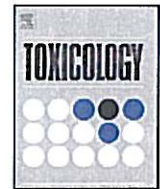




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Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines

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ABSTRACT

Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic *in vivo* regulatory studies. We measured cytotoxicity with three assays (Alamar Blue[®], MTT, ToxiLight[®]), plus genotoxicity (comet assay), anti-estrogenic (on ER α , ER β) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24 h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in food, feed or in the environment has thus to be considered, and their classifications as carcinogens/mutagens/reprotoxics is discussed.

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1. Introduction

Today, the production and dissemination of xenobiotics in the environment increase, and humans are exposed daily to many of these, but also their metabolites, which are present as pollutants (Feron et al., 2002). They act as mixtures having compensatory, multiplicative, or synergistic effects as we have shown (Benachour et al., 2007a). Among them, glyphosate (G)-based herbicides belong to the first herbicides used worldwide, and are major pollutants of rivers and surface waters (Cox, 1998; IFEN, 2006). They can contaminate organisms, including humans, but also food, feed and ecosystems (Takahashi et al., 2001; Acquavella et al., 2004; Contardo-Jara et al., 2008). Their use and presence in the food chain are further increased again with more than 75% of genetically modified edible plants that have been designed to tolerate high levels of these compounds (Clive, 2009), commercialized in various

formulations. The question of the active toxic threshold of these substances *in vivo* is still open; but it is now well demonstrated that mixtures formulated with G and adjuvants are themselves not environmentally safe, in particular for aquatic life (UE classification). They can even enhance heavy metals toxicity (Tsui et al., 2005). Their *in vivo* carcinogen, mutagen and reprotoxic (CMR) actions are discussed in this paper for two reasons. First, *in vivo* effects on reproduction of G-based herbicides on reproduction, such as sperm production or pregnancy problems and outcomes are already published (Yousef et al., 1995; Savitz et al., 1997; Daruich et al., 2001; Beuret et al., 2005; Dallegre et al., 2007; Oliveira et al., 2007; Cavalcante et al., 2008). Secondly, cellular mutagenic and toxic effects are now explained occurring at very low doses in cells involved in reproduction such as embryonic, fetal and placental ones (Marc et al., 2002, 2004; Richard et al., 2005; Dimitrov et al., 2006; Bellé et al., 2007; Benachour et al., 2007b; Benachour and Séralini, 2009). Since numerous CMR are also endocrine disruptors (ED), harmful for the environment and thus the object of specific legislations, the objective of this study was to test for the first time the ED capacities of these major pollutants on human cells. Androgen and estrogen receptors were examined using tran-

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scriptional activation assays, as well as aromatase activity. We have also measured a potential genotoxic activity for the most active formulation.

The human liver cell line HepG2 has been chosen since it constitutes the best characterized human liver cell line, moreover it is used as a model system to study xenobiotic toxicity (Urani et al., 1998; Knasmüller et al., 2004; Westerink and Schoonen, 2007). The defined phase I and phase II metabolism, covering a broad set of enzymes forms in HepG2 cells, offers the best hope for reduced false positive responses in genotoxicity testing (Kirkland et al., 2007). In addition, the liver is the major detoxification organ exposed to food or drinks contaminants. R has been demonstrated to damage carp or rat hepatocytes at low levels (Szarek et al., 2000; Malatesta et al., 2008). The objective of this study was also to compare the actions of four mainly used G-based Roundup (R) formulations, and G alone as control, on different enzymatic pathways and cellular endpoints. The endocrine mechanism was checked not only on three different sexual steroid receptors (estrogen receptors ER α , ER β , androgen receptors AR) but also on aromatase, the enzyme responsible for the irreversible androgen to estrogen conversion (Simpson et al., 1994, 2002). If these parameters are disturbed this will be in turn crucial for sexual and other several cell differentiations, bone metabolism, liver metabolism (Hodgson and Rose, 2007), reproduction, pregnancy and development, but also behaviour and hormone-dependent diseases such as breast or prostate cancer (Séralini and Moslemi, 2001). Few data have thus far been obtained yet at this level (Hokanson et al., 2007; Oliveira et al., 2007). This is important since chronic and genetic diseases can be provoked in humans and children by environmental pollution (Edwards and Myers, 2007) as well as by endocrine disruption (Rogan and Ragan, 2007).

2. Materials and methods

2.1. Chemicals

N-Phosphonomethyl glycine (glyphosate, G, PM 169.07), as well as most other compounds, otherwise specified, were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France (F)). Roundup herbicide formulations (Monsanto, Anvers, Belgium) were available on the market: Roundup Express[®] 7.2 g/L of G, homologation 2010321 (R7.2), Bioforce[®] or Extra 360 at 360 g/L of G, homologation 9800036 (R360), Grands Travaux[®] 400 g/L of G, homologation 8800425 (R400), Grands Travaux plus[®] 450 g/L of G, homologation 2020448 (R450). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), L-glutamine at 200 mM, phosphate sodium buffer (PBS), EDTA trypsin (0.05%) came from Invitrogen (Cergy-Pontoise, F). Alamar Blue was from Biosources (Camarillo, USA). Lysis buffers (RLB 5X, CCLR 5X), Luminol, agar NMP, enzymes and reagents for RT-PCR are from Promega, F, primers from (Eurobio, Les Ulis, F), Dextran from Pharmacia (Orsay, F), Chlorophenolred- β -D-galactopyranoside (CPRG) from Roche Diagnostics (Mannheim, Deutschland). Bradford solution came from BioRad (Munich, Deutschland). [3 H] androstenedione was from PerkinElmer (Courtabouef, F).

2.2. Cell cultures

The hepatoma cell line HepG2 (from a 15-year-old Caucasian boy hepatoma) was provided by ECACC (Salisbury, UK). Cells were grown in flasks of 75 cm² surface from Dutscher (Brumath, F) in phenol red-free EMEM (Abcys, Paris, F) containing 2 mM L-glutamine, 1% non-essential amino acid, 100 U/ml of antibiotics (mix of penicillin, streptomycin, fungizone) and 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, F), 10% fetal bovine serum (PAA, Les Mureaux, F). For anti-estrogenic activity, HepG2 cells were grown only in phenol red-free MEM (Fischer Bioblock, Illkirch, F) and without antibiotics.

The MDA-MB453-kb2 cell line was obtained from ATCC (Molsheim, F). This cell line possesses a high level of androgen receptor (Hall et al., 1994) and was stably transfected with the pMAMneo-Luc plasmid which contains the sequence of androgen-responsive luciferase reporter plasmid driven by the Mouse Mammary Tumor Virus (MMTV) (Wilson et al., 2002). MDA-MB453-kb2 cells were grown in culture 75 cm² flask (Dutscher, Brumath, F) in Leibovitz-15 (L15) medium (Fischer Bioblock, Illkirch, F) supplemented with 10% of fetal calf serum (Invitrogen, Cergy-Pontoise, F). Cells were incubated at 37 °C and the medium was removed every 48 h. In order to check the toxicity of the different compounds the neutral red assay was performed (Borenfreund and Puerner, 1984).

2.3. Toxicity tests

A 2% Roundup solution and an equivalent solution of glyphosate to Roundup Bioforce[®] were prepared in serum-free medium and adjusted to the pH 5.8 of the 2% Roundup Bioforce[®], they have been used for consecutive dilutions up to 10⁻⁷. The mitochondrial activity measure is based on the MTT test by cleavage of MTT into a blue colored product (formazan) by the mitochondrial enzyme succinate dehydrogenase (Mosmann, 1983; Denizot and Lang, 1986; Scatena et al., 2004). This assay was used to evaluate human cell viability. MTT was prepared at a 5 mg/mL stock solution in PBS, filtered at 0.22 μ m, and diluted to 1 mg/mL in a serum-free medium. After cell treatments, the supernatants were recovered for the ToxiLight[®] bioassay and adherent cells were washed with serum-free medium and incubated with 120 μ L MTT per well after each treatment. The 48-well plates, with 50,000 cells per well, were incubated for 3 h at 37 °C and 120 μ L of 0.04 N-hydrochloric acid containing isopropanol solution was added to each well. The plates were then vigorously shaken in order to solubilize the blue formazan crystals formed. The optical density was measured at 570 nm using a luminometer Mithras LB 940 (Berthold, Thoiry, F).

The bioluminescent ToxiLight[®] bioassay (Lonza, Saint Beauzire, F) is a non-destructive cytotoxicity highly sensitive assay designed to measure cell membrane damage. It quantitatively measured the release of Adenylate Kinase (AK) from the membranes of damaged cells (Crouch et al., 1993; Squirrel and Murphy, 1997). AK is a robust protein present in all eukaryotic cells, which is released into the culture medium when cells die. The enzyme actively phosphorylates ADP and the resultant ATP is then measured using the bioluminescent firefly luciferase reaction with the ToxiLight reagent. The advantage of this assay is that the cell lysis step is not necessary. After 24 h of different treatments, 50 μ L of cell supernatants were deposited in a 96-well plate. Then 50 μ L of the AK Detection Reagent (AKDR) were added by well. The plates (Dutscher, Brumath, F) were then placed under agitation for 15 min safe from the light, and then luminescence was measured using the luminometer Mithras LB 940 (Berthold, Thoiry, F) at 565 nm. The serum-free medium was the negative control, and a positive control was the active reagent AKDR mixed with cells treated in the serum-free medium to determine the basal activity.

The caspases 3/7 activities were measured with the Caspase-Glo[®] 3/7 assay (Promega, Paris, F) in 96-well white plates (Dutscher, Brumath, F). It was a luminescent method designed for automated high-throughput screening of caspases activity, or apoptosis induction (O'Brien et al., 2000). The assay provides a pro-luminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate was cleaved to release amino-luciferin, a substrate of luciferase used in the production of light. The Caspase-Glo[®] 3/7 reagent was added (50 μ L per well) after cell treatments by 24 or 48 h of R450. Plates were then agitated during 15 min and incubated 45 min at room temperature safe from the light, to stabilize the signal before measuring the glow-type luminescence provoked by the caspase cleavage of the substrate. The negative control is the serum-free medium, the positive control is the active reagent mixed with cells treated in the serum-free medium to determine the basal activity of the caspases 3/7. Luminescence was measured using the luminometer Mithras LB 940 (Berthold, Thoiry, F).

The Alamar Blue[®] assay was performed according to the procedure described by O'Brien et al. (2000). About 30,000 HepG2 cells per well were grown 24 h in 96-well plates and then exposed to 250 μ L of different treatments for 24 h, pH adjusted to 7.4. After treatment, 100 μ L of the 10% Alamar Blue solution were added in each well and incubated for 2 h at 37 °C. Measurement of the optical density at 540 and 620 nm was performed using a spectrophotometer Multiskan EX (Thermo Fisher Scientific, Courtabouef, F). The viability was expressed as a percentage of the control results (medium only).

The neutral red assay was performed according to the procedure described by Borenfreund and Puerner (1984). About 50,000 MDA-MB453-kb2 cells per well were seeded in 24-well plates and grown 24 h (37 °C). After 24 h of different treatments (1 mL firstly adjusted to pH 7.4), cells were washed with PBS. Then, 1 mL of neutral red solution (50 μ g/mL) was added in each well for 3 h (37 °C). For the last time, cells were washed and 1 mL acid acetic/ethanol (1/50, v/v) was added in each well, and the plate was shaken for 10 min before measuring the neutral red release by fluorescence (emission filter: 580 nm and excitation filter: 535 nm). The viability was expressed as a percentage of controls (medium only).

2.4. Genotoxicity test

The very sensitive comet assay is also known as the single-cell gel electrophoresis (SCGE) assay. The underlying principle is the ability of denatured DNA fragments to migrate during electrophoresis that can be carried out under highly alkaline conditions (pH > 12.6), in order to detect single- and double-strand breaks and alkali-labile lesions. The assay was adapted from Singh et al. (1988) with some modifications for cell preparation (Valentin-Severin et al., 2003). Shortly, after 24 h treatment, cell suspensions were prepared by washing the cells with PBS and treating them with trypsin/EDTA for 5 min at 37 °C. Samples from 8 wells were pooled, centrifuged (100 \times g, 5 min, 4 °C) and resuspended in 100 μ L PBS. Fifty microliter of cells (5 \times 10⁴ cells) prepared for analysis were then mixed with 75 μ L of 0.5% low melting-point (LMP) agarose at 37 °C. The cell suspension was rapidly spread onto a pre-coated slide, covered with a 25 mm² cover slip and placed at 4 °C for 5 min. Cover slips were removed and the slides treated with lysis solution for at least 1 h at 4 °C. After lysis, slides were exposed to alkaline electrophoresis buffer (pH 13) for 40 min and

subjected to electrophoresis for 20 min (300 mA, 25 mV). Then, the alkali was neutralized with Tris buffer, the slides rinsed with cold ethanol 96%, and dried at room temperature. For analysis, slides were recovered with 70 μ L iodure propidium and placed under a cover slip. Reading was performed with a fluorescence microscope (40 \times). Nuclei observed were classified into 4 classes: 0 (undamaged), 1 (minimum damage), 2 (medium) and 3 (maximum damage) according to Collins (2004) and Collins et al. (2008).

2.5. Aromatase disruption

Aromatase activity was evaluated according to the tritiated water release assay (Thompson and Siiteri, 1974) with a slight modification as previously described (Dintinger et al., 1989). This method is based on the stereo-specific release of 1β -hydrogen from the androstenedione substrate, which forms tritiated water during aromatization. The HepG2 cells were exposed to non-toxic concentrations of glyphosate alone or Roundup, and were washed with serum-free EMEM and incubated for 90 min with 200 nM [1β - 3 H] androstenedione at 37 °C (5% CO₂, 95% air). The reaction was stopped by centrifugation at 2700 \times g at 4 °C for 10 min. After adding 0.5 mL of charcoal/dextran T-70 suspension, the mixture was centrifuged similarly. Supernatant fractions were assessed for radioactivity by scintillation counting (Packard, Liquid scintillation counter 1600LR, USA).

Aromatase mRNA levels were measured by semi-quantitative RT-PCR. Total RNA was extracted (RNAagents method, Promega, F) from HepG2 cells and checked at 260, 280 nm and by electrophoresis on agarose gel (1.5%) stained with ethidium bromide. Five microns were reverse-transcribed (RT) using 200 U MMLV-RT (Moloney murine leukemia virus reverse transcriptase) at 42 °C for 60 min in the presence of 0.2 μ g oligo dT, 500 μ M of each dNTP and 20 U RNasin in a total volume of 40 μ L. The cDNA obtained were used for PCR. For each run, a master mix was prepared with 1.5 U Taq DNA polymerase in PCR buffer containing 200 mM dNTP, 1.5 mM MgCl₂, and 25 pmols of each primer in a total volume of 50 μ L. The PCR primers were EXIII sense, 5' TGA GGT CAA GGA ACA CAA GA 3' and EXIII antisense 5' ATC CAC AGG AAT CTG CCG TG 3' (Corbin et al., 1988). The thermal cycling conditions consisted of an initial step at 95 °C for 2 min and then 35 cycles of 95 °C for 30 s and 60 °C for 60 s. Aromatase mRNA levels were normalized with the control housekeeping gene GAPDH. The primers used for PCR was for the sense primers 5' CCA TCA CCA TCT TCC AGG AGC 3' and for the antisense 5' GGA TGA TGT TCT GGA GAG CC 3'. The resulting PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Gels were photographed using photoprint Vilbert Lourmat (F) system and analyzed with image J computer program.

2.6. Anti-estrogenic activity

Five plasmids were used for the transient transfections of the HepG2 cell line. Plasmids ERE-TK-Luc, hER α and hER β were kindly provided by Dr D. McDonnell (Ligand Pharmaceutical, San Diego, USA); pCMV β Gal and pSG5 were used for the normalization of luciferase activity (Cabaton et al., 2009). ERE-TK-Luc is a 6.7 kb expression vector containing a single copy of the estrogen response element of the

vitellogenin with a minimal thymidine kinase promoter driving firefly luciferase (Tzukerman et al., 1994). Plasmids hER α and hER β are built from the plasmid pRST-ER (Rous Sarcoma Virus/T7 promoter; Hall and McDonnell, 1999) and encode the human wild-type estrogen receptor α or β . The pCMV β Gal contains β -galactosidase gene and is used in order to control the transfection efficiency. Finally, pSG5 is used to obtain an appropriate DNA concentration for the transfection.

HepG2 cells were transiently transfected using Exgen 500 procedure (Euromedex, Mundolsheim, F). 120,000 cells per well were grown at 37 °C (5% CO₂, 95% air) in MEM supplemented with 2 mM glutamine, 1% non-essential amino-acid and 10% of dextran-coated charcoal fetal calf serum in 24-well plates. The microplates were then incubated for 24 h. For transfections, all plasmids were first diluted in 0.15 M NaCl to a final concentration of 100 ng/ μ L and then mixed: 100 ng ERE-TK-Luc, 100 ng hER α or β , 100 ng pCMV β Gal and 200 ng pSG5. Then 2 μ L of Exgen 500 diluted in NaCl 0.15 M were added to DNA. The mix was centrifuged and incubated at least 10 min at room temperature. The mixture was added to OptiMEM and distributed into the wells (300 μ L/well). After 1 h of incubation (37 °C, 5% CO₂), the medium was removed and replaced by 1 mL of treatment medium without fetal calf serum for 24 h. To observe an anti-estrogenic activity, cells were co-treated with xenobiotics and 17β -estradiol 10^{-8} M. ICI 182 \times 780 (10^{-8} M) was used as positive control. At the end of the treatment, cells were lysed with Reporter Lysis Buffer (Promega) and frozen at -80 °C for at least 30 min. Then they were scraped and placed into microtubes before three freezing (liquid nitrogen)/thawing (37 °C water bath) cycles and centrifuged 5 min at 10,600 \times g.

For luciferase activity measurement, 10 μ L of lysate were mixed with 50 μ L of luciferase assay reagent (Promega) into a white 96-well plate. The mixtures were immediately analysed using a luminometer (TopCount NT, Packard). The β -Galactosidase activity was measured using chlorophenol-red β -D-galactopyranoside (Roche Diagnostics GmbH, Mannheim, Germany). The chlorophenol-red product was measured with a spectrophotometer at 570 nm (MRX Dynex). Protein concentration determination was performed using 2 μ L of the lysate according to Bradford (1976) on a spectrophotometer at 595 nm. Luciferase activity for each treatment group was normalized to β galactosidase activity and protein level (Luc \times Prot/Gal) was compared to the control (17β -estradiol 10^{-8} M) set at 100%.

2.7. Anti-androgenic activity

MDA-MB-453-kb2 cells were seeded in 24-well plates and 50,000 cells per well were grown in L-15 medium without phenol-red supplemented with 5% dextran-charcoal fetal calf serum for 24 h (37 °C without CO₂). After 24 h incubation, medium was removed and cells were washed with 500 μ L PBS and exposed to roundup solutions in co-treatment with DHT (4×10^{-10} M) for 24 h in medium without fetal calf serum. Nilutamide (10^{-6} M) was used as positive control. For luciferase activity measurement, 10 μ L of lysate were mixed with 40 μ L of luciferase assay system (Promega) into a white 96-well plate. The mixtures were immediately analysed using a luminometer (TopCount NT, Packard). Results were expressed as a percentage of the data obtained with the androgen DHT (4×10^{-10} M).

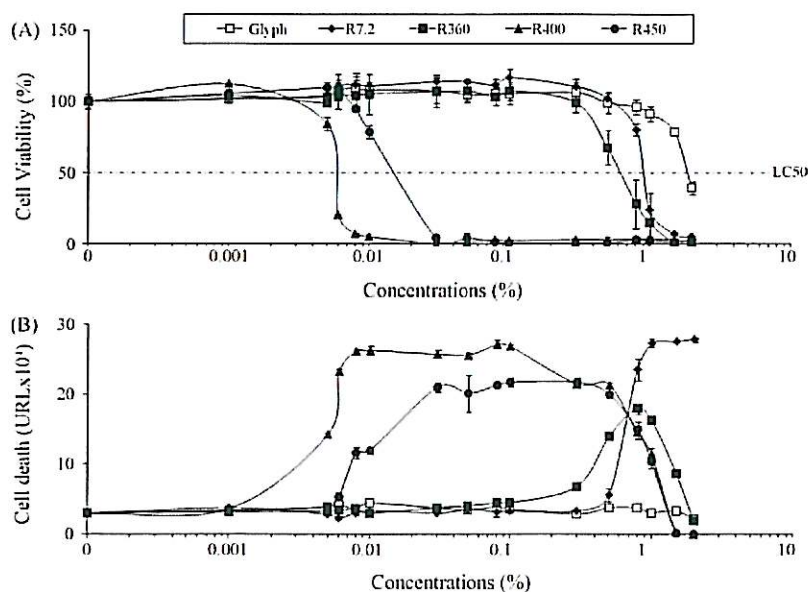


Fig. 1. Dose-dependent effects of glyphosate (G) and four glyphosate-based formulations (Roundup containing 7.2–450 g/L G) on HepG2 cells viability after 24 h of exposure. These effects were evaluated by the MTT test (A) or the ToxiLight assay (B). The results are presented in % comparatively to non-treated cells (100% viability, A) or in relative levels to non-treated cells (URL: 1, B). Cells were grown at 37 °C (5% CO₂, 95% air) in medium EMEM with 10% serum during 48 h to 80% confluence in 48-well plates for MTT test or 96-well plates for ToxiLight, and then exposed to the products for 24 h without serum. All experiments were repeated 4 times in triplicates.

Table 1
Comparative initial toxicities and LC50 of glyphosate-formulations measured by three different ways (described in Section 2) on HepG2 cell line.

Products	Alamare blue test (%)		MTT test (%)		ToxLight assay (%)
	Initial toxicity	LC50	Initial toxicity	LC50	
G	1	2.78	1	1.8	>2
R7.2	0.2	0.36	0.8	0.86	0.8
R360	0.1	0.22	0.5	0.65	0.3
R400	0.0005	0.0012	0.005	0.0055	0.005
R450	0.005	0.006	0.008	0.017	0.006

The initial toxicities correspond to the % of product provoking the first significant effects (around 10% toxicity) for glyphosate alone (G) or at different concentrations (7.2–450 g/L) in different Roundup formulations (R).

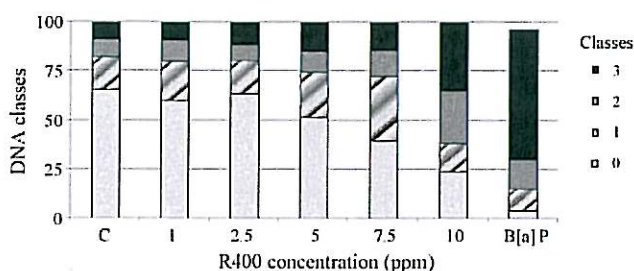


Fig. 2. DNA damages (increasing from classes 1–3, Comet assay) after HepG2 cells exposure to R400 during 24 h at different concentrations (0: control C, 1–10 ppm). Benzo[a]pyrene (50 μ M, B[a]P) was used as positive control. All experiments are repeated 3 times in duplicate for 100 cells.

2.7.1. Statistical analysis

All data were presented as the mean \pm standard error (S.E.M.). Statistical differences were determined by a Student *t*-test using significant levels of 0.01 (**), or 0.05 (*) with GraphPad Prism 4 software.

3. Results

HepG2 cells, in our experiments, generally show a growth rate around 32 h in control medium. All glyphosate-based formulations, by contrast to glyphosate alone (toxic from 1% in MTT assay), induce a rapid decrease in cell viability according to the formulation and the test, within 24 h only (Fig. 1 and Table 1). Several endpoints were reached: mitochondrial respiration and activity (MTT Fig. 1A and Alamar blue, the most sensitive assay, Table 1) or cellular membrane damage (Fig. 1B). Mortality is dose-dependent for all R in formulations, but there is no dose-dependency to G concentration. This is confirmed for the first time by three specific methods. The most cytotoxic formulation (400 g/L of G) does not contain the highest concentration of G. The two first formulations demonstrate similar middle toxicities (7.2 and 360 g/L of G), the two others show 20–200 times higher toxicity (400 and 450 g/L of G, Fig. 1). The dif-

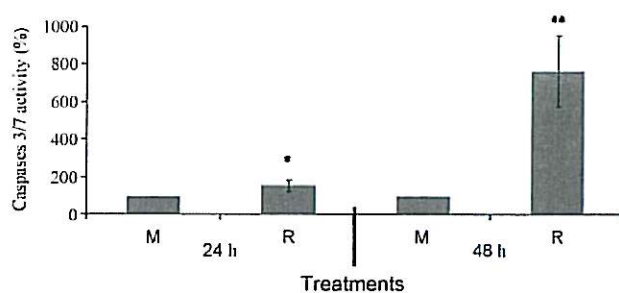


Fig. 3. Time-dependent apoptosis through caspases 3/7 induction by Roundup (R) on HepG2 cells. The relative caspases 3/7 activities (% control with serum-free medium M) are presented after 24 h of R at 60 ppm or 48 h. Cells reached 80% confluence with serum before being treated.

Table 2

Comparative IC50 for different glyphosate-based formulations on steroid receptors in HepG2 cells.

IC50	R7.2	R360	R400	R450
ER α % μ M G	0.203 86.5	0.145 3087.5	0.0006 14.2	0.002 53.2
ER β % μ M G	0.246 104.8	0.16 3406.9	0.0003 7.1	N.D. N.D.
AR % μ M G	0.077 32.8	0.031 660.1	0.00009 2.13	0.002 53.2

Glyphosate is at 7.2, 360, 400 or 450 g/L in the four Roundup (R), in % R for the first line, and in equivalent G concentration (μ M) on the second line. This is tested on estrogen receptors (ER α and β) transfected-HepG2 and in the breast cancer cell line MDA-MB453-kb2.

ferent values of LC50 and initial statistically significant toxicities (around LC10) for the various formulations are in the same range whatever the assay: R400 > R450 > R360 > R7.2 (Table 1).

Effects of R400 on HepG2 DNA after 24 h exposure are illustrated in Fig. 2. In our conditions, we observed around 50% DNA strand breaks at 5 ppm (25% class 1, 11% class 2 and 15.5% class 3). This effect is dose-dependent with a drastic increase in classes 2 (27%) and 3 (36%), revealing major damages at 10 ppm, corresponding to 24 μ M G dissolved in specific adjuvants. This provokes around 75% DNA fragments in comparison to 35% in negative controls. The positive control, the well-known promutagen Benzo[a]Pyrene, induces 95% damages, but at about 2 times higher concentrations (50 μ M). This result clearly shows that the DNA of the human hepatoma cell line is damaged by a G-based herbicide.

The caspases 3/7 are significantly activated with non toxic doses of R450 (60 ppm, Fig. 3) up to 156% in 24 h. Their levels are considerably enhanced to 765% within 48 h, R is able to induce apoptosis.

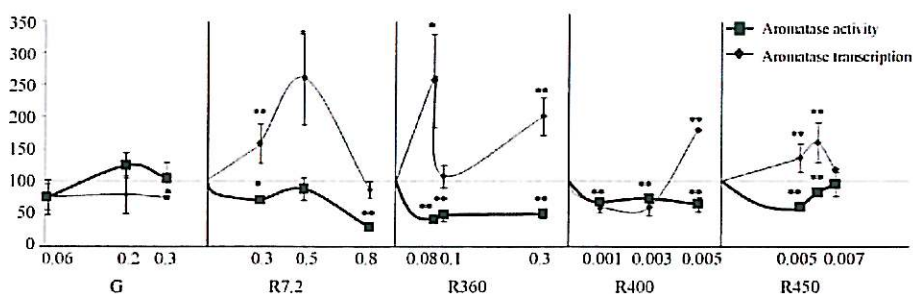


Fig. 4. Dose-dependent effects of glyphosate (G) and the four Roundup formulations on aromatase activity (bold line) and mRNA levels in HepG2. These effects below toxic levels were evaluated in % controls respectively, by tritiated water release during aromatization, and semiquantitative RT-PCR. Cells were grown as in Fig. 1 and then exposed for 24 h to xenobiotics. All experiments were repeated 3 times in triplicates. Statistically significant differences are indicated for $p < 0.01$ (**) and $p < 0.05$ (*).

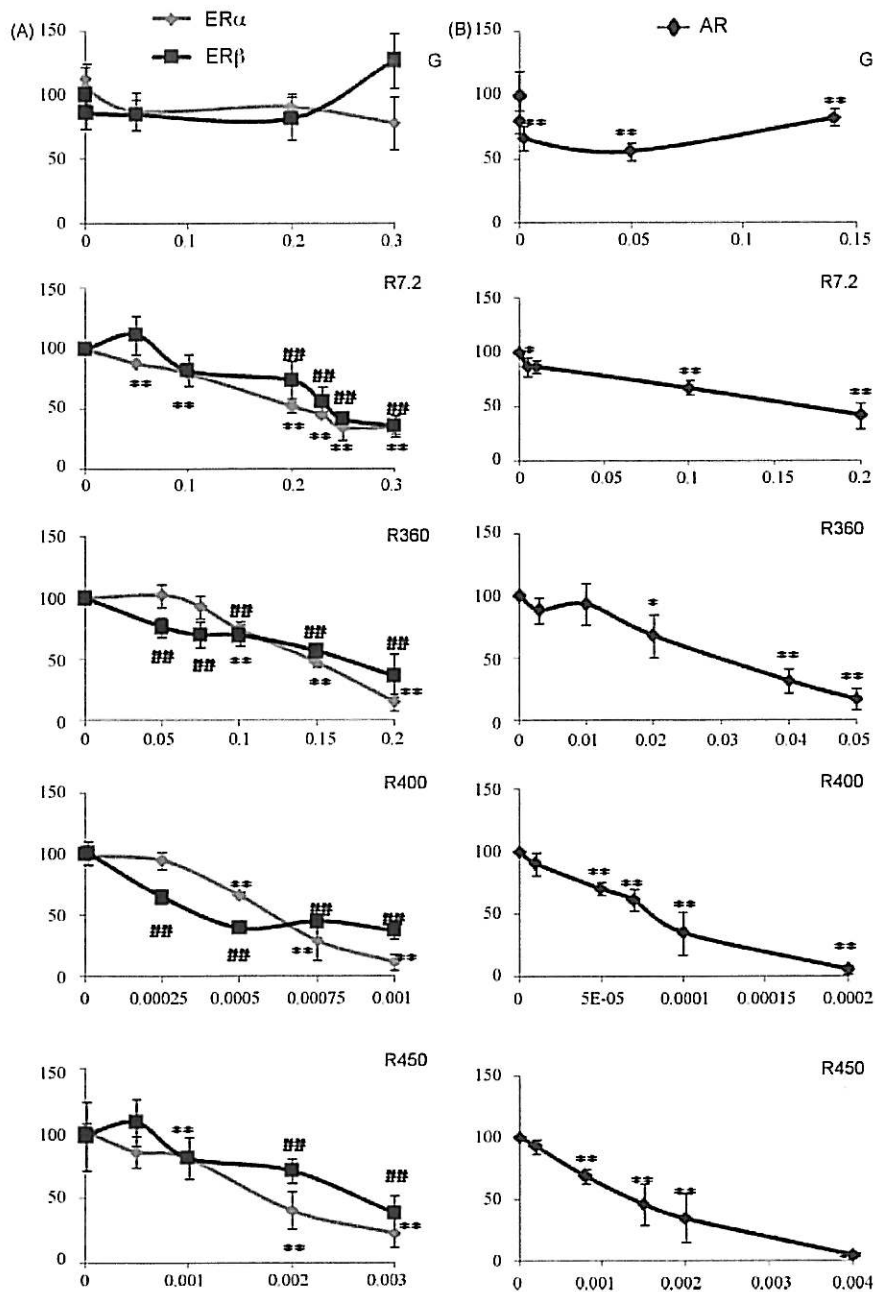


Fig. 5. Dose-dependent effects of Glyphosate (G) and the four Roundup formulations on ER α , ER β (A, left column) transcriptional activities in HepG2 transiently transfected (ERE-TK-Luciferase) and AR (B, right, measured in MDA-MB453-kb2 cells). These effects below toxic levels (except last dose on the scale) were evaluated after 24 h in % controls respectively, activated by 10^{-8} M estradiol 17 β for ER and 10^{-10} M DHT for AR. All experiments were repeated 3 times in triplicates. Statistically significant differences are indicated for $p < 0.01$ (** for ER α and AR, ## for ER β).

We have obtained interferences of G-based herbicides with human cell endocrine activities, below initial toxic doses (which are around LC10), known for at least two out of three cytotoxicity tests. We began to study the gene expression variations of the irreversible sexual steroid conversion, aromatase. Both enzymatic activity and specific mRNA levels were assessed (Fig. 4). G alone is always inactive, while all the formulations inhibited androgen to estrogen conversion, below all LC50 and always in 24 h. In the meantime, biphasic effects were seen on the aromatase mRNA levels for all formulations, with increases 130–250% followed by a return to normal in most cases. An inhibition was seen for R400 then followed by the increase. These effects were thus neither linear nor G-proportional.

Furthermore we also observed at lower doses disruptions of estrogen and androgen dependent transcriptional activities. These were quite linear and dose-dependent (for R not for G) in the case of each formulation, in the range of values tested, after 24 h of exposure (Fig. 5). The corresponding IC50 were determined (Table 2). For all G-based herbicides, common anti-estrogenic profiles for both ER and anti-androgenic ones were revealed, according to the slopes of the curves (Fig. 5A and B). G alone had no anti-estrogenic activity but was clearly anti-androgenic at sub-agricultural and non cytotoxic dilutions. Even if data showed that both ER transcriptional activities were comparably affected, there were some formulations specificities: R400 is clearly 2 times more active on ER β , and R450 on ER α . The most toxic formulations are the

most inhibitors at lower non cytotoxic doses, on cell endocrine activities (Fig. 5). All formulations except R450 appeared more anti-androgenic than anti-estrogenic. We can classify the R inhibition efficiencies: from R400 > R450 > R360 > R72, with a 300–800 times difference between the strongest inhibitor and the lowest (Table 2).

4. Discussion

This work evidences the toxic effects of four formulations of the major herbicide worldwide (R) on an human hepatic cell line HepG2, a pertinent model for xenobiotic actions (Knasmüller et al., 2004). This is also because the liver is the first detoxification organ, and very sensitive to dietary pollutants. We tested sub-agricultural dilutions and noticed the first toxic effects at 5 ppm, and the first endocrine disrupting actions at 0.5 ppm, which is 800 times lower than the level authorized in some food or feed (400 ppm, US EPA, 1998). This confirms and enhances the potential toxic action of G-based herbicides that we observed on human placental and embryonic cell lines, and on fresh umbilical cord cells (Richard et al., 2005; Benachour et al., 2007b; Benachour and Séralini, 2009). Their mechanistic time and dose-dependent actions on mitochondria, plasma membrane, caspases 3/7 and DNA fragmentation has been previously demonstrated. Here we obtain for the first time their relative LC50 by three different methods, but also their genotoxicity, and endocrine disruption potentials from lower levels on three different sexual steroid receptors on human cell lines. The mixtures in formulations in this work are always the most toxic in comparison to G alone, as previously underlined (Richard et al., 2005), and also observed in aquatic communities (Relyea, 2008). We confirm that the nature of the adjuvants changes the toxicity more than G itself, not only in embryonic or neonate cells (Benachour and Séralini, 2009) but also in human cell lines (HepG2 and MDA-MB453-kb2) from young or adult. This allows deleterious actions at very low levels that have no more herbicide properties. This creates environmental concerns of contaminating authorized amounts found in rivers, soils or food and feed within 24 h only. The time-amplified effects have also been previously described (Benachour et al., 2007b). Our three different methods measuring in particular simultaneously FAD, NAD and NADPH dehydrogenases, mitochondrial succinate dehydrogenase and plasma membrane degradation gave consistent results with comparable differential toxicities profiles, with the four G-based herbicides, even if one test was obviously more sensitive than the others (Alamar Blue).

We demonstrate here for the first time the DNA damages of a G-based herbicide on a human cell line at residual levels corresponding to 120 nM of G. An association was previously suggested with a multiple myeloma incidence in agricultural workers (De Roos et al., 2005). However, there was still a serious doubt about direct genotoxicity in mammals (Williams et al., 2000; Dimitrov et al., 2006), that was recently questioned in mice (Heydens et al., 2008), after contradictory results. G was known to be genotoxic alone on human cells, but at 10^6 higher levels (mM, Monroy et al., 2005) in comparison to this study. It was similar for AMPA alone, a G metabolite (Mañas et al., 2008). DNA damages were already induced by G and synergistic oxidative stress in human fibroblasts (Leuken et al., 2004), and thus combined mutagenic effects of adjuvants and G, plus its metabolites, appear obvious at minute doses in the present work. It is noticed that the biotransformation of xenobiotics results in the production of reactive intermediates such as reactive oxygen species which are toxic and can cause oxidative damage to DNA (Cadet et al., 2003). In addition, R, with its adjuvants, has been previously demonstrated to provoke DNA adducts in the kidneys and livers of mice (Peluso et al., 1998) and DNA lesions in tadpoles, bovine cells, drosophila, fish, or caimans (Clements et al., 1997; Lioi

et al., 1998; Kaya et al., 2000; Cavas and Könen, 2007; Cavalcante et al., 2008; Poletta et al., 2008). The comet is a very sensitive assay but not specific. Two other endpoints must be taken into account using this method: apoptosis and DNA repair. During the apoptotic process, DNA is broken down into nucleosome-sized pieces. Comet equivalent to class 4 (DNA in the tail and small head) can reveal cells in the earliest stages of apoptosis, this class was not taken into account in this study. Caspases 3/7 activations characteristic of apoptosis were demonstrated recently by some of us to be provoked by similar R formulations in other human cells (Benachour and Séralini, 2009). In this study, R450 is able to induce caspases at 60 ppm. As Comet equivalent to class 4 (DNA in the tail and small head) can reveal cells in the earliest stages of apoptosis, then this class was not taken into account here (Collins et al., 2008). Experiments are running in the lab to check if at this lower concentration of R400 these DNA damages can be really due to a repair process.

We then tested the potential endocrine disruption below the toxic levels described above in human cells. This was done by measuring not only the capacity of G-based herbicides to disrupt androgenic or estrogenic transcriptional activities, but also to modify a crucial irreversible androgeno-estrogenic steroid metabolism, through aromatase gene expression measurement. A constitutive but low aromatase activity inhibition was observed in this work with all formulations as suggested previously (Richard et al., 2005; Benachour et al., 2007b) due to the combined effects of G plus adjuvants here. Low levels of aromatase inhibition resulted in intersexed gonads and possible female reproductive impairment at adulthood in amphibians (Olmstead et al., 2009). Comparable hypotheses have been proposed for humans (Séralini and Moslemi, 2001), even for other xenobiotics (Moslemi and Séralini, 2005; Salaberria et al., 2009). It becomes obvious that the direct enzymatic effect of G (Richard et al., 2005) does not exclude a transcriptional disruption as it was observed in mouse and urchin eggs (Walsh et al., 2000; Marc et al., 2002, 2005). The biphasic profile of this aromatase transcription disruption could be either due to a direct DNA interaction of R compounds (Peluso et al., 1998) or to a receptor-mediated interaction like it was shown on ER-mediated transcription for other pesticides (Sheleer et al., 2000). In order to test this hypothesis, we studied the interaction with three different steroid receptors able to bind steroid-like structures as well as aromatase, which is indeed regulated by estrogens and androgens in mammals (Bourguiba et al., 2003).

Steroid receptors may be involved in xenobiotic receptor pathways of action (Mattews and Gustafsson, 2006; Rokutanda et al., 2008). They are even disrupted by several xenobiotics, like other ER α or even steroid membrane receptors in various animals (Watson et al., 2006), for instance the pesticide methoxychlor upregulates ER β in the bass (Blum et al., 2008). Even surfactants, adjuvants, plasticizers or pesticides have been proven to interfere with AR (Paris et al., 2002; Wilson et al., 2008). The *in vivo* consequences may be obvious for sexual differentiation and reproduction (Sultan et al., 2001; Martin-Skilton et al., 2008). R itself may affect male genital organs in drakes (Oliveira et al., 2007) or estrogen-regulated genes in human cells (Hokanson et al., 2007). It was then logic to test the ED potential on ER α , ER β and AR. Here we prove for the first time for four G-based formulations their dose-dependent interactions with these receptors. Their IC50 are measured in μ M higher than those of well known inhibitors such as raloxifen or tamoxifen for estrogen receptors (Sibley et al., 2003; Ozcan-Arican and Ozalpan, 2007), and flutamide for androgen receptors (Simard et al., 1986), which have IC50 in the nM range. The G in adjuvants has comparable properties than other ED (Xu et al., 2005). Moreover the various adjuvants change obviously the shape or at least the bioavailability, penetration and bioaccumulation of G at this level, and/or anyway its receptor interactions, with the results we have described.

The non G-linear cytotoxic effects, and at lower levels ED effects, demonstrate also the major role of adjuvants in biological disruptions. Moreover the direct interaction of G with the aromatase catalytic site previously demonstrated (Richard et al., 2005) and confirmed by an aromatase disruption here, has now to be considered with the present interaction demonstration with three steroid receptors. Since G is designed to inhibit in plants the enzyme EPSPS involved itself in essential aromatic amino acids metabolism (Amrhein et al., 1980; Franz, 1985), it is possible that G (in R) could fit in a binding site for a molecule with an aromatic cycle, such as those in steroid receptors or steroid metabolizing enzymes (Walsh et al., 2000). It is also possible that, as suggested for other xenobiotics, these herbicides bind to more than one site on steroid receptors (Arnold et al., 1997).

In conclusion, according to these data and the literature, G-based herbicides present DNA damages and CMR effects on human cells and *in vivo*. The direct G action is most probably amplified by vesicles formed by adjuvants or detergent-like substances that allow cell penetration, stability, and probably change its bioavailability and thus metabolism (Benachour and Seralini, 2009). These detergents can also be present in rivers as polluting contaminants. The type of formulation should then be identified precisely in epidemiological studies of G-based herbicides effects (Acquavella et al., 2006). Of course to drive hypotheses on *in vivo* effects, not only dilution in the body, elimination, metabolism, but also bioaccumulation and time-amplified effects (Benachour et al., 2007b) should be taken into account. These herbicides mixtures also present ED effects on human cells, at doses far below agricultural dilutions and toxic levels on mitochondrial activities and membrane integrity. These doses are around residual authorized levels in transgenic feed, and this paper is the first clear demonstration of these phenomena in human cells. The *in vivo* ED classification of G-based herbicides with this molecular basis must be now carefully assessed.

Conflict of interest statement

None.

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Roundup[®] in genetically modified plants: Regulation and toxicity in mammals¹

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Context

Among the 134 million hectares of genetically modified plants growing worldwide in 2009, more than 99.9 % are described as pesticide plants (Clive 2009). Around 80 % are tolerant to Roundup, a glyphosate based herbicide. Its use on GMOs is thus amplified, and this phenomenon shed a new light on the problem of herbicide residues in plants. This is because these GM plants have been modified so that they can contain high levels of Roundup. They are modified to behave normally after several treatments with this herbicide, which were not allowed at such levels on regular plants before. The latest generation, like Smartstax crops, even cumulate a tolerance up to 2 herbicides and a production of 6 insecticides. By this widespread use and the known potential hazards of pesticides, their residues are a major concern for health and the environment. Moreover the new metabolism that they could trigger in GMOs remains to be studied. A debate on international standards is ongoing on their capacity to predict and avoid adverse effects of the herbicide residues at environmental or nutritional exposures, particularly in GMOs.

As far as Roundup is concerned, the formulations of which are mixtures of only one proposed active ingredient (glyphosate) with various adjuvants, up to 400 ppm of residues are authorized in some Genetically Modified food and feed (EPA 2008). It is also recognized by regulatory agencies that these residues are found in meat and products generated from livestock fed with glyphosate tolerant soya or maize (EFSA 2009).

Review on Roundup toxicity studies

Surprisingly, more and more studies have revealed unexpected effects of Roundup, including carcinogenic and endocrine disrupting effects. This is at lower doses than those authorized for residues found in Genetically Modified Organisms (GMOs). For example, Roundup altered the spermatogenesis of rats exposed in utero to 50 ppm per day (Dallegrave et al. 2007). Even a tumour promoting potential is observed on mice

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exposed to 25 ppm per day (George et al. 2010). Alterations of rat testicular morphology and testosterone levels occur at doses of 5 ppm per day (Romano et al. 2009). In our laboratory we have observed endocrine disruption on human cell lines; it was a disruption of aromatase, of the androgen and estrogen receptors in 24 hours, starting from 0.5 ppm Roundup. This corresponds to glyphosate concentrations 2000 times less than the authorized levels in GMOs (Gasnier et al. 2009). Furthermore, we have shown that Roundup inhibited cellular respiration, and that it also caused membrane damages. Last but not least, Roundup showed genotoxic effects, as well as it induced apoptosis and necrosis in human cells (Benachour & Séralini 2009). Most of these effects are amplified with time. This is preoccupying, and it does highlight the limits of the Acceptable Daily Intake concept for long term exposures.

Debate on health risks

In all these studies, toxic effects were not detected with the so-called active ingredient glyphosate alone at these doses; they were more related to the formulations of the herbicide and its adjuvants. These remain confidential and their residues are not measured. Out of the 20 tests required (or conditionally required) to register a pesticide in the United States, only 7 short-term acute toxicity tests use the whole formulation; the others are done using the sole active ingredient (Cox & Sorgan 2006). The problem of pesticide registration is indeed very old, and it is only the active ingredient that is tested in chronic mammalian toxicity tests (generally for 2 years on rats). Moreover there is generally only one 2-year test worldwide on a mammal per pesticide, performed by the company commercializing this pesticide. Adjuvants are often considered to be inert in the assessment process. This is a major issue. Such a simplistic approach of pesticides hazards bypasses the potential effects of adjuvants and their mixtures with the active ingredient on chronic risks. This issue is even more crucial with GMOs which are designed to tolerate the formulations that enter the edible plant cells.

Nevertheless, it is well known that adjuvants are mixed with the active ingredient in order to increase the efficiency of formulations. In medicine, adjuvants are also used to increase the molecule absorptions, or the effectiveness of vaccines. In chemical products such as pesticides, they are used to increase targeted toxicity (for example penetration in leaves or insects), but they do have an effect also on non specific targets too. Some known adjuvants of Roundup such as polyethoxylated tallowamine (or POEA) showed more toxic effects than glyphosate in various models, and even more than Roundup in some cases on aquatic life for example (Tsui & Chu 2003; Marc et al. 2005) or on human cells (Benachour & Seralini 2009).

By only considering the active ingredient, regulatory thresholds seem to guarantee the safety of residues, however we conclude that it is not the case with the whole formulations, in particular those specific to GMOs. In conclusion, confidentiality on the composition of formulations must be lifted, as announced recently by the U.S. Environmental Protection Agency following our work (EPA 2009). People consuming GMOs are thus

exposed to residues of many formulations which are themselves mixtures of different chemicals. The long term combined effects have never been evaluated, not even in laboratory animals. We suggest that regulatory agencies change their paradigms and integrate modern knowledge, in order to guarantee the safety of pesticides residues, in particular when associated with genetically modified plants.

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