toxic than glyphosate, and we separated experimentally three groups of formulations differentially toxic according to their concentrations in ethoxylated adjuvants. Among them, POE-15 clearly appears to be the most toxic principle against human cells, even if others are not excluded. It begins to be active with negative dose-dependent effects on cellular respiration and membrane integrity between 1 and 3 ppm, at environmental/occupational doses. We demonstrate in advance that POE-15 induces necrosis when its first micellization process occurs, by contrast to glyphosate which is known to promote endocrine disrupting effects after entering cells. Altogether, these results challenge the establishment of guidance values such as the acceptable daily intake of glyphosate, when these are mostly based on a long term in vivo test of glyphosate alone. Since pesticides are always used with adjuvants that could change their toxicity, the necessity to assess their whole formulations as mixtures becomes obvious. This challenges the concept of active principle of pesticides for non-target species.

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1. Introduction

Pesticide formulations are mixtures of adjuvants and so-called “active principles” on plants for herbicides, and insects for insecticides, etc. The supposed specificity of active principles on their targets does not mean a priori that they are the most toxic compounds of the formulations on human cells. Numerous mammalian (Colborn et al., 1993) and other animal studies (Hawthorne and Dively, 2011) evidenced side effects for pesticides. The toxicology of mixtures cannot be fully understood without knowing the differential toxicity of the various compounds of the formulations and their combined effects. Surprisingly, to measure their side effects, the active principles of pesticides are generally tested alone at a regulatory level in long-term mammalian trials, although their adjuvants are developed at least to enhance their stability and penetration into cells. However, most of the adjuvants are classified as inert.

Here we tested the differential and combined cytotoxicity of the major pesticides in the world which are glyphosate-based herbicides (GBH), and analyzed their composition and mechanisms of action. The residues of the GBH such as Roundup (R) are also among the first contaminants of ground and surface waters (IFEN, 2006), and of some food and feed because they are present since more than 15 years in around two third of genetically modified (GM) cultivated edible plants, because they are designed at least to tolerate R (James, 2011). Glyphosate (G) is toxic in plant cells by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase used as a first step in aromatic amino acid synthesis (Boocock and Coggins, 1983). Adjuvants considered as inert include, according to the formulations, surfactants like POEAs (polyethoxylated alkylamines,
Fig. 1, isobutane, light petroleum distillate, etc. that may induce among other DNA damages (Cox, 2004). However G is still generally hypothesized to be the active ingredient for non-target side effects. Unexpected side effects of G-based formulations were evidenced on non-target species, among other endocrine disruptions during spermatogenesis or pregnancy (Beuret et al., 2005; Clair et al., 2012; Dallegrave et al., 2007; Daruich et al., 2001; Oliveira et al., 2007; Romano et al., 2011; Savitz et al., 1997; Yousef et al., 1995). This may be related to adjuvants in formulation. They are indeed more and more considered as responsible for GBH toxicity (Mesnage et al., 2010; Williams et al., 2012), but the mechanistic and the nature of the cytotoxic agent(s) on human cells are still unknown. This is a general question that can arise for all pesticides.

The detailed known composition indicates that major adjuvants are ethoxylated, such as POEA which are themselves mixtures of di-ethoxylates of tallowamines characterized by their oxide/tallowamine ratio. POEA commonly used in GBH is the POE (15) tallowamine (POE-15). We thus compared the toxicity and the composition of 9 formulations varying in adjuvants contents: Roundup Ultra, Roundup GT, Roundup GT+, Roundup Bioforce, Roundup 3plus, Glyphophos, Topglypho 360, Clinic E.V., and Bayer GC. For controls, we tested a formulation containing POE-15 without G (Genamin T200), and POE-15 alone. The compositional analysis of these products was performed by a non-quantitative mass spectrometry (MALDI-TOF MS/MS), considered as the best way to analyze pesticides formulations (Corbela et al., 2010; Cserháti and Forgács, 1997). Physico-chemical properties of POE-15 were approached by the measurements of its critical micelle concentration (CMC), determined by absorption changes in its presence of Comassie blue CBB R-250.

We used HEK293, JEG3 and HepG2 cell lines, three models where unexpected effects of GBH have already been demonstrated (Benachour and Seralini, 2009; Gasnier et al., 2009). JEG3 cells are a useful model for examining placental toxicity (Letcher et al., 1999), and HepG2 for hepatic toxicity (Urani et al., 1998). HEK293 were chosen because of the sensitivity of embryonic cells, Roundup causing pregnancy outcomes (Savitz et al., 1997). Moreover, we have demonstrated that these cell lines are even less sensitive than primary cells (Benachour and Seralini, 2009; L’Azou et al., 2005), and therefore are possibly representative of a real cellular toxicity. For cytotoxicity measurements, we assayed mitochondrial succinate dehydrogenase (SD) activity (MTT assay), and its formulations are indeed known to target mitochondria (Astiz et al., 2009; Peixoto, 2005). Cytotoxicity was also characterized by the measurement of apoptosis and necrosis, respectively by caspases 3/7 activation (Liu et al., 2005) and adenylate kinase leakage after membrane alterations (Crouch et al., 1993).

Overall, we questioned if an active toxic principle in a target species may be always generalized as such in a non target one, and thus if the regulatory toxicological tests on active principles alone are relevant.

2. Materials and methods

2.1. Chemicals

Glyphosate (N-phosphonomethyl glycine, G, CAS: 1071-83-6) was purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). GBH formulations available on the market were by alphabetical order: Bayer GC (12.5% of G, 1–5% of POE-15, homologation 59873567), Clinic EV (42% of G, 11% of POE-15, homologation 9900039), Genamin T200 (50–80% of POE-15, homologation 8500170), Glyphophos (39–43% of G, 13–18% of POE-15, homologation 9100537), Roundup Grand Travaux (400 g/L of G, RCT, homologation 8800425), Roundup Grand Travaux plus (450 g/L of G, 90 g/L of ethoxylated ethylalkylamine (EO-EA), RGT, homologation 2020448), Roundup Ultra (41.5% of G, 16% surfactant, homologation 9700259), Roundup Bioforce (360 g/L of G, homologation 9800036), Roundup 3plus (170 g/L of G, 8% surfactant homologation 9300241), Topglypho 360 (360 g/L of G, homologation 2000254), POE-15 (CAS: 61791-26-2) was purchased from ChemService (West Chester, PA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other compounds, otherwise noticed, were obtained from Sigma–Aldrich. MTT was prepared as a 5 mg/mL stock solution in phosphate-buffered saline, filtered through a 0.22 μm filter before use, and diluted to 1 mg/mL in a serum-free medium.

2.2. Cell lines and treatments

The human embryonic kidney 293 cell line (HEK 293, ECACC 85120602), was provided by Sigma–Aldrich (Saint Quentin Fallavier, France). The heptoma cell line HepG2 was provided by ECACC (85011430). JEG3 cell line (ECACC 92120308) was provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM (Abcs, Paris, France) containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (a mixture of penicillin, streptomycin and fungizone) (Lonza, Saint Beaurisse, France) 10 mg/mL of kanamycin (Dominique Dutscher, Brumath, France) and 10% Fetal Bovine Serum (PAA, les Mureaux, France). JEG3 cells were supplemented with 1 mM sodium pyruvate. Cells were grown with this medium at 37°C (5% CO2, 95% air) during 48h to 80% confluence, and then washed and exposed 24h with serum-free EMEM to various chemicals. This model was validated (Benachour et al., 2007) since cytotoxic effects were similar in presence with serum but delayed by 48h. The dilutions of formulated herbicides, adjuvants and G alone were prepared in serum free medium as stock solutions at a similar pH.

2.3. Cytotoxicity biomarkers

After treatments, the following tests were applied: succinate dehydrogenase (SD) activity assay (MTT) (Mossmann, 1983). Integrity of mitochondrial dehydrogenase enzymes indirectly reflects the cellular mitochondrial respiration. The optical density was measured at 570 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The bioluminescent Toxlitight bioassay (Lonza, Saint Beaurisse, France) was applied for the membrane degradation assessment, by the intracellular adenylate kinase (AK) release in the medium; this is described as a necrosis marker (Crouch et al., 1993). Finally, the apoptotic cell death was evaluated by the Caspase-Glo 3/7 assay (Promega, Paris, France). Luminescence was measured using a Mithras LB 940 luminometer (Berthold, Thoiry, France). These methods were previously described (Benachour and Seralini, 2009).

2.4. Mass spectrometry (MS)

MS experiments were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF TOF ion optics and an OptiBeamMS™ on-axis laser irradiation with 1000 Hz repetition rate. The system was calibrated immediately before analysis with a mixture of des-Arg-Bradykinin, Angiotensin I, Glu1-Fibrinopeptide B, ACTH (18–39), ACTH (7–38) and mass precision was better than 50 ppm. A 0.8 μL volumes of the GBH solution diluted 100 times in water was mixed with 1.6 μL volumes.
of solutions of α-cyano-4-hydroxycinnamic acid matrix prepared in 50% ACN with 0.1% TFA. The mixture was spotted on a stainless steel Opti-TOFTM 384 targets; the droplet was allowed to evaporate before introducing the target into the mass spectrometer. Acquisitions were taken in manual and automatic modes. A laser intensity of 3000 was typically employed for ionizing. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range from 100 to 2000 Da. MS/MS spectra were acquired in the positive MS/MS reflector mode by summarizing a maximum of 2500 single spectra (10 × 250) with a laser intensity of 3900. For the tandem MS experiments, the acceleration voltage applied was 1 kV and air was used as the collision gas. Gas pressure medium was selected as settings.

2.5. Critical micelle concentrations (CMC) determinations

CMC determinations were performed and adapted according to Samsonoff et al., 1986. CMC was measured by the incorporation of Coomassie brilliant blue R-250 (CBB-R250) in micelles formed by serial dilutions of detergents. The CBB-R250 reagent was prepared as previously described (Bradford, 1976). Varying concentrations of adjuvants were added in a volume of 1 ml, 100 μl of CBB-R250 was added to make a final concentration of 80 μg/mL. Solutions were shaken and distributed in 96 well-plates in triplicate. Absorption was then measured against a water blank at 600 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The validation of the technique was performed with triton X-100, with a CMC of 0.15–0.20 mM (Courtney et al., 1986).

2.6. Statistical analysis

The experiments were repeated at least 3 times in different weeks on 3 independent cultures (n = 9). LC50 values were calculated by a nonlinear regression using sigmoid (5-parameters) equation with the GraphPad software. All data were presented as the means ± standard errors (SEMs). Statistical differences were determined by Student’s t-test using significant levels with p < 0.01 (**) and p < 0.05 (*).

3. Results

Here we studied for the first time the precise involvement of the adjuvants and G in GBH induced toxicity, on three human cell lines from different embryonic origins (kidney, liver, and placenta) in order to test their specificities. We first compared mitochondrial respiration (SD activity) in presence of 9 formulated mixtures of G and adjuvants, G alone, formulating agents without G (Genamin), and a major adjuvant of some formulations, POE-15 (Fig. 2). All chemicals are cytotoxic, inducing similar dose-dependent patterns on HEK293, HepG2, and JEG3 in 24 h. JEG3 were up to 2-fold more sensitive to treatments than HEK293 and HepG2 in comparison to control. We observed for all cell lines different ranges of toxicities.
allowing the classification of the products tested as follows. The most toxic were the adjuvants alone POE-15 (LC50 ~ 1–2 ppm; agricultural dilutions: 1–2% of the herbicide formulation containing adjuvants) and Genamin, themselves around 100-fold more toxic than a middle group with the majority of formulations (6, with among them R GT and GT+). This middle group is again 100-fold more toxic than the third one which includes R Ultra, R Bioforce, R 3 plus and finally G alone. Moreover, POE-15 diluted to the concentration at which it is present in Clinic E.V. (a formulation from the middle group) presented a similar toxicity than this GBH and to the middle group in general. It thus appears to be the toxic principle in human cells. In addition, we also demonstrate that two formulations claiming a similar concentration of G (360 g/L) and different adjuvants (16% of POEA or other adjuvants), Glyphogan and R Ultra respectively, exhibited very different toxicities, 150-fold stronger on average for Glyphogan on the 3 cell lines (Fig. 2). Thus some other adjuvants appear also to have some toxicity.

To check the composition in adjuvants we studied all the formulations by MALDI-TOF MS/MS (Fig. 3). Knowing that the specificities of MALDI-TOF ionization did not detect G but adjuvants, we separated 4 groups of adjuvants: (A) with a spectrum centered on 900 m/z, POE-15 and Genamin, and those present in 4 formulations of the middle group thus containing also POE-15, (B) those contained in the third less toxic group with a spectrum centered on 600 m/z corresponding to another common adjuvant, and (C) and (D), two other adjuvants in the formulations of the middle group, respectively in (C) R GT+ (500 m/z) and (D) R GT (300 m/z). The belonging of each product to each group was further confirmed by analysis of fragmentation spectra, giving for instance for ions of group A: 840.6, 858.7, 884.7, 902.8 m/z. All these spectra corresponded to the family of alkylamines. The POE-15 had a peak increment of 44 (delta) like all group A (Table 1). The same delta in C and D were characteristic of an ethoxylated chain. C was an ethoxylated ethalylamine. D was confirmed by fragmentation to be identical to POE-2 and a delta of 58 corresponded to another non ethoxylated adjuvant in group B. We summarized these findings with LC50 values (Table 1).

We then tested the linearity of the toxicity in function of G or ethoxylated adjuvants concentrations (Fig. 4). The cytotoxicity induced by GBH is not linear to G concentrations (R2 ~ 0.3, Fig. 4A), but only to the 3 ethoxylated adjuvants (R2 > 0.93, Fig. 4B), and not to the non-ethoxylated one, and this is obtained with all cell lines. Ethoxylated adjuvants can thus be considered as the active principle of the toxicity of GBH in human cells.

In order to understand the mechanism of action of adjuvants, three other experiments were performed. First, the critical micelle concentration (CMC) of POE-15 was determined by absorption changes of GBH R-250 (Fig. 5). The method was validated by the measurement of the CMC of the triton X-100 (0.15–0.20 mM (Courtney et al., 1986)). We evidenced a micellization of POE-15 beginning at 3 ppm, similarly to toxicity thresholds (Fig. 2). POE-15 thus appears to be able to disrupt the cellular membranes by micellization with the lipid bilayer around the CMC. This was even better understood by the differential measurement of the cytotoxicity through membrane disruption or caspases activation (Fig. 6). For the three cell lines, results are almost comparable: POE-15 and R GT+ (containing also an ethoxylated adjuvant) induced more necrosis (Fig. 6A) by membrane alterations rather than apoptosis (Fig. 6B), even if present. By contrast, G induced only apoptosis at higher levels. Ethoxylated adjuvants are thus not inert at all but cell membrane disruptors, and then induce severe mitochondrial alterations.

### 4. Discussion

This study unravels the differential nature and cytotoxicity of the main compounds from the major herbicide formulations in the world. These formulations are conceived to enhance the pesticide activity through mixtures of adjuvants and G. The latter is the active principle toxic in plants; in this study we checked how this

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Products tested</th>
<th>Glyphosate (g/L)</th>
<th>Adjuvants m/z (MS)</th>
<th>Delta (MS)</th>
<th>LC50 HepG2 (ppm)</th>
<th>LC50 HEK293 (ppm)</th>
<th>LC50 JEG3 (ppm)</th>
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<td>900</td>
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<td>89</td>
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<tr>
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<td>44</td>
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<td>2</td>
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<tr>
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<td>16% nk</td>
<td>600</td>
<td>58</td>
<td>11,000</td>
<td>6395</td>
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<tr>
<td></td>
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<td>nk</td>
<td>600</td>
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<td>6106</td>
<td>5043</td>
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<tr>
<td></td>
<td>R 3 plus</td>
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<td>nk</td>
<td>600</td>
<td>58</td>
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<td>24,000</td>
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<td>C</td>
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<td>7.5% Eto-OA</td>
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<td>44</td>
<td>145</td>
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<tr>
<td></td>
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<td>POE-2</td>
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active principle is differentially toxic on non-target organisms in comparison to the so-called inert adjuvants in numerous formulations.

Here we demonstrate that all formulations are more toxic than G alone on three human cell lines as previously underlined (Benachour and Seralini, 2009; Richard et al., 2005). Then for the first time we separated experimentally three groups of formulations differentially toxic according to the amount of ethoxylated adjuvants. The 3 less toxic formulations (like G alone) were demonstrated to contain no ethoxylated adjuvants by mass spectrometry, and are around 10,000 times less toxic on mitochondrial activity than POE-15 alone, the major adjuvant. All the other formulations were toxic proportionally to the dilutions of POE-15 or other ethoxylated adjuvants in the formulations, in a linear manner to some extent; in fact G does not buffer or amplify direct POE-15 toxicity.

Thus POE-15 appears to be clearly the toxic principle in human cells. It begins to be active with negative effects on cellular respiration and membrane integrity between 1 and 3 ppm, when its first micellization process occurs in this work. This membrane disruption then lead to the necrotic adjuvant-linked effects observed, amplifying the necrosis/apoptosis ratio by contrast to G at higher levels as shown. Accordingly, it was found (Chamel and Gambonnet, 1997) that a CMC of the C12NEO30 congenier of a POEA is around 2 ppm. Its partition coefficient measured at around 1.7 confirmed its lipophilic character and its ability to penetrate the cells. It is known that ethoxylated adjuvants can insert in cells membranes, disrupting their structure and functions as previously shown in bacteria (Nobels et al., 2011). This is a general property of surfactants (Boeije et al., 2006). We notice that among different class of surfactants, ethoxylated adjuvants are of the more toxic, even potentially genotoxic (Nobels et al., 2011). Importantly, this is not only observed in vitro because when rats are treated with G, R and POEA, the latter was also found to be the most toxic compound (Adam et al., 1997), even in other animal models (Marc et al., 2005). This was demonstrated for other pesticides (Eddleston et al., 2012). Generally, the question of the toxicity of adjuvants in pesticides is more and more recognized (Brausch and Smith, 2007; Krogh et al., 2003; Tsui and Chu, 2003).

This does not exclude cellular endocrine disruptions below these levels that may not be due to POE-15 alone (or other ethoxylated adjuvants), but that occur through glyphosate entering in aromatase active site for instance (Richard et al., 2005) or in androgen receptor which is inhibited from 0.2 ppm of G in adjuvants (Gasnier et al., 2009). It should not be forgotten that G has its own toxicity and may also exert long term or chronic toxicity. The active principle G alone has been evidenced to cause oxidative stress (Astiz et al., 2009; Cavusoglu et al., 2011), endocrine disruption (Clair et al., 2012), or developmental effects (Marc et al., 2005). G was even recently described as a teratogen (Paganelli et al., 2010). In this case we have a model of multiple combined negative effects (through different cellular metabolic endpoints) caused by the main pesticide mixtures, which are the formulations themselves. This is true even if the activities of ethoxylated adjuvants on endocrine disruption must be still detailed in the future.

These results were obtained in vitro; cellular cultures replace whenever it is possible animal experimentation (Hartung, 2009). Our study was performed during 24 h and does not anticipate the elimination or the possible bioaccumulation and long term combined effects with other xenobiotics. R human cellular effects indeed increased according to time (Benachour et al., 2007) and radiolabeled G accumulated in cells within 48 h, suggesting a
bioaccumulation of low concentrations of G (Gasnier et al., 2011). R adjuvants may also form adducts and link to DNA avoiding a direct elimination (Peluso et al., 1998).

However, our lowest thresholds of toxicities and endocrine disruptions may be comparable to the range of environmental/occupational exposures. A farmer or a gardener spraying a GBH may be punctually exposed to 5000 ppm, and even regularly by occupational exposure. As a matter of fact G varied from 3 to 233 ppb in farmers urine (Acquavella et al., 2004), this may be in addition to a chronic dietary/drink exposure of G found up to 70 ppb in serum of non-occupationally exposed women (Aris and Leblanc, 2011).

In conclusion, pesticide formulations should be studied as mixtures for toxic effects. The multiple combined effects could induce pathologies on a long term. Here we can question the use of ethoxylated adjuvants in herbicide formulations, since they appear as active principles for human cell toxicity. This leads also to challenge guidance values such as the acceptable daily intake (ADI) of G, which is calculated with pure G in long term toxicological tests in vivo (German Federal Agency CPFS, 1998), while G is always used with adjuvants that are not immediately biodegradable (Banduhn and Frazier, 1978) and could change its toxicity. This will be also important for other active principles of pesticides, and thus their ADI can be overestimated. The necessity of studying formulations as mixtures is common to all pesticides. The pathological consequences of exposure to chronic toxicities of whole formulations could be tested with mammals over a 2-year period. This implies a complete shift in the concepts underlying chemical Toxicology, which could come from mixtures studies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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