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Comparative study of cytotoxic and genotoxic effects induced by herbicide *S*-metolachlor and its commercial formulation Twin Pack Gold® in human hepatoma (HepG2) cells

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ABSTRACT

The *in vitro* effects of *S*-metolachlor and its formulation Twin Pack Gold® (96% a.i.) were evaluated in human hepatoma (HepG2) cells. Cytokinesis-blocked micronucleus cytome (CBMN-cyt) and MTT assays as well as Neutral Red uptake were employed for genotoxicity and cytotoxicity evaluation. Activities were tested within the concentration range of 0.25–15 μg/ml *S*-metolachlor for 24 h of exposure. Both compounds rendered a minor reduction in the NDI although not reaching statistical significance. Results demonstrated that the *S*-metolachlor was not able to induce MNs. On the other hand, 0.5–6 μg/ml Twin Pack Gold® increased the frequency of MNs. When cytotoxicity was estimated, *S*-metolachlor was not able to induce either a reduction of lysosomal or mitochondrial activity. Contrarily, whereas 1–15 μg/ml Twin Pack Gold® induced a significant reduction of mitochondrial activity, all tested concentrations of the formulated product induced a significant decrease of lysosomal performance as a function of the concentration of the *S*-metolachlor-based formulation titrated into cultures. Genotoxicity and cytotoxicity differences obtained with pure *S*-metolachlor and the commercial *S*-metolachlor-based formulation indicate that the latter may contain additional unsafe xenobiotics and support the concept of the importance of evaluating not only the active principle but also the commercial formulation when estimating the real hazard from agrochemicals.

1. Introduction

Nowadays, nearly 25% of currently used pesticides are chiral, and this ratio is increasing with the introduction of more structure-complex pesticides (Ye et al., 2010).

S-metolachlor is one of the most intensively used chloroacetamide herbicides in agriculture for the control of preemergent annual grasses and many broadleaf weeds in a variety of crops, such as corn, sunflower, soybean, potato, and horticultural crops (CASAFE, 2011). *S*-Metolachlor is physically and chemically equivalent to metolachlor, but is more active at the site of action of susceptible plants (O'Connell et al., 1998). It has been reported the relationship between *S*-metolachlor and metolachlor showing that the use of *S*-metolachlor reduces the risk of contamination to workers, consumers, and the environment (O'Connell et al., 1998).

Toxicological information for *S*-metolachlor has been documented previously. It has been reported that the herbicide does not reveal genotoxic, carcinogenic, or neurotoxic potential in rodents (USEPA, 1997). In mutagenicity studies, *S*-metolachlor was neither mutagenic to microbial cells nor clastogenic in animals (USEPA, 1997). Similarly, there was no evidence of DNA damage/repair in the hepatocytes recovered from treated rats (USEPA, 1997). However, whereas low-dose *S*-metolachlor treatments induced a marked increase in hepatocyte proliferation, indicating that the herbicide reached the target organ, higher concentrations induced hepatotoxic but not genotoxic effects (USEPA, 1997). Tests for the herbicide were negative in rat hepatocytes when the *in vitro* mammalian cell gene mutation assay was employed (USEPA, 1997). When *in vivo* studies were performed, Liu et al. (2006) revealed that *S*-metolachlor in chronic studies was much less toxic to *Daphnia magna* than metolachlor. Pereira et al. (2009) demonstrated that the herbicide affected the respiratory activity of *Bacillus stearothermophilus* and the bioenergetics of rat liver

Abbreviations: CP, cyclophosphamide; CBMN-cyt, cytokinesis-blocked micronucleus cytome assay; ETOH, ethanol; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; NDI, nuclear division index; MN, micronucleus; NR, Neutral Red.

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mitochondria, although the authors suggested that the compound was safer than other pesticides they have previously studied. However, the toxicology information for *S*-metolachlor is incomplete, and its effects on nontarget organisms, including humans, have not yet been completely elucidated. Recent studies have revealed that the herbicide caused changes in the reproductive endocrinology of male rats (Mathias et al., 2012). Furthermore, *S*-metolachlor induced damage in the thymus, measured by thymic plaques in tadpoles of *Rana pipiens* (Hayes, 2006).

Genotoxicity and cytotoxicity assessment through the use of several end points are the most used tools to reveal whether a xenobiotic can jeopardize target and nontarget organisms. The micronucleus (MN) assay is considered a good indicator of genotoxicity and is now widely used for pesticide genotoxicity estimation (Fenech, 2000, 2007). Micronuclei are whole or partial chromosomes that have not been incorporated into the daughter nucleus following mitosis due to the chromosome breaking (clastogenic process) or mitotic spindle dysfunction (aneugenic process) (Fenech, 2000, 2007). The assay has proved to be useful for measuring DNA damage in *in vitro* and *in vivo* cells in the presence of genotoxic compounds (Ali et al., 2011; González et al., 2011; Nikoloff et al., 2012; Soloneski et al., 2008; Vera-Candioti et al., 2013).

Different cell types have been used to determine the genotoxicity and cytotoxicity of a large number of compounds, including agrochemicals (Elliott, 2005; González et al., 2011; Nikoloff et al., 2012; Soloneski et al., 2007). Since many xenobiotics require metabolic activation to react with DNA, the use of cells that possess endogenous biotransforming activity can reduce some of the problems associated with the use of exogenous activation mixtures such as S9 mix (Valentin-Severin et al., 2003). The human hepatoma (HepG2) cell line appears to be a practical alternative for assessing genotoxicity and cytotoxicity (Tuschl and Schwab, 2003). HepG2 cells are easy to handle and contain several phase I and phase II enzymes, which are necessary for the activation of various xenobiotics (Tuschl and Schwab, 2003).

Cytotoxicity assays are widely used in *in vitro* toxicological studies. The Neutral Red (NR) and MTT assays have been used extensively to study the toxic effects of chemicals on a variety of different cell types grown in monolayer cultures (Borenfreund and Puerner, 1985; Molinari et al., 2009; Papis et al., 2011). Both of them have been introduced as alternative cell viability indicators and used to estimate the basal cytotoxicity of chemicals on cultured cells (Mazzotti et al., 2001). The NR is a quantitative colorimetric bioassay based on the ability of viable cells to incorporate and bind NR neutral red within lysosomes. NR is a weak cationic dye that readily penetrates the cell membrane and accumulates intracellularly in lysosomes, where it binds with anionic sites to the lysosomal matrix. Changes of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible (Borenfreund and Puerner, 1985). MTT assay is a sensitive quantitative colorimetric methodology measuring the reduction of a yellow tetrazolium dye MTT that is converted into formazan, a violet compound, by the activity of the enzyme succinate dehydrogenase present in the inner mitochondrial membrane of mammalian cells. Since the reduction of MTT to formazan takes place in metabolically active cells, the amount of formazan produced is directly correlated with the number of viable cells (Kosmider et al., 2004).

The aim of this study was to evaluate the genotoxicity and cytotoxicity exerted by the herbicide *S*-metolachlor as an active ingredient and one of its commercial formulations, Twin Pack Gold (96% a.i.), on HepG2 cells. In this report, we employ the cytokinesis-blocked micronucleus cytome (CBMN-cyt) assay, MTT and NR uptake as end points to analyze the effect exerted by the herbicide on malignant human liver cells.

2. Materials and methods

2.1. Chemicals

S-Metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)-2-methoxy-1-methylethyl]acetamide; CAS 87392-12-9] was obtained from Sigma Chemical Co. (St. Louis, MO). Twin Pack Gold® (96% *S*-metolachlor; excipient c.s.) was kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina). Cytochalasin B from *Dreschslera dematioidea* (CAS 14930-96-2), cyclophosphamide (CAS 6055-19-2), ethanol (CAS 64-17-5), Neutral Red (CAS 553-24-2), and MTT (CAS 57360-69-7) were purchased from Sigma Chemical Co. (St. Louis, MO).

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

HepG2 cells (HB-8065, American Type Culture Collection, Rockville, MD) 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 CBMN-cyt assay was performed following the protocol of Chiang (Chiang et al., 2011) with minor modifications. Experiments were set up with cultures at the log phase of growth. Cells were seeded onto precleaned 22 × 22 mm coverslips 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. Afterwards, cells were treated with *S*-metolachlor and Twin Pack Gold® within the concentration range of 0.25–6 μg/ml. Prior to use, *S*-metolachlor and Twin Pack Gold® were diluted directly in culture medium. Both compounds were diluted so that addition of 100 μl into 2.9 ml of culture would allow herbicides to reach the required concentration ranges. Cells were cultured for 24 h, 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, as recommended previously (Chiang et al., 2011). Negative controls (untreated cells) and positive controls (0.2 mg/ml cyclophosphamide) 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 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, cells were treated with a cold hypotonic solution (0.075 m KCl, 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 coverslips were air dried and then placed down onto precleaned slides using Depex mounting medium. For the MN analysis, 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; Nikoloff et al., 2012). 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. Neutral Red (NR) assay

The NR assay is based on the protocol described by Borenfreund and Puerner (1985). 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 *S*-metolachlor and Twin Pack Gold® within the 0.25–15 μg/ml concentration range for 24 h. Five percent ethanol-treated cells were used as positive controls. Following exposure with test compounds, cells were incubated for an additional 3 h period in the presence of 100 μg/ml NR dye dissolved in saline buffered phosphate solution (PBS). Then the cells were washed with PBS, and the dye was extracted in each well. Absorbance at 550 nm was measured with a microplate spectrophotometer (Sunrise Absorbance Reader, Tecan Austria GmbH, Salzburg, Austria). Results are expressed as the mean percentage of cell viability from three independent experiments performed in parallel.

2.4. MTT assay

The procedure was performed following the techniques described by Kosmider et al. (2004) with slight modification. Briefly, HepG2 cells (10^5 cells/ml) were seeded in MEM complete culture medium for 24 h on 96-well microplates. Afterward, the culture medium was removed, and the cells were treated with *S*-metolachlor and Twin Pack Gold® within a range of 0.25–15 μg/ml for 24 h. Five percent ethanol-treated cells were used as positive controls. Following exposure with tested compounds, 20 μl of MTT was added for an additional 3 h period. Then the formazan crystals were dissolved in 100 μl of DMSO. Absorbance at 550 nm was measured with a microplate spectrophotometer (Sunrise Absorbance Reader, Tecan Austria GmbH, Salzburg, Austria). Results are expressed as the mean percentage of cell viability from three independent experiments performed in parallel.

2.5. Statistical analysis

The data were analyzed using Statgraphics 5.1 Plus software. Differences between MN and NDI in HepG2-treated and control culture data were evaluated by χ^2 test. The two-tailed Student's t test was used to compare MTT and NR data between treated and control groups. To check for a concentration-dependent response to the treatments, Spearman's rank order linear correlation analysis was also performed. The chosen level of significance was 0.05, unless indicated otherwise.

3. Results

The NDI data from *S*-metolachlor- and Twin Pack Gold®-cytokinesis-blocked HepG2 cells are presented in Fig. 1. Overall, both compounds rendered a minor reduction in the NDI although not reaching statistical significance ($p > 0.05$). Fig. 1 also shows the results of the analysis of herbicide-induced MNs in binucleated cytokinesis-blocked HepG2 cells. An increased frequency of MNs was observed in those cyclophosphamide-treated cultures (positive control) compared to control values ($p < 0.001$). The frequency of MNs was not significantly increased when HepG2 cells were exposed to *S*-metolachlor, regardless of the herbicide concentration titrated into cultures ($p > 0.05$). On the other hand, Twin Pack Gold® increased the frequency of MNs compared to control cultures in those 0.5–6 μg/ml-treated cells ($0.05 > p < 0.001$) (Fig. 1). A regression analysis showed a concentration-dependent increase in the frequency of Twin Pack Gold® -induced MNs ($r = 0.91$, $p < 0.01$).

Data obtained from the alteration of lysosomal activity performed with the NR assay are presented in Fig. 2. Ethanol-treated cultures (positive controls) produced a statistically significant toxicity in HepG2 cells compared to negative controls ($p < 0.001$). Results revealed a significant cell growth inhibition when HepG2 cells were exposed to 0.25–15 μg/ml Twin Pack Gold® ($p < 0.001$). No statistical alteration in the lysosomal activity was observed when HepG2 cells were exposed to all assayed concentrations of *S*-metolachlor ($p > 0.05$). A regression analysis showed that lysosomal activity decreased as a function of the concentration of Twin Pack Gold® titrated into cultures ($r = -0.76$, $p < 0.001$). Overall, the NR assay demonstrated that Twin Pack Gold® exerted a higher cytotoxic effect than *S*-metolachlor did.

Data of the alterations in the energetic cell metabolism induced by *S*-metolachlor and Twin Pack Gold® are depicted in Fig. 3. Ethanol-treated cultures (positive controls) exerted a statistically significant toxicity enhancement in HepG2 cells compared with negative controls ($p < 0.001$). Fig. 3 shows that cellular metabolism was not altered by *S*-metolachlor treatments regardless of the concentration ($p > 0.05$). On the other hand, an inhibition was achieved when HepG2 cells were exposed to 1–15 μg/ml Twin Pack Gold® ($0.05 > p < 0.001$). A regression analysis showed that the inhibition increased as a function of the concentration of Twin Pack Gold® ($r = -0.94$, $p < 0.001$). Overall, the MTT assay demonstrated that Twin Pack Gold® exerted a higher cytotoxic effect than S-metolachlor did in HepG2 cells.

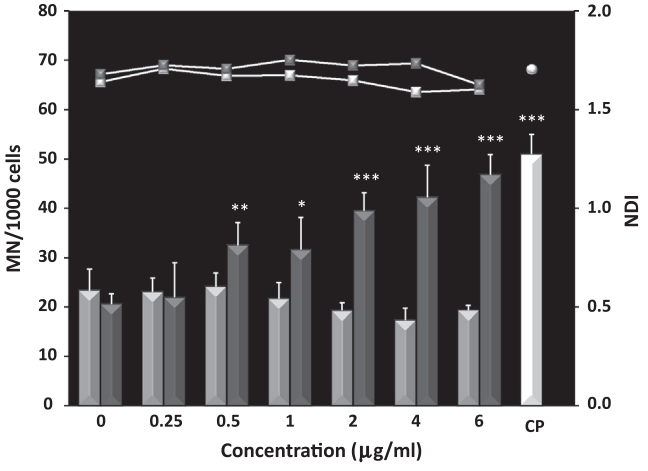

Fig. 1. Effect of *S*-metolachlor (light grey) and the *S*-metolachlor-based formulation Twin Pack Gold® (dark grey) on MN induction (bars) and NDI (squares) in binucleated cytokinesis-blocked HepG2 cells. Results are presented as the mean number of MNs per 1000 binucleated cytokinesis-blocked cells from pooled data from three independent experiments ± SE of the mean. Cyclophosphamide (0.2 mg/ml; CP, white bar, white circle) was used as a positive control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values.

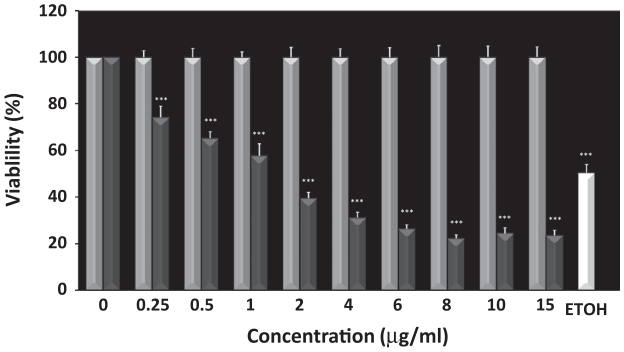

Fig. 2. Lysosomal activity assessed with the NR assay in HepG2 cells treated with *S*-metolachlor (light grey bars) and the *S*-metolachlor-based formulation Twin Pack Gold® (dark grey bars). Cultures were incubated for 3 h with NR dye after 24 h of herbicide treatment. Results are expressed as the mean percentage of cell viability from three independent experiments performed in parallel (*y*-axis) and plotted against the herbicide concentration (0–15 μg/ml concentration range; *x*-axis). Five percent ethanol-treated (white bar) cells were used as positive controls. $^{***}p < 0.001$, significant differences with respect to control values.

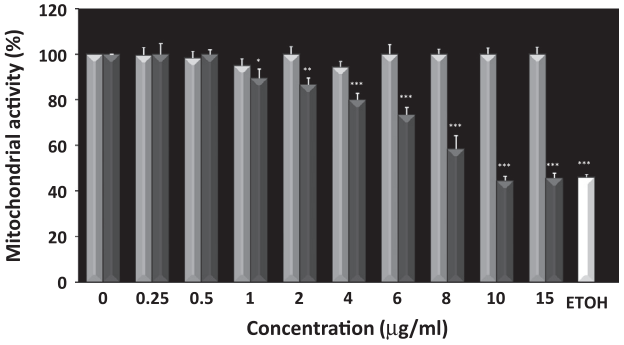

Fig. 3. Cellular metabolism inhibition evaluated by MTT assay in HepG2 cells treated with *S*-metolachlor (light grey bars) and the *S*-metolachlor-based formulation Twin Pack Gold® (dark grey bars). Cultures were incubated for 3 h with MTT after 24 h of herbicide treatment. Results are expressed as the mean percentage of cell viability from three independent experiments performed in parallel (*y*-axis) and plotted against the herbicide concentration (0–15 μg/ml concentration range; *x*-axis). Five percent ethanol-treated (white bar) cells were used as positive controls. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values.

4. Discussion

In the present report, the genotoxicity and cytotoxicity of the herbicide *S*-metolachlor and the *S*-metolachlor-containing technical formulation Twin Pack Gold® were evaluated *in vitro* in HepG2 cells by analyzing different end points, i.e., MN induction, NDI, and MTT and NR uptake. The results demonstrated that both compounds rendered a minor reduction in the NDI although not reaching statistical significance. They also revealed that *S*-metolachlor was not able to induce MNs. On the other hand, a concentration range of 0.5–6 μg/ml of Twin Pack Gold® increased the frequency of MNs. Furthermore, the pure ingredient was not able to induce either a reduction of lysosomal or mitochondrial activity.

Contrarily, whereas 1–15 μg/ml Twin Pack Gold® induced a significant reduction of mitochondrial activity, all tested concentrations of the formulated product induced a significant decrease of lysosomal performance. Therefore, the results demonstrated that all *in vitro* bioassays were sensitive enough to detect both genotoxic and cytotoxic properties exerted by the commercial formulated product, namely, Twin Pack Gold®, at least on HepG2 cells.

Although numerous studies that assess the genotoxicity and cytotoxicity of the racemic form of metolachlor have been reported (Clements et al., 1997; Greenlae et al., 2004; Grisolia and Ferrari, 1997; Hill et al., 1997; Roloff et al., 1992), accessible information on the genotoxic and cytotoxic properties of *S*-metolachlor is scarce. To date, a single report is available about pure herbicide *S*-metolachlor-induced genotoxicity and embryotoxicity. Mai et al. (2012) recently reported that DNA damage and embryotoxic effects were significantly induced in Pacific oyster embryos (*Crassostrea gigas*) following 24 h exposure to 0.01–10 μg/l pure *S*-metolachlor. According to this study, within the dose range assayed, there was a dose-dependent increase of DNA damage, estimated by comet assay in embryo cells of treated oysters, in tail DNA from 12.1% to 18.3%. Furthermore, an increase in abnormal D-shell larvae, measured as D-shell larvae presenting mantle and/or shell abnormalities, was also observed (Mai et al., 2012). On the other hand, our current results highlight that *S*-metolachlor is unable to induce genotoxicity as reported by the U.S. EPA (1997), at least when the MN assay is employed as a cytogenetic end point. The apparent lack of sensitivity of the MN assay to the genotoxicity of the herbicide *S*-metolachlor compared to the comet assay might be attributable to the generation of a particular type of damage. Comparative investigations between comet and MN assays employing several compounds have been performed previously, showing that the MN technique seems to be less sensitive than single cell gel electrophoresis assay for assessing DNA damage potential (Goethem et al., 1997; He et al., 2000; Nikoloff et al., 2012; Tafazoli and Volders, 1996). Nevertheless, it should be taken into account that the difference in the sensitivity of these two end points may be attributable to the type of *S*-metolachlor-induced lesions differentially estimated by these assays. Whereas the comet assay can mostly detect single-strand DNA breaks and alkaline labile sites, the MN assay detects those lesions arising from chromatid or chromosomal fragments induced by clastogens and/or vagrant chromosomes at anaphase produced by aneugens (OECD, 1997). It is a valuable and sensitive technique characterized by restricting the analysis to cells that have passed through their first division cell cycle (Fenech, 2007). Accordingly, it could be suggested that *S*-metolachlor can only induce single-strand DNA breaks but not any other type of macrolessions leading to MN production in mammalian cells *in vitro*, at least when HepG2 cells are employed as the target. Furthermore, the possibility that HepG2 cells are able to repair the damage induced by *S*-metolachlor cannot be dismissed. Similarly, the possibility that the most severely damaged cells are unable to proliferate after injury and enter into mitosis could be ruled out, since no concentration of *S*-metolachlor induced cellular growth inhibition in HepG2 cultures, at least when estimated by the CBMN-cyt bioassay.

In the present study, S-metolachlor showed neither clastogenic nor cytotoxic effects on HepG2 cells cultures during a 24 h exposure. However, the formulated product Twin Pack Gold® increased the MN frequency in micronucleated cells when concentrations within the 0.5–6 μg/ml range were tested. In addition, cytotoxicity was also induced in HepG2 cells after treatment the *S*-metolachlor-based formulation. A loss of lysosomal activity, indicated by a significant decrease in the uptake of NR, was observed when HepG2 cells were exposed to 0.25–15 μg/ml Twin Pack Gold®. Furthermore, alteration in energetic cell metabolism induced by Twin Pack Gold®, measured by the ability of mitochondrial succinic dehydrogenase, was further analyzed by the MTT assay. The results obtained showed a marked decrease in MTT dye reduction to an insoluble violet formazan product at 1–15 μg/ml Twin Pack Gold®. Accordingly, our findings reveal the ability of the *S*-metolachlor-based formulation Twin Pack Gold® to enhance cytotoxicity in the HepG2 cell line, as demonstrated by the NR and the MTT bioassays.

It is well known that pesticides are usually applied in their formulated forms, where the active ingredient is combined with organic solvents, emulsifying and wetting agents, which affect the pesticide penetration and performance (WHO, 1990). The additives may synergize or antagonize the toxicity of the active ingredient. Consequently, the WHO emphasized the necessity of evaluating the toxic hazards of formulated pesticides (WHO, 1990). Although additive compounds frequently comprise part of a commercial pesticide formulation, they are not usually included in any discussion of the effects on living cells, and their adverse effects may exceed those of the active ingredients. Although pesticides are developed through very strict regulation processes to function with reasonable certainty and minimal impact on human health and the environment, serious concerns have been raised about health risks resulting from occupational exposure and from residues in food and drinking water (WHO, 1990). Several investigations have demonstrated that the additive compounds present in pesticide commercial formulations have the ability to induce cellular damage by themselves, separate from the active ingredient (Cox and Surgan, 2006; González et al., 2006, 2007; Kaya et al., 1999; Lin and Garry, 2000; Mann and Bidwell, 1999; Molinari et al., 2009, 2010; Rayburn et al., 2005; Soloneski et al., 2001, 2002, 2003, 2007, 2008; Soloneski and Larramendy, 2010; Sorensen et al., 2003; Zeljezic et al., 2006). Unfortunately, the identities of the additive compounds present in the commercial formulation Twin Pack Gold® were not made available to us by the manufacturers. It should be mentioned that according to our Argentinean administration, the excipients present in any agrochemical are not required to be listed on the agrochemical data sheet and can be kept as a "trade secret." Years ago, USEPA (1982) claimed that the acute toxicity of a technical active ingredient can differ significantly from that of the end-use formulation containing that active ingredient. Our results are in total agreement with this concept.

Our current observations clearly demonstrate that the MN induction as well as cytotoxic effects exerted by Twin Pack Gold® are most probably due to deleterious effect(s) induced by xenobiotics included in the excipient of the *S*-metolachlor-based herbicide, with marked genotoxic as well as cytotoxic properties.

Conflict of Interest

The authors declare no conflict of interests.

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