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Assessment of DNA damage, cytotoxicity, and apoptosis in human hepatoma (HepG2) cells after flurochloridone herbicide exposure

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ABSTRACT

In vitro effects of flurochloridone (FLC) and its formulations Twin Pack Gold® [25% active ingredient (a.i.)] and Rainbow® (25% a.i.) were evaluated in HepG2 cells. Whereas cytokinesis-blocked micronucleus cytome (CBMN-cyt) and single-cell gel electrophoresis (SCGE) assays were employed for genotoxicity, MTT, neutral red, and apoptosis detections were used for cytotoxicity evaluation. Activities were tested within the concentration range of 0.25–15 μg/ml FLC. Results demonstrated that neither FLC nor Rainbow® was able to induce MNs. On the other hand, 5 μg/ml Twin Pack Gold® only increased MN frequency. Furthermore, 10 and 15 μg/ml of both formulations resulted in cellular cytotoxicity demonstrated by alterations in the nuclear division index and cellular death. A marked increase in the genetic damage index was observed after treatment with all compounds. SCGE assay appeared to be more sensitive bioassay for detecting primary DNA strand breaks at lower concentrations of FLC than did MN. Our results reveal that FLC and its two formulations trigger apoptosis on HepG2 cells. The results represent the first experimental evidence of the *in vitro* apoptogenic role exerted on mammalian cells by FLC and the FLC-based formulations Rainbow® and Twin Pack Gold®, at least on HepG2 cells.

1. Introduction

Flurochloridone (FLC) is a pyrrolidone selective herbicide applied worldwide, including in Argentina, to control many broad-leaf weeds and annual grasses in several crops. FLC interferes with carotenoid biosynthesis, causing bleaching of the leaves (Klíčová et al., 2002; Lay et al., 1985). The exact mechanism(s) of action of the herbicide are not totally clarified. However, it has been recently demonstrated that FLC exposure caused important biochemical changes on leaves of *Vicia sativa*, increasing the activities of glutathione-*S*-transferases and the content of tripeptide glutathione and a reduction of glutathione reductase (Kaya and Yigit, 2012). Accordingly, the involvement of reactive oxygen species (ROS) in the mechanism(s) of action has been suggested (Kaya and Yigit, 2012).

According the European Food Safety Authority, FLC has no genotoxic, carcinogenic or neurotoxic potential, and it is unlikely to be genotoxic (EFSA, 2013). However, while considerable information is accessible about the environmental and ecological effects of FLC (EFSA, 2013; WHO, 1990, 2009), scarce information on genotoxicity and/or cytotoxicity has been reported. Yüzbasioglu et al. (2003) reported previously that FLC induced abnormal cell-cycle progression and cellular mitodepressive activity in *Allium cepa* root meristematic cells. c-Metaphase, multipolarity, polyploidy, chromosome lagging, chromosomal stickiness, chromosome breaks, bridges, fragments, sister union, and micronucleus (MN) were the most frequently observed alterations after herbicide exposure (Yüzbasioglu et al., 2003). Recently, we reported the genotoxic and cytotoxic effects of the pure herbicide and its formulations Twin Pack Gold® and Rainbow® employing several end points in hamster CHO-K1 cells (Nikoloff et al., 2012b). For all compounds, our observations revealed a significant increase in sister chromatid exchange frequencies, a delay in cell-cycle progression, alterations in mitotic activity, as well as cellular growth inhibition, measured by a reduction in mitochondrial activity after 24 h of continuous exposure (Nikoloff et al., 2012b). Furthermore, by using the same *in vitro* cellular system, we recently demonstrated the ability of FLC to induce DNA single-strand breaks measured by the comet assay (Nikoloff et al., 2012b).

Induction of DNA single-strand breaks and DNA macrolesions evaluated by single-cell gel electrophoresis (SCGE) and cytokinesis-blocked micronucleus cytome (CBMN-cyt) bioassays,

Abbreviations: CP, cyclophosphamide; CBMN-cyt, cytokinesis-blocked micronucleus cytome assay; ETOH, ethanol; FLC, flurochloridone; GDI, genetic damage index; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; NDI, nuclear division index; MN, micronucleus; NR, neutral red; SCGE, single cell gel electrophoresis assay.

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respectively, are widely used end points to identify in different eukaryotic cells the genotoxic properties of a variety of xenobiotics, including agrochemicals (Ali et al., 2011; Fenech, 2007; González et al., 2003, 2007; Molinari et al., 2009; Nikoloff et al., 2012b). Furthermore, the MN test is a required end point by regulatory agencies and has emerged as one of the preferred methods for the assessment of both clastogenic and aneugenic damage as well as a valid alternative methodology for chromosomal aberration analysis (ICH, 2011; OECD, 1997, 2007).

However, the above-mentioned assays cannot give an indication of the mechanism of cytotoxicity. It is well known that toxic effects of environmental chemicals can lead to passive cell death or necrosis, or otherwise result in the active mechanism via apoptotic cell signaling. Apoptotic cell death can be induced by a variety of stimuli, e.g., ligation of cell surface receptors, starvation, growth factor/survival factor deprivation, heat shock, hypoxia, or DNA damage (Circu and Aw, 2010). The dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions, such as immunodeficiency, autoimmune diseases, neurodegenerative diseases, and cancer, among others (Circu and Aw, 2010).

One of the major problems of *in vitro* genotoxicity and cytotoxicity bioassays is the lack of a drug-metabolizing system in the cell lines currently used for routine testing of xenobiotics. Besides, exogenous activation mixtures isolated from rodent livers only partly mimic the biotransformation of a test compound in the *in vivo* condition. Several xenobiotics require metabolic activation to react with DNA; thus the use of *in vitro* cells possessing endogenous biotransforming activity can avoid the use of exogenous activation mixtures. Human hepatoma cell lines, HepG2 being the most promising cell line among them (Aden et al., 1979), appear to be a practical and suitable alternative for assessing genotoxicity. HepG2 cells are easy to handle and contain several enzymes responsible for the activation of various xenobiotics, including phase I and II enzymes (Knasmüller et al., 2004).

Since few studies have reported the effects of FLC on mammalian cells, the objective of this report is to investigate the genotoxicity and cytotoxicity exerted by the herbicide as an active ingredient and two of its commercial formulations most commonly used in Argentina, Rainbow® [25% active ingredient (a.i.)] and Twin Pack Gold® (25% a.i.) in HepG2 cells by the CBMN-cyt assay, SCGE, neutral red (NR), and MTT end points. Furthermore, the cellular response of HepG2 cells in terms of the induction of the apoptosis process by the herbicide was also assayed.

2. Materials and methods

2.1. Chemicals

Flurochloridone [3-chloro-4-(chloromethyl)-1-[3-(trifluoromethyl) phenyl]-2-pyrrolidone; CAS 61213-25-0] was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Twin Pack Gold® (25% a.i.) and Rainbow® (25% a.i.) were kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina) and Magan Argentina S.A. (Buenos Aires, Argentina), respectively. Acetone (ACTN) and hydrogen peroxide were purchased from Merck KGaA (Darmstadt, Germany). Cytochalasin B from *Dreschslera dematioidea* (CAS 14930-96-2), cyclophosphamide (CP; CAS 6055-19-2), ethanol (CAS 64-17-5), neutral red (CAS 553-24-2), MTT (CAS 57360-69-7), and propidium iodide (CAS 25535-16-4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC was purchased from Invitrogen Molecular Probes® (Carlsbad, CA, USA).

2.2. Cell cultures and herbicide treatment for the cytokinesis-blocked micronucleus cytome (CBMN-cyt) assay

HepG2 (HB-8065; American Type Culture Collection, Rockville, MD) cells were grown in MEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin (all from Gibco, Grand Island, NY) at 37 °C in a 5% CO_2 atmosphere. The MN assay was performed following the protocol of Chiang et al. (2011) with minor modifications. Experiments were set up with cultures at the log phase of growth. The cells were seeded onto precleaned 22 × 22 mm cover slips in 35 mm Petri dishes at a density of 1.5×10^5 cells in a final media volume of 3 ml per dish for 24 h. Cells were treated with FLC, Twin Pack Gold® and Rainbow® within the concentration range of 0.25–15 μg/ml. Prior to use, FLC was first dissolved in ACTN and then diluted in culture medium, whereas Twin Pack Gold® and Rainbow® were diluted directly in culture medium. Both compounds were diluted so that addition of 100 μl into 2.9 ml of cultures would allow herbicides to reach the required concentration ranges. Cells were cultured for 24 h as recommended previously (Chiang et al., 2011) and then washed twice with PBS, treated with cytochalasin B (3 μg/ml), and incubated at 37 °C in a 5% CO_2 atmosphere for an additional 16 h period until harvesting. The final solvent concentration was less than 1% for all treatments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (0.2 mg/ml CP) were run simultaneously with herbicide-treated cultures. None of the treatments produced pH changes in the culture medium (range, 7.2–7.4). Each experiment was repeated three times, and the cultures were performed in duplicate for each experimental and time point. The same batches of culture medium, serum, and reagents were used throughout the study. At the end of the culture period, the cells were treated with a cold hypotonic solution (KCl 0.075 M, 4 °C, 5 min), prefixed with methanol at −20 °C for 20 min, and fixed with methanol at −20 °C for 20 min. Afterward, slides were stained with 3% aqueous Giemsa solution for 10 min. The cover slips were air dried and then placed down onto precleaned slides using Depex mounting medium. For the MN assay, at least 1000 binucleated cells per experimental point from each experiment were blind-scored at 1000× magnification according to our previous report (González et al., 2011). The number of binucleated cells with zero, one, two, or three MNs was determined in binucleated cytokinesis-blocked cells following the examination criteria reported by Fenech (2007). A minimum of 500 viable cells per experimental point were scored to determine the percentage of cells with one, two, and three or more nuclei, and the nuclear division index (NDI) was calculated for each experimental point according to the method of Eastmond and Tucker (1989).

2.3. Cell cultures and herbicide treatment for SCGE assay

Prior to herbicide treatment, exponentially growing HepG2 cells were detached with a rubber policeman, centrifuged, and then resuspended in complete culture medium. Afterward, aliquots containing 2×10^5 cells/ml were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere in a final media volume of 1 ml containing the test compounds. All compounds were used at a final concentration between 1 and 15 μg/ml. The final solvent concentration was <1% for all the treatments in all experiments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (0.2 mg/ml CP) were run simultaneously with herbicide-treated cultures. None of the treatments produced pH changes in the culture medium (pH 7.2–7.4). The SCGE and cell viability assays were performed immediately after 2 h short treatment period. Each experiment was repeated three times, and the cultures were performed in duplicate for each experimental and time point. The SCGE assay was performed following the alkaline procedure described by Singh et al. (1988) with minor modifications as reported elsewhere (Nikoloff et al., 2012a). Analysis of the slides was performed under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and nonoverlapping cells. DNA damage was classified in four classes (I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavas and Konen, 2007) (Fig. 1). Results are expressed as the mean number of damaged nucleoids (sum of classes II, III, and IV) and the mean comet score for each treatment group. Additionally, a genetic damage index (GDI) was calculated for each test compound using the formula $GDI = [(I) + 2(II) + 3(III) + 4(IV)]/N_{(I–IV)}$, where I–IV represent the nucleoid type, and N_I–N_{IV} represent the total number of nucleoids scored (Pitarque et al., 1999).

2.4. Cell viability assay

At the end of the culture period, cell viability was determined using the ethidium bromide/acridine orange assay (McGahon et al., 1995). Briefly, 10 μl of a 1:1 freshly prepared mixture of ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml) was mixed with 10 μl of the cell suspension under study. Afterward, the cells were analyzed using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. Each experiment was repeated three times, and the cultures were performed in duplicate for each experimental and time point. The cell viability was monitored at 2 h. At least 500 cells were counted per experimental point, and results are expressed as the percentage of viable cells among all cells.

2.5. Neutral red and MTT assays

Briefly, 1×10^5 HepG2 cells/ml were cultured in MEM complete culture medium on 96-well microplates for 24 h. Afterward, the culture medium was removed, and the cells were treated with FLC, Twin Pack Gold®, or Rainbow® within the 0.25–15 μg/ml concentration range for 24 h. Five percent ethanol-treated and