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Genotoxicity of mixtures of glyphosate and atrazine and their environmental transformation products before and after photoactivation

A. Roustan *, M. Aye, M. De Meo, C. Di Giorgio

Laboratoire de Mutagenèse et Toxicologie Environnementales, IMBE – UMR CNRS 7263/IRD 237, Faculté de Pharmacie, Aix-Marseille Université, 27 Bd Jean Moulin, 13385 Marseille Cedex 05, France

HIGHLIGHTS

- The cytogenetic effects of pesticide mixtures were evaluated on CHO cells.
- A 20-fold enhanced activity was observed in mixture with the four pesticides.
- It was 100-fold increased after light-irradiation, through oxidative stress.
- It highlighted the importance of cocktail effects in environmental matrices.
- It showed the limits of usual strategies to estimate environmental risks.

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ABSTRACT

The photo-inducible cytogenetic toxicity of glyphosate, atrazine, aminomethyl phosphoric acid (AMPA), desethyl-atrazine (DEA), and their various mixtures was assessed by the in vitro micronucleus assay on CHO-K1 cells.

Results demonstrated that the cytogenetic potentials of pesticides greatly depended on their physico-chemical environment. The mixture made with the four pesticides exhibited the most potent cytogenetic toxicity, which was 20-fold higher than those of the most active compound AMPA, and 100-fold increased after light-irradiation. Intracellular ROS assessment suggested the involvement of oxidative stress in the genotoxic impact of pesticides and pesticide mixtures.

This study established that enhanced cytogenetic activities could be observed in pesticide mixtures containing glyphosate, atrazine, and their degradation products AMPA and DEA. It highlighted the importance of cocktail effects in environmental matrices, and pointed out the limits of usual testing strategies based on individual molecules, to efficiently estimate environmental risks.

1. Introduction

Since their discovery in the 1940s, pesticides have greatly contributed to improve the yield and quality of plants, and to ensure crop production. As a consequence, the global consumption of pesticides, which was lower than 0.5 kg Ha^{-1} in the 1960s, has constantly increased to reach 2 Kg Ha^{-1} in 2004. Today, modern agriculture is very dependent on pesticides, since 4.6 million tons of chemical pesticides are annually sprayed into the environment, corresponding to an approximate global sale of 300 billion dollars (Zhang et al., 2011). Despite various international regulations, pesticides have contaminated all the environmental compartments worldwide (Vecchia et al., 2009; Dubois and Lacouture, 2011; Mouvet, 2007). In France, 35% of the soil surface is subjected to pesticide contamination, and fifteen pesticides are commonly quantified in soils and waters. They mostly correspond to substances which have been banned since the 2000s, or to their degradation products and metabolites (Dubois and Lacouture, 2011).

Atrazine and glyphosate have been among the most widely used agricultural pesticides (Zeljezic et al., 2006; Mañas et al., 2009a). With nearly 650 000 tons annually widespread, glyphosate is the best-selling chemical pesticide in the world. It is also the top ranked herbicide in the European Union, as it has been used in 40% of agricultural land (Zhang et al., 2011). Atrazine is the most popular triazine pesticide worldwide (Pathak and Dikshit, 2012). In the early 90s, it was spread on approximately 67% of all corn acreage, 65% of sorghum acreage, and 90% of sugar-cane acreage. Today,

* Corresponding author. Tel.: +33 4 91 83 56 91; fax: +33 4 86 13 68 33.
E-mail addresses: audrey.roustan@laposte.net (A. Roustan), ayebaratier.melanie@yahoo.fr (M. Aye), michel.de-meo@univ-amu.fr (M. De Meo), carole.di-giorgio@univ-amu.fr (C. Di Giorgio).

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glyphosate, atrazine and their main breakdown products aminomethyl phosphoric acid (AMPA) and desethyl-atrazine (DEA), are responsible for an almost universal contamination of surface water and groundwater (Dubois and Lacouture, 2011).

Because of their intrinsic toxicity and their persistent nature, pesticides have greatly contributed to degrade ecosystems (Joy et al., 2005). As a consequence, their residual presence in plants and animals has resulted in a global contamination of food, which represents an important health risk to human populations. Indeed, occupational exposure to pesticides has led to a significantly increased risk of degenerative diseases such as neoplasia (Bolognesi et al., 2009; Barry et al., 2011) and neuro-endocrine disruptions (Cooper et al., 2007). Moreover, environmental exposure to pesticides has been suspected to generate long-term transmissible effects, since recent studies have demonstrated that some of them could interfere with DNA metabolism (Mineau, 2005). Concerning glyphosate and atrazine, several experiments have attempted to assess and quantify their genotoxic/mutagenic properties. In spite of conflicting results mostly due to the different methodologies used, some studies have established their genotoxic potential. Glyphosate has been shown to induce in vitro increases of sister chromatid exchanges, chromosome aberrations (Lioi et al., 1998a; Lioi et al., 1998b) and DNA lesions in human cells (Monroy et al., 2005; Mladinic et al., 2009; Mañas et al., 2009a). It has also produced in vivo genotoxic and clastogenic damages (Prasad et al., 2009; Mañas et al., 2009a). Atrazine has been classified as "possibly carcinogenic to human" (Group 2B) by the International Agency for Research on Cancer (1999). It has been shown to induce both in vitro and in vivo cytogenetic damages (Biradar and Rayburn, 1995; Gebel et al., 1997). On the contrary, very few studies have been conducted on their main degradation products AMPA and DEA. Moreover, most of the assays have been performed in laboratory conditions, which considerably differed from those observed in the environment. In particular, the possible photo-inducible properties of such compounds have certainly been underestimated, as well as the possible enhanced effects of pesticide mixtures.

In the present study, the in vitro micronucleus assay was used to evaluate the cytogenetic toxicity of glyphosate and atrazine, and their degradation products AMPA and DEA. Then, to mimic the environmental conditions that may occur in soils or waters, different pesticide mixtures were prepared with glyphosate, atrazine, AMPA and DEA, and assessed for their cytogenetic toxicity. For each pesticide or pesticide mixture, assays were performed in the dark with and without metabolic activation, and with sunlight irradiation. Intracellular ROS amounts were also analyzed in the dark to evaluate oxidative stress.

2. Materials and methods

2.1. Reagents and cell culture

Atrazine ($C_8H_{14}ClN_5$, CAS No. 1912-24-9), glyphosate (N-(phosphonométhyl)glycine, $C_3H_8NO_5P$, CAS No. 1071-83-6), DEA (desethyl-atrazine, 2-Amino-4-chloro-6-isopropylamino-1,3,5-triazine, $C_6H_{10}ClN_5$, CAS No. 6190-65-4) and AMPA (Aminomethyl phosphonic acid, $NH_2CH_2P(O)(OH)_2$, CAS No. 1066-51-9) were purchased from Sigma–Aldrich Chemical Company (St Quentin-Fallavier, France). Atrazine and DEA were dissolved into dimethyl sulfoxide DMSO (Sigma) while glyphosate and AMPA were dissolved in phosphate buffer saline PBS (Sigma).

To fully explore the combinatory effects of pesticides, all the possible combinations of the four pesticides were assessed. Six different mixtures of two pesticides were made: glyphosate/AMPA, glyphosate/atrazine, glyphosate/DEA, AMPA/atrazine, AMPA/DEA and atrazine/DEA. For each mixture, a stock solution containing 10 mg mL^{-1} of the sum of the different compounds (5 mg mL^{-1} of each pesticide) was obtained, and several dilutions of this mixture were performed. Four different mixtures of three pesticides were made: glyphosate/AMPA/atrazine, glyphosate/AMPA/DEA, glyphosate/atrazine/DEA and AMPA/atrazine/DEA. For each mixture, a stock solution containing 10 mg mL^{-1} of the sum of the different compounds (3.33 mg mL^{-1} of each pesticide) was obtained, and several dilutions of this mixture were performed. A mixture was made with the four pesticides: glyphosate/AMPA/atrazine/DEA. A stock solution containing 10 mg mL^{-1} of the sum of the different compounds (2.5 mg mL^{-1} of each pesticide) was obtained, and several dilutions of this mixture were performed.

Experiments were performed on the well-established Chinese Hamster Ovary cell line, CHO-K1 cells (ATCC-LGC Standards Sarl, Molsheim, France). This cell line is characterized by a good genetic stability and by a short generation time, it has been well validated for genetic toxicology assays (Westerink et al., 2011). CHO-K1 cells were maintained in McCoy's 5A medium (Sigma, St Quentin-Fallavier, France) supplemented with 10% bovine calf serum, 1 mM glutamine (1 mM, Eurobio, Paris, France), and 100 U mL^{-1}–10 μg mL^{-1} penicillin–streptomycin (Eurobio, Paris, France). They were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Metabolic activation mixture (S9 Mix)

To examine the possible clastogenic effects of pesticide metabolites resulting from the cytochrome-based P450 oxidation systems in mammalian livers, an exogenous activation system (S9 Mix) was used. This metabolic activation system (S9) was a 9000-g centrifuged supernatant of a 10% liver homogenate and was prepared from male Sprague–Dawley rats treated with a single injection of Aroclor 1254 (500 mg kg^{-1} body weight), 5 d before they were killed. The protein concentration in the S9 homogenate was 26 mg mL^{-1}. In the micronucleus assay, S9 Mix contained 10% S9, 5 mM glucose-6-phosphate, 4 mM NADP, 33 mM KCl and 8 mM $MgCl_2$ diluted in PBS.

2.3. Photoactivation

To reproduce the photo-inducible phenomenon experienced by exposition to pesticides in the environment, the micronucleus assay was performed with a photoactivation procedure. Irradiation of cell cultures was carried out 1 h after the addition of pesticides. Irradiation was performed with a solar simulator Suntest CPS+ (Atlas Material Testing Technology BV, Moussy le Neuf, France) apparatus equipped with a xenon arc lamp (1100 W), a special glass filter restricting transmission of light below 290 nm and a near IR-blocking filter. The irradiance for the photoactivation was fixed at 750 W m^{-2} throughout the experiments and the combined light dose was 4.5 J cm^{-2} for one minute irradiation (0.03 J cm^{-2} of UVB, 0.41 J cm^{-2} of UVA and 4.06 J cm^{-2} of visible light). This irradiation dose corresponded to 1–3 min period of solar exposure during a clear summer day in the United Kingdom (Diffey, 2002).

The temperature of the samples was kept at 4 °C using a water cooling circuit in the irradiation chamber. UVA-visible light (320–800 nm) was obtained using the solar ID65 filter plus a window glass filter.

2.4. Micronucleus assay

Micronuclei are defined as chromosome fragments or whole chromosomes that lag during cell division due to the lack of a centromere or to a defect in cytokinesis (Kirsch-Volders et al.,