Toxicology in Vitro 29 (2015) 834–844

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Carbamates: A study on genotoxic, cytotoxic, and apoptotic effects induced in Chinese hamster ovary (CHO-K1) cells

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ARTICLE INFO

Article history:
Received 14 November 2014
Accepted 17 March 2015
Available online 24 March 2015

Keywords:
Apoptosis
Carbamates
CBMN-cyt assay
CHO-K1 cells
Neutral red
MTT assays

ABSTRACT

In vitro effects of the carbamates pirimicarb and zineb and their formulations Aficida® (50% pirimicarb) and Azzurro® (70% zineb), respectively, were evaluated in Chinese hamster ovary (CHO-K1) cells. Whereas the cytokinesis-blocked micronucleus cytome assay was employed to test for genotoxicity, MTT, neutral red (NR), and apoptosis evaluation were used as tests for estimating cell viability and succinic dehydrogenase activity, respectively. Concentrations tested were 10–300 μg/ml for pirimicarb and Aficida®, and 1–50 μg/ml for zineb and Azzurro®. All compounds were able to increase the frequency of micronuclei. A marked reduction in the nuclear division index was observed after treatment with 5 μg/ml of zineb and Azzurro® and 10 μg/ml of Azzurro®. Alterations in the cellular morphology not allowing the recognition of binucleated cells exposed to 300 μg/ml pirimicarb and Aficida® as well as 10–50 μg/ml zineb and Azzurro®. All four compounds induced inhibition of both cell viability and succinic dehydrogenase activity and trigger apoptosis in CHO-K1 cells, at least when exposed for 24 h. The data herein demonstrate the genotoxic and cytotoxic effects exerted by these carbamates and reveal the potential risk factor of these pesticides, still extensively used worldwide, for both human health and the environment.

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1. Introduction

Pesticides are chemicals of fundamental importance in the fight against diseases, widely used for pest control in agriculture, industrial farming, gardening, homes, and soil treatments. Although attempts to decrease pesticide use through organic agricultural practices and the other alternative technologies to control pests are very well known (Larramendy and Soloneski, 2011), at present, continued exposure to pesticides via a number of routes, e.g., occupational exposure, home and garden use, spray drifts, and residues in household dust, food, soil, and drinking water, among others, remains a serious problem worldwide (Fenner-Crisp, 2001). Furthermore, several reports have demonstrated that when grain and crops are grown in the presence of pesticides and then employed to feed livestock, pesticide residues can accumulate in the animals' fatty tissue and milk (Ciscato et al., 2002; Nag and Raikwar, 2011). Risk assessment plays a crucial role in the process of taking decisions about the use and control of pesticides, both new and existing (USEPA, 2004). Accumulating experience suggests that postmarket epidemiological surveillance of pesticide safety is essential to ensure public health and the quality of our environment (USEPA, 2004). Epidemiological studies suggested that pesticides currently on the market may cause cancer in nontarget species, including humans, and that a lot of occupational and agricultural workers worldwide experience unintentional pesticide poisoning each year (Alavanja et al., 2005; IARC, 2003). In addition to causing environmental damage, pesticide exposure frequently affects wild nontarget species because they possess physiological or biochemical similarity to the target organisms (Lee et al., 2004).

Carbamates are chemicals extensively applied in modern agriculture throughout the world as insecticides, fungicides, herbicides, nematicides, and/or sprout inhibitors (USEPA, 2004). These chemicals are part of a large group of synthetic pesticides that have been developed, produced, and used on a large scale within the last 50 years. Additionally, they are used as biocides for industrial and other applications as surface sprays or as baits in garden and home products for the control of household pests (IARC, 1976). Thus,

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http://dx.doi.org/10.1016/j.tiv.2015.03.011
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carbamates are potentially harmful to the health of different kinds of organisms (USEPA, 2004). They are toxicants that are easily absorbed and tend to accumulate in soil, plants, food, and ground and surface waters, and some of them have clear genotoxic properties (USEPA, 2004). Among carbamates, members differ in their spectrum of activity, their range of toxicity (from low to moderate), and their degree of persistence in different environmental matrices (IARC, 1976). Among all classes of pesticides, carbamates are the most commonly used compounds because organophosphates and organochlorines are extremely toxic and have delayed neurotoxic effects (Hour et al., 1998). Like the organophosphates, their mode of action is the inhibition of cholinesterase enzymes, affecting nerve impulse transmission, and exposure can occur by several routes in the same individual due to their multiple uses (USEPA, 2004). Additionally, carbamates are relatively unstable compounds that break down in the environment within weeks or months (IARC, 1976).

Pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate) is a dimethylcarbamate insecticide member with both contact and systemic activity, and is widely used against aphids in agriculture and fruit growing (USEPA, 1974b). Based on its acute toxicity, it has been classified as moderately hazardous (Class II) by the World Health Organization (http://www.who.int/ipcs/publications/pesticides hazard/en/), and slightly to moderately toxic (Category II–III) by U.S. EPA (1974a). However, the exact mechanism of pirimicarb-induced DNA damage has not been elucidated so far.

Zineb [ethylene bis(dithiocarbamate) zinc] is a widely employed foliar dithiocarbamate fungicide with primarily agricultural and industrial applications (USEPA, 1996). Although zineb has mainly been registered for use on a large number of fruit, vegetable, and field crops, ornamental plants, and for the treatment of seeds, it has also been registered for use as a fungicide in paints and for mold control on fabrics, leather, linen, painted surfaces, surfaces to be painted, and paper, plastic, and wood surfaces (USEPA, 1996). Zineb has been classified as a practically nontoxic compound (Class IV) by the U.S. EPA (2006) based on its potency by oral and inhalation exposure routes. The available data on the deleterious effects of zineb do not allow a definitive evaluation of its carcinogenic potential, and it has been not classified as a carcinogenic agent for humans (Category III) by the IARC (1976). It has been reported previously that the mechanism of action of several ethylenebisdithiocarbamate fungicides like mancozeb, maneb, and zineb is related to the formation of cyanide, which reacts with thiol compounds within cells (Hayes, 2007; USEPA, 1996).

Both pirimicarb and zineb have been evaluated using *in vitro* and *in vivo* mutagenicity, cytotoxicity, and genotoxicity assays (USEPA, 1974a,b, 1996). They have been generally recognized as nonmutagenic in bacteria, yeast, and fungi, as well as in mammalian cells (IARC, 1976; USEPA, 1974b). When the induction of chromosomal damage was evaluated on pirimicarb-exposed mammalian cells *in vitro* and *in vivo*, negative results were reported in rat bone marrow cells (Jones and Howard, 1989) as well as in human lymphocytes with or without S9 metabolic activation (Wildgoose et al., 1987). However, positive results were obtained when the chromosomal aberrations and sister chromatid exchange (SCE) bioassays were performed on Chinese hamster ovary (CHO-K1) cells (Soloneski and Larramendy, 2010). In *in vivo* systems, pirimicarb did not induce chromosomal alterations in bone marrow cells of Wistar male rats after oral administration (Anderson et al., 1980). Contrarily, a significant increase of chromosomal aberrations in peripheral blood lymphocytes from occupational workers was observed after pirimicarb exposure (Pilinskaia, 1982). Finally, when the micronucleus (MN) induction end point was employed, positive results were reported in *in vivo* erythrocytes from the fish *Cnesterodon decemmaculatus* and *Rhinella arenarum* anuran tadpoles by Vera Candioti et al. (2010a,b).

Effects at the chromosomal level following exposure to zineb revealed its genotoxic potential, through increases in the frequency of chromosomal aberrations, SCEs, and MNs in human lymphocytes and CHO cells (Soloneski et al., 2001, 2002a,b). Not only are zineb as well as the zineb-containing technical formulation Azzurro® able to induce MNs in human lymphocytes *in vitro*, but such induction is also restricted to B $CD20^+$ and T suppressor/cytotoxic $CD8^+$ cell subsets (Soloneski et al., 2002b). Besides, Enninga (1986) showed that zineb induced structural chromosomal aberrations in CHO cells, both with and without the S9 microsomal fraction. Similar responses were obtained in *in vivo* studies. Positive results were reported for lymphocytes from workers occupationally exposed to zineb, in which an increased frequency of structural chromosome aberrations was observed (Pilinskaia, 1974). Finally, in contrast to these studies, it was reported that zineb did not induce MNs in bone marrow cells of Wistar male rats after oral administration (Huntingdon Research Centre, 1985).

Previous investigations demonstrated that the use of *in vitro* systems is a valuable method for evaluating the inherent genotoxicity and cytotoxicity after a xenobiotic exposure (Bolognesi, 2003; Bradley et al., 1981; Knasmüller et al., 2004). The use of *in vitro* systems in short toxicity studies provides the opportunity not only for extrapolation from *in vitro* to *in vivo* systems, but also to obtain information on biological responses at higher levels of biological organization (Kirsch-Volders et al., 2003). The use of the CHO-K1 cell line is highly recommended by the OECD for *in vitro* genotoxicity tests of numerous xenobiotics (OECD, 2010). For genotoxicity screening, the cytokinesis-blocked micronucleus cytome (CBMN-cyt) assay in different eukaryotic cells is widely used in both molecular epidemiology and cytogenetics to evaluate the occurrence and the proportions of chromosomal damage after exposure to numerous xenobiotic agents, including agrochemicals (Ali et al., 2009; Fenech, 2008; González et al., 2003, 2007). MNs are whole or partial chromosomes that have not been incorporated into the daughter nucleus following mitosis due to the chromosome breaking (clastogenic) or mitotic spindle dysfunction (aneugenic) processes (Fenech, 2000, 2008). Furthermore, MN induction is an end point required by regulatory agencies, and the MN assay 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, 2007).

Cytotoxicity assays are widely used in *in vitro* studies, and they are useful for predicting acute toxicity. The neutral red (NR) uptake and tetrazolium salt (MTT) assays are two of the most frequently used methods for the preliminary screening of the cytotoxic effects of chemicals on a variety of different cell types grown in monolayer cultures (Borenfreund and Puerner, 1985; Molinari et al., 2009). 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). Apoptosis, or programmed cell death, occurs both in normal developmental processes as well as in disease, and it is the principal mechanism by which cells are physiologically eliminated in metazoan organisms (Elmore, 2007). Apoptosis is associated with programmed events, including morphological and biochemical changes, which are necessary for the differentiation and development of organs and organisms (Elmore, 2007). The death receptor-mediated pathway and the mitochondria-mediated pathway are the two major cellular apoptotic pathways (Elmore, 2007). Toxic effects of environmental pollutants can lead to passive cell death or necrosis, or result in the active mechanism of apoptosis (Circu and Aw, 2010). It is now

evident that many substances exert their toxicity via apoptotic cell signaling 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).

In this paper, we explore the effects of two carbamates, namely, pirimicarb and zineb, on mammalian CHO-K1 cells. The present experiments were designed to investigate comparatively the genotoxicity and cytotoxicity of the pure compounds and their commercial formulations most commonly used in Argentina, Aficida® (50% pirimicarb) and Azzurro® (70% zineb), by the CBMN-cyt, NR, and MTT assays. Additionally, the cellular responses to the toxic carbamate action were examined to monitor apoptotic and necrotic cellular status.

2. Materials and methods

2.1. Chemicals

Pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate, CAS 23103-98-2, purity 99.5%) and zineb [ethylene bis(dithiocarbamate) zinc; CAS 12122-67-7, purity 97.0%] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Aficida® (50% pirimicarb, excipients c.s.) and Azzurro® (70% zineb, excipients c.s.) were kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina) and Chemiplant (Buenos Aires, Argentina), respectively. Dimethyl sulfoxide (DMSO, CAS 67-68-5), cytochalasin B (Cyt-B) from *Dreschslera dematioidea* (CAS 14930-96-2), 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, USA). Annexin V-FITC and propidium iodide (PI) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Bleomycin (BLM; Blocamycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina). All other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co.

2.2. Cell cultures and carbamate treatment for the CBMN-cyt assay

CHO-K1 (American Type Culture Collection, Rockville, MD) cells were grown in Ham's F10 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin (all from Gibco, Grand Island, NY, USA) at 37 °C in a 5% CO_2 atmosphere. Experiments were set up with cultures at the log phase of growth. The cells were seeded onto precleaned 22 × 22 mm tested for cell culture cover slips (Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany) in 35 mm Petri dishes at a density of 1.2×10^4 cells/dish. Treatments with the test compounds were performed 24 h after plating as recommended previously (Nikoloff et al., 2014; Soloneski et al., 2002a; Soloneski and Larramendy, 2010). Prior to use, pure pirimicarb and zineb were first dissolved in DMSO and then diluted in culture medium, whereas Aficida® and Azzurro® were directly diluted in culture medium. Both compounds were diluted so that addition of 100 μl into 2.9 ml of culture would allow pesticides to reach the required concentration ranges. Cultures were treated within the range of 10–300 μg/ml for pirimicarb and its pirimicarb-based formulation Aficida® and within the range of 1–50 μg/ml for zineb and Azzurro®. The concentration ranges employed were selected according to our previous genotoxic reports (Soloneski et al., 2002a; Soloneski and Larramendy, 2010). The final solvent concentration was less than 1% for all treatments. Immediately after treatments, 3 μg/ml Cyt-B was incorporated into cultures, and then cells were incubated at 37 °C in a 5% CO_2 atmosphere under a safety light for an additional 24 h period until harvesting. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (BLM, 1.0 μg/ml) were run simultaneously with agrochemical-treated cultures. None of the treatments produced significant 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 CBMN-cyt assay, at least 1000 binucleated cells per experimental point from each experiment were scored blindly at 1000× magnification as previously reported (Nikoloff et al., 2014). 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).

2.3. Examination of slides and assessment of the nuclear division index

Slides were coded and scored blind by one researcher at 600× magnification. Examination of the slides was performed following the recommendations suggested by Fenech (2007). A minimum of 500 viable cells per experimental point was 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) using the formula $NDI = [(N_1) + 2(N_2) + 3(N_{3+})]/N$, where N_1–N_{3+} represent the number of cells with one to three or more nuclei, and N is the total number of viable cells scored.

2.4. Neutral red and MTT assays

Briefly, 1×10^5 CHO-K1 cells/ml were cultured in Ham's F10 complete culture medium on 96-well microplates for 24 h. Afterward, the culture medium was removed, and the cells were treated with pirimicarb and Aficida® (10–300 μg/ml concentration range) or zineb and Azzurro® (1–50 μg/ml concentration range) for 24 h. Five percent ethanol-treated and 0.075% DMSO-treated cells were used as positive and vehicle controls, respectively. The protocol described by Borenfreund and Puerner (1985) was employed for the NR assay. Following exposure with test compounds, cells were incubated for an additional 3 h period in the presence of 100 μg/ml neutral red dye dissolved in PBS. Then the cells were washed with PBS, and the dye was extracted in each well. The procedure of the MTT assay was performed following the techniques described by Miki et al. (1993). Following exposure to 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 540 nm was measured with a microplate spectrophotometer (Sunrise Absorbance Reader, Tecan Austria GmbH, Salzburg, Austria). Results were expressed as the mean percentage of cell viability and succinic dehydrogenase activity from three independent experiments performed in parallel.

2.5. Cell cultures and pesticide treatment for flow cytometry analysis

Briefly, CHO-K1 cells in the log phase of growth were seeded in 24-well microplates at a density of 3.5×10^4 cells/ml and treated for 24 h after plating to detect apoptotic and necrotic cells among the cell population. Pirimicarb and Aficida® were diluted so that the addition of up to 20 μl into cultures allowed the final concentrations of 100 and 200 μg/ml to be reached. Similarly, both zineb and Azzurro® were diluted so that the addition of up to 20 μl into cultures allowed the final concentrations of 10 and 15 μg/ml to be

Table 1
Micronucleus (MNs) induction and nuclear division index (NDI) values for control, pirimicarb-, and pirimicarb-based technical formulation Aficida®-treated binucleated cytokinesis-blocked Chinese hamster ovary (CHO-K1) cells.[a]

Concentration (μg/ml)	Pirimicarb					Aficida® (50% Pirimicarb)				
	MN frequencies[b]	Micronucleated cell distribution[c]			NDI[d]	MN frequencies[b]	Micronucleated cell distribution[c]			NDI[d]
		1 MN	2 MN	3 MN			1 MN	2 MN	3 MN	
0	29.0 ± 0.33	21.7 ± 0.51	2.7 ± 0.19	0.7 ± 0.00	2.13 ± 0.04	31.0 ± 0.84	20.0 ± 0.30	4.0 ± 0.51	1.0 ± 0.00	2.19 ± 0.03
10	48.5 ± 1.35***	31.0 ± 2.50*	5.3 ± 0.38	2.5 ± 0.19*	2.31 ± 0.14	42.3 ± 1.86*	28.7 ± 2.39	4.3 ± 0.19	1.7 ± 0.51	2.29 ± 0.02
50	49.0 ± 1.20***	30.7 ± 1.20*	4.7 ± 0.58	3.0 ± 0.33**	2.25 ± 0.06	41.7 ± 3.01*	26.7 ± 0.39	4.0 ± 0.33	2.3 ± 0.58	2.11 ± 0.04
100	51.0 ± 1.20***	38.7 ± 1.20***	3.7 ± 0.58	1.7 ± 0.33*	2.09 ± 0.02	42.0 ± 1.58*	27.7 ± 1.00	5.7 ± 0.69	1.0 ± 0.33	1.98 ± 0.02
200	62.7 ± 1.20***	41.3 ± 1.20***	6.7 ± 0.58***	2.7 ± 0.33*	2.05 ± 0.01	57.3 ± 1.58***	31.7 ± 1.00**	8.3 ± 0.69*	3.0 ± 0.33*	2.00 ± 0.02
300	ND		ND		ND	ND		ND		ND
BLM[e]	71.0 ± 1.20***	47.3 ± 1.20***	7.3 ± 0.58***	3.0 ± 0.33***	2.11 ± 0.06	71.0 ± 1.20***	47.3 ± 1.20***	7.3 ± 0.58***	3.0 ± 0.33***	2.11 ± 0.04

[*] $p < 0.05$ significant difference with respect to control values.
[**] $p < 0.01$ significant difference with respect to control values.
[***] $p < 0.001$ significant difference with respect to control values.
[a] CHO-K1 cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later.
[b] Results are presented as mean MNs/1000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.
[c] Results are presented as number of cells carrying 1, 2 or 3 MNs among 3000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.
[d] Results are presented as mean value of pooled data from three independent experiments ± S.E. of the mean.
[e] Bleomycin (BLM, 1.0 μg/ml) was used as positive control. ND, Not determined.

reached. The final solvent concentration was <1% in all experiments. Negative controls (untreated cells and DMSO solvent vehicle-treated cells) were run simultaneously with carbamate-treated cultures. None of the treatments produced pH changes in the culture medium (pH 7.2–7.4). For the experiments, the concentrations were selected according our previous cytotoxic results (Soloneski et al., 2002a; Soloneski and Larramendy, 2010). After an incubation period of 24 h (37 °C, 5% CO_2), cells were processed for flow cytometry using annexin V-FITC/PI double staining according to the manufacture's protocol. The treated cells were washed with PBS and resuspended in 100 μl of binding buffer, supplemented with 5 μl of FITC-annexin V and 5 μl of PI, and incubated for 15 min at room temperature in the dark. The cell populations were discriminated as viable (annexin V negative/PI negative), early apoptotic (annexin V positive/PI negative), late apoptotic (annexin V positive/PI positive), and necrotic (annexin V negative/PI positive). Flow cytometric analysis was performed with an Accuri C6 flow cytometer (BD Biosciences). Each experiment was repeated three times, and cultures were performed in duplicate for each experimental point. The results are expressed as the mean percentages of alive, early apoptotic, late apoptotic, and necrotic cells among all cells from three independent experiments performed in parallel.

2.6. Statistical analysis

The data were analyzed using Statgraphics 5.1 Plus software. MN data were compared by applying one-way ANOVA. Variables were tested for normality with the Kolmogorov–Smirnov test, and homogeneity of variances between groups was verified by the Levene test. Pairwise comparisons between the different groups were made using the post hoc least significant difference test. Differences in the NDI and apoptosis in treated and control cells 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

Table 1 shows the results of the analysis of insecticide pirimicarb- and Aficida®-induced MNs in binucleated cytokinesis-blocked CHO-K1 cells. An increased frequency of MNs was observed in BLM-treated cultures (positive control) compared to control values ($p < 0.001$). A significant induction of MNs was observed after treatments with 10–200 μg/ml pirimicarb ($p < 0.001$) and Aficida® ($p < 0.05$ and $p < 0.001$). A correlation test showed that the total frequency of MNs increased as a function of the concentration of pirimicarb ($r = 0.82$, $p < 0.01$) or Aficida® ($r = 0.89$, $p < 0.01$) titrated into cultures (Table 1). Statistical analysis revealed that when cells were treated with 10–200 μg/ml pirimicarb, the total MN frequency enhancement was due to an increase in the frequency of binucleated cells carrying one MN ($p < 0.05$ and $p < 0.001$) as well as an increase in binucleated cells carrying three MNs ($p < 0.05$). In addition, damaged binucleated cells containing two MNs were observed at the highest pirimicarb concentration tested ($p < 0.001$). Finally, only in 200 μg/ml Aficida®-treated cultures was the frequency of cells carrying one, two, or three MNs significantly higher than that of control cultures ($0.05 > p < 0.01$). When the cultures were treated with 300 μg/ml pirimicarb or Aficida®, evident alterations in cell morphology were induced which did not allow monitoring MN frequencies. The NDI values induced by pirimicarb and Aficida® treatments are presented in Table 1. Regardless of the concentration, no significant alterations in the NDI were found in cultures treated with either compound compared to controls ($p > 0.05$).

Table 2 shows the results of the analysis of fungicide zineb- and Azzurro®-induced MNs in binucleated cytokinesis-blocked CHO-K1 cells. An increased frequency of MNs was observed in BLM-treated cultures (positive control) compared to control values ($p < 0.001$). A significant induction of MNs was observed after treatments with 1–5 μg/ml zineb and Azzurro® ($p < 0.001$). Statistical analysis revealed that when cells were treated with 1 μg/ml zineb or Azzurro®, the total MN frequency enhancement was due to an increase in the frequency of binucleated cells carrying two MNs ($p < 0.001$) as well as by an increased frequency of binucleated cells carrying three MNs ($p < 0.001$). When the cells were treated with 5 μg/ml zineb or Azzurro®, the frequency of cells carrying one, two, or three MNs was significantly higher than that of control cultures ($0.05 > p < 0.001$). Finally, Azzurro® increased the frequency of MNs compared to control cultures of cells treated with 10 μg/ml ($p < 0.001$; Table 2). When the cultures were treated with 10 μg/ml zineb or 15–50 μg/ml zineb or Azzurro®, evident alteration in cell morphology was induced which did not allow monitoring of MN frequencies. A significant reduction of the NDI was observed only in 5 μg/ml zineb-treated ($p < 0.01$) and in 5

Table 2
Micronucleus (MNs) induction and nuclear division index (NDI) values for control, zineb-, and zineb-containing technical formulation Azzurro®-treated in binucleated cytokinesis-blocked Chinese hamster ovary (CHO-K1) cells.[a]

Concentration (μg/ml)	Zineb					Azzurro® (70% Zineb)				
	MN frequencies[b]	Micronucleated cell distribution[c]			NDI[d]	MN frequencies[b]	Micronucleated cell distribution[c]			NDI[d]
		1 MN	2 MN	3 MN			1 MN	2 MN	3 MN	
0	27.0 ± 0.33	26.3 ± 0.51	0.3 ± 0.19	0.00 ± 0.00	2.18 ± 0.04	29.3 ± 0.84	26.7 ± 0.30	1.3 ± 0.51	0.0 ± 0.00	2.26 ± 0.03
1	40.7 ± 1.35***	28.3 ± 2.50	2.7 ± 0.38***	2.3 ± 0.19***	2.09 ± 0.14	43.3 ± 1.86***	28.0 ± 2.39	5.7 ± 0.19***	1.3 ± 0.51***	2.18 ± 0.02
5	66.0 ± 1.20***	47.0 ± 1.20***	5.0 ± 0.58***	3.0 ± 0.33***	1.80 ± 0.06**	53.3 ± 3.01***	36.3 ± 0.39*	6.0 ± 0.33***	3.0 ± 0.58***	1.96 ± 0.04*
10	ND		ND		ND	90.0 ± 1.58***	50.7 ± 1.00***	16.7 ± 0.69***	2.0 ± 0.33***	1.48 ± 0.02***
15	ND		ND		ND	ND		ND		ND
50	ND		ND		ND	ND		ND		ND
BLM[e]	70.0 ± 1.20***	46.3 ± 1.20***	7.3 ± 0.58***	3.0 ± 0.33***	2.11 ± 0.06	70.0 ± 1.20***	46.3 ± 1.20***	7.3 ± 0.58***	3.0 ± 0.33***	2.11 ± 0.04

** $p < 0.01$ significant difference with respect to control values.
*** $p < 0.001$ significant difference with respect to control values.
[a] CHO-K1 cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later.
[b] Results are presented as mean MNs/1000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.
[c] Results are presented as number of cells carrying 1, 2 or 3 MNs among 3000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.
[d] Results are presented as mean value of pooled data from three independent experiments ± S.E. of the mean.
[e] Bleomycin (BLM, 1.0 μg/ml) was used as positive control. ND, Not determined.

and 10 μg/ml Azzurro®-treated cultures ($p < 0.05$ and $p < 0.001$, respectively). A correlation analysis showed that the NDI decreased in a concentration-dependent manner when Azzurro® ($r = -0.99$, $p < 0.001$) was titrated into cultures (Table 2).

Data of cell viability obtained after pesticide treatment are presented in Fig. 1. Statistically significant loss of cell viability was observed between negative (untreated and DMSO-treated cells) and positive controls ($p < 0.001$) (Fig. 1A and B). The data revealed a significant reduction of cell viability when CHO-K1 cells were exposed to 300 μg/ml pirimicarb ($p < 0.001$) and 200–300 μg/ml Aficida® ($0.01 > p < 0.001$) (Fig. 1A). A correlation test showed that cell viability decreased as a function of the concentration of pirimicarb ($r = -0.89$, $p < 0.001$) or Aficida® ($r = -0.94$, $p < 0.001$) titrated into cultures. Overall, the NR assay demonstrated that Aficida® exerted a more cytotoxic effect than the active ingredient in the CHO-K1 cell line within the 10–300 μg/ml concentration range (Fig. 1A). When the fungicide was employed, the statistical analysis revealed a significant CHO-K1 cell viability inhibition after exposure to 10–50 μg/ml zineb or Azzurro® ($p < 0.001$) (Fig. 1B). A correlation test showed that cell viability decreased as a function of the concentration of zineb ($r = -0.76$, $p < 0.01$) or Azzurro® ($r = -0.85$, $p < 0.001$) titrated into cultures.

The results obtained on the alterations of the succinic dehydrogenase activity are presented in Fig. 2. Ethanol-treated cultures (positive controls) produced a statistically significant toxicity in CHO-K1 cells compared with negative and DMSO-treated cultures ($p < 0.001$). The data presented in Fig. 2A show a significant succinic dehydrogenase inhibition only for CHO-K1 cells exposed to 300 μg/ml pirimicarb or Aficida® ($p < 0.001$). A correlation test revealed that succinic dehydrogenase activity decreased as a function of the concentration of pirimicarb ($r = -0.84$, $p < 0.01$) or Aficida® ($r = -0.81$, $p < 0.001$) titrated into cultures. The statistical analysis revealed a significant succinic dehydrogenase inhibition after exposure to 10–50 μg/ml zineb or Azzurro® ($p < 0.001$) (Fig. 2B). A correlation test showed that succinic dehydrogenase activity decreased as a function of the concentration of zineb ($r = -0.84$, $p < 0.01$) or Azzurro® ($r = -0.90$, $p < 0.001$) titrated into cultures. Overall, both the NR and MTT bioassays demonstrated that the active ingredient zineb exerted a markedly higher cytotoxicity than the commercial formulation product Azzurro® in CHO-K1 cells within the 1–50 μg/ml concentration range (Figs. 1B and 2B).

Flow cytometry analyses of cells treated with 100 and 200 μg/ml pirimicarb and Aficida® are presented in Fig. 3. The frequencies of live, early apoptotic, late apoptotic, and necrotic cells observed in the DMSO-treated cultures were consistent with the routinely observed frequency for negative control cultures, and pooled data are presented for control cultures. The results indicate that only 200 μg/ml pirimicarb induced a slight significant increase in the frequency of late apoptosis ($p < 0.05$) (Fig. 3D). A significant increase in early apoptosis was found when the two concentrations of the commercial formulation were employed ($p < 0.05$). Finally, significant increases in the frequencies of late apoptotic ($p < 0.001$) and necrotic ($p < 0.05$) cells were observed only in 200 μg/ml Aficida®-treated cultures. Overall, a correlation analysis revealed that the frequency of apoptotic cells increased as a function of the concentration of pirimicarb or Aficida® titrated into cultures ($r = 0.99$, $p < 0.001$) (Fig. 3D).

Fig. 4 shows the flow cytometry analyses in cells treated with both 10 and 15 μg/ml zineb and Azzurro®. The results indicate that 10 and 15 μg/ml zineb induced a significant increase in the frequency of both early and late apoptotic cells ($p < 0.001$) (Fig. 4D). Only a significant increase in late apoptosis was found when the commercial formulation was used at concentrations of 10 and 15 μg/ml ($p < 0.001$). Finally, a significant increase in the frequency of necrotic cells ($p < 0.05$) was observed in Azzurro®-treated cultures. Overall, correlation analyses revealed that the frequency of apoptotic cells increased as a function of the concentrations of both zineb ($p < 0.01$) and Azzurro® ($r = 0.99$, $p < 0.001$) titrated into cultures (Fig. 4D).

4. Discussion

The aim of this study was to evaluate and compare the *in vitro* genotoxic and cytotoxic effects induced by two active pure carbamates and some of their technical formulations in CHO-K1 mammalian cells. The CBMN-cyt assay was employed to analyze the damage inflicted by pirimicarb and zineb and their commercial formulations Aficida and Azzurro®, respectively. Additionally, the NDI, NR, and MTT bioassays, as well as apoptosis induction, were used to determine quantitatively whether carbamate treatments were able to induce cytotoxicity. All concentrations of the insecticides pirimicarb and Aficida® increased the MN frequency in CHO-K1 cells after 24 h of exposure. The capacity of pirimicarb and Aficida® to induce MNs was found to be equivalent for all concentration assayed. In addition, the fungicides zineb and Azzurro® increased the frequency of MNs when cells were exposed within the 1–5 μg/ml and 1–10 μg/ml concentration ranges to zineb and

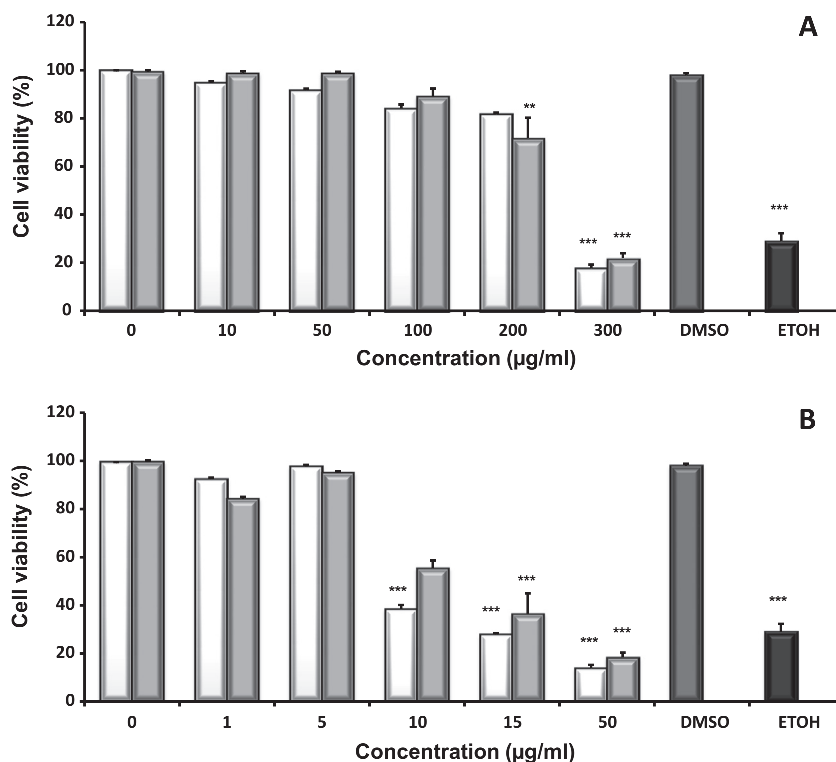

Fig. 1. Cell viability assessed with the NR assay in pure ingredient- (white bars) and formulated product-treated (gray bars) CHO-K1 cells. Cultures were incubated for 3 h with NR dye after 24 h treatment. Results are expressed as the mean percentage of cell viability from three independent experiments performed in parallel (*y*-axis) and plotted against the carbamate concentration (*x*-axis) employing 0–300 µg/ml concentration-range of pirimicarb and Aficida® (A) or 1–50 µg/ml concentration-range of zineb and Azzurro® (B). Five percent ethanol-treated (black bar) and 0.075% DMSO-treated (gray black bar) cells were used as positive and solvent controls, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values.

Azzurro®, respectively. On the other hand, 300 µg/ml of the insecticides as well as 10–50 µg/ml of the fungicides resulted in severe cellular cytotoxicity as shown by alterations in the NDI and cellular death. Overall, the results demonstrate that both the pure ingredients and the formulated products are able to reduce both cell viability and cellular metabolism, as measured by succinic dehydrogenase activity at equivalent levels when higher concentrations of xenobiotics are employed. As shown by the results, MTT and NR colorimetric bioassays appear more sensitive to the carbamate zineb-induced cytotoxicity than that induced by pirimicarb. Finally, the results indicate that both pure and formulated products were able to induce a significant increase in the frequency of apoptotic cells after 24 h of treatment.

According to the U.S. EPA (1974b), pirimicarb is an insecticide with medium to high environmental persistence and with moderate toxic potency for humans. Nevertheless, the organization has allowed its extensive usage, even though various effects of its subchronic/chronic toxicity have been reported (USEPA, 1974b). In the present study we report the genotoxic and apoptotic potential of pure pirimicarb and its formulation Aficida® on mammalian CHO-K1 cells. So far, this is the first report demonstrating the ability of both compounds to induce MNs and apoptosis in mammalian cells, at least in CHO-K1 cells. We have demonstrated that the insecticide induces genotoxicity in a dose-dependent manner. Furthermore, we observed that the capacity of pirimicarb and Aficida® to induce MNs was equivalent for all concentrations assayed. These results are in concordance with our previous studies indicating an equivalent increase in the frequency of structural chromosomal aberrations exerted by both compounds in CHO-K1 cells (Soloneski and Larramendy, 2010). The current results are consistent with previous findings depicting the genotoxic potential of pirimicarb through the induction of chromosomal damage in human lymphocytes *in vivo* in occupationally exposed workers (Pilinskaia, 1982) as well as single-strand DNA breaks revealed by the comet assay in human lymphocyte cells without metabolic activation (Ündeger and Basaran, 2005). In agreement with the positive genotoxic effects found in our current study, we have observed previously the ability of the formulated product Aficida® to increase the frequency of MNs in erythrocytes of the fish *C. decemmaculatus* and *R. arenarum* tadpoles (Vera Candioti et al., 2010a,b). Additionally, our results demonstrate that a significant number of binucleated cells contained two or more MNs after insecticide pirimicarb and Aficida exposure. The significant increase in the number of MNs we observed after *in vitro* exposure could suggest that high concentrations of pirimicarb can cause breakage at the chromosome level. A considerable number of intercalating agents have proved to be potent clastogens in mammalian cells, and they are involved in carcinogenic processes (Ferguson and Denny, 2007; Kulling and Metzler, 1997; Shahabuddin et al., 2005). In effect, Zhang et al. (2011) investigated the binding interactions between the pure pirimicarb and DNA. They observed that pirimicarb can bind to DNA, and the most important interaction mode is intercalative binding (Zhang et al., 2011). This mode of action could explain the results found by us, and then a clastogenic effect for the insecticide could be suggested. Therefore, further research studies should be focused on determining a plausible clastogenic origin of the MN induction by pirimicarb.

Although zineb is unlikely to contaminate groundwater (USEPA, 1996) and it is considered nonmutagenic in bacterial systems (IARC, 1976), the fungicide hydrolyzes to a highly water soluble environmental metabolite, namely, ethylenethiourea, and some

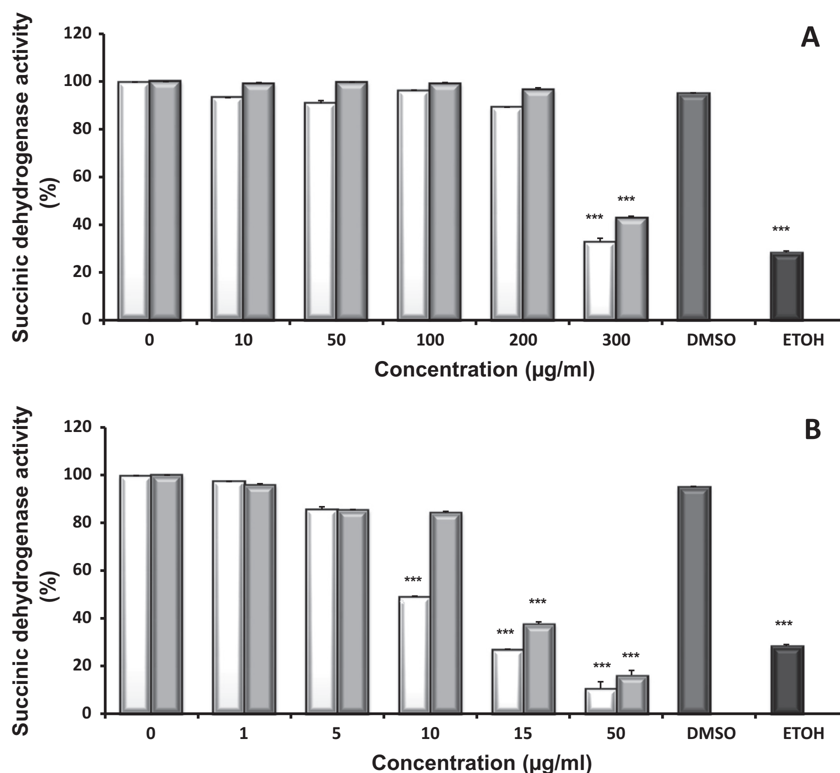

Fig. 2. Succinic dehydrogenase activity assessed with the MTT bioassay in pure ingredient- (white bars) and formulated product-treated (gray bars) CHO-K1 cells. Cultures were incubated for 3 h with MTT dye after 24 h treatment. Results are expressed as the mean percentage of succinic dehydrogenase activity from three independent experiments performed in parallel (*y*-axis) and plotted against the carbamate concentration (*x*-axis) employing 0–300 µg/ml concentration-range of pirimicarb and Aficida® (A) or 1–50 µg/ml concentration-range of zineb and Azzurro® (B). Five percent ethanol-treated (black bar) and 0.075% DMSO-treated (gray black bar) cells were used as positive and solvent controls, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values.

concern surrounding its use is due to the formation of this carcinogenic degradation product (USEPA, 1996). Residuals of this kind of compound can cause environmental and human health problems, and they have been reported to be hepatotoxic as well as carcinogenic for mammals (USEPA, 1996). Our present findings showing the ability of the fungicide to induce MNs *in vitro* confirm the genotoxic potential of zineb and the zineb-containing formulation Azzurro®. Using the MAC (Morphology, Antibody, Chromosomes) methodology, we were able to demonstrate years ago an increased frequency of MNs in stimulated human lymphocytes exposed *in vitro* to both zineb and Azzurro® (Soloneski et al., 2002b). In our current experimental conditions, MN induction is produced by cellular exposure to low concentrations of both compounds, whereas higher concentrations result in a cytotoxic effect demonstrated by alterations in the NDI and cellular death. The zineb concentrations used herein are known to cause DNA damage estimated by SCE and comet bioassays in CHO-K1 cells (Soloneski et al., 2002a), and mitotic spindle disturbances in transformed and nontransformed mammalian cells *in vitro* (Soloneski et al., 2003a). In agreement with the positive genotoxic effects here observed, we found previously that zineb and Azzurro® induced chromosome aberrations, SCEs (Soloneski et al., 2001), and MNs in human peripheral blood lymphocytes *in vitro* (Soloneski et al., 2002a). In addition, structural chromosomal aberrations in CHO cells (Enninga, 1986) and MNs in human lymphocytes of agricultural workers *in vivo* (Pilinskaia, 1974) were also reported after zineb exposure. It has been reported previously by us that zineb most probably induces DNA damage via free radical reactions and production of reactive oxygen species (Soloneski et al., 2003b). We observed that CHO-K1 cells preincubated with an antioxidant system such as α-tocopherol supplementation had a significantly reduced frequency of SCEs after exposure to both zineb and Azzurro® (Soloneski et al., 2003b). Moreover, Astiz et al. (2012) revealed that zineb induced changes in antioxidant status with concomitant increases in nitric oxide levels in plasma, liver, brain, kidney, and testes from exposed rats. Similarly, oxidative stress was recently reported to be the event induced by zineb exposure in V79 cells, where a decrease in the activity of both glutathione peroxidases and cytoplasmic superoxide dismutase and a concomitant increase in catalase activity were observed (Grosicka-Maciąg et al., 2013). Finally, it was also reported previously that the mechanism of action of zineb is related to the formation of cyanide (Hayes, 2007; USEPA, 1996). Further investigations are required to determine the roles of oxidative DNA damage and/or cyanide production in the chromosomal breakage exerted by zineb.

Cytotoxicity bioassays have proved most useful as tools to clarify the biochemical or metabolic alterations involved in the mechanisms of toxic action of several xenobiotics at the cellular level such as free radicals, irritants, and/or genotoxic compounds (Mazzotti et al., 2001). Here, we estimate the early cytotoxic response exerted by the carbamates pirimicarb and zineb by NR uptake and MTT bioassays. Our results indicate that both the two pure carbamates and their formulated products induce cytotoxicity in CHO-K1 cells by decreasing both cell viability and succinic dehydrogenase activity. Additionally, our findings highlight that both pure carbamate compounds more actively induce cytotoxic effects in CHO-K1 cells than their corresponding commercial formulations, especially when Azzurro® is employed. Accordingly, it can be assumed that the deleterious effect(s) of pure carbamates or their active metabolites by themselves are more directly responsible for their cellular cytotoxicity profile than any plausible xenobiotic(s) present within their technical formulations.

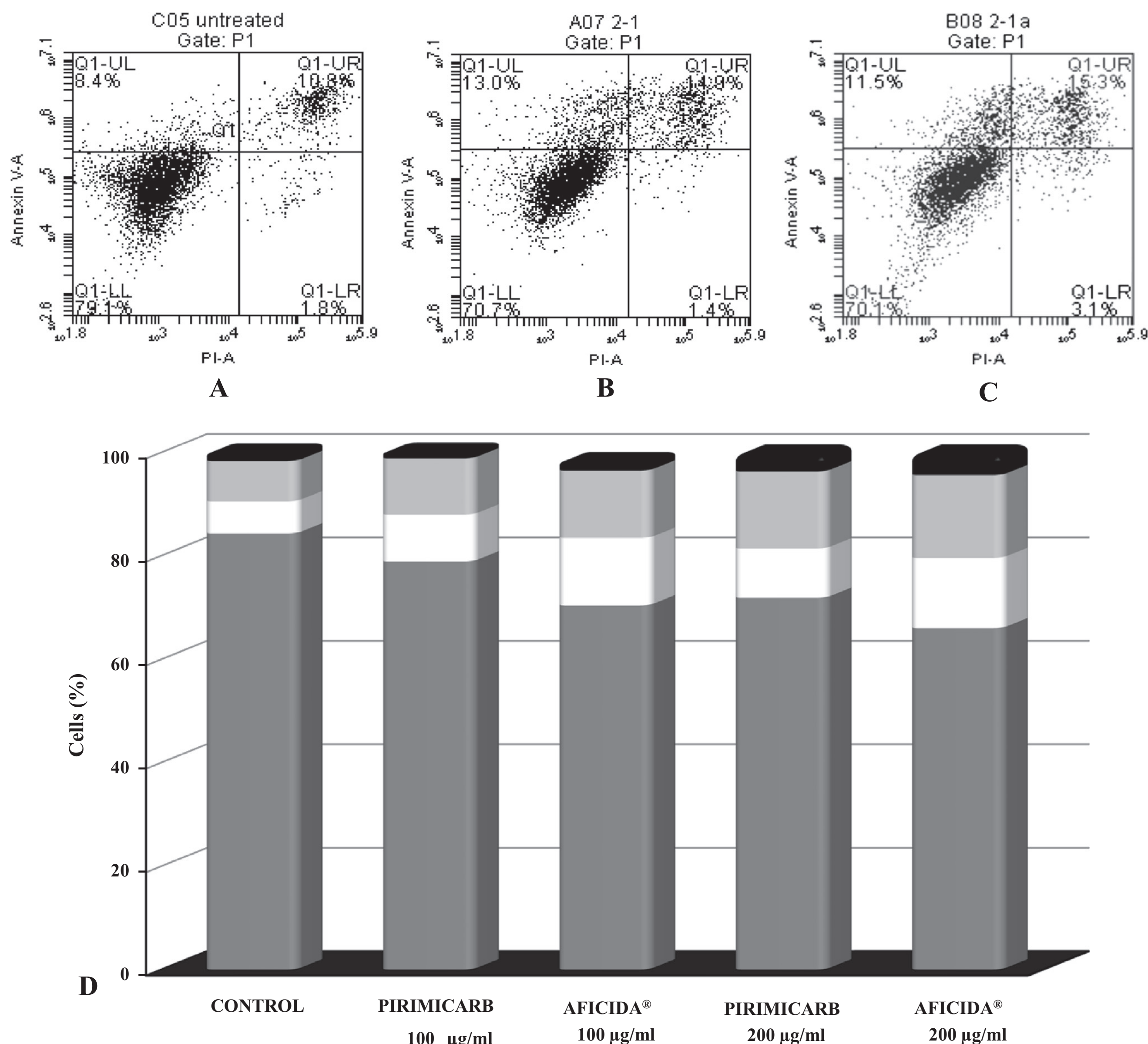

Fig. 3. Pirimicarb and the pirimicarb-based commercial formulation Aficida® induce apoptosis in CHO-K1 cells. Cells were treated with test compounds and processed 24 h later with the Annexin V/PI staining for quantification of the incidence of apoptosis cells by flow cytometry. A–C, representative dot plot analyses of control (A), 100 µg/ml pirimicarb-treated (B), and 200 µg/ml pirimicarb-treated (C) cultures. The cell populations were discriminated in each quadrant as viable cells in the lower left (LL; Annexin V negative/PI negative), early apoptotic cells in the upper left (UL; Annexin V positive/PI negative), late apoptotic cells in the upper right (UR; Annexin V positive/PI positive), and necrotic cells in the lower right quadrant (LR; Annexin V negative/PI positive). The percentages of cells from four independent experiments after flow cytometry analysis are shown in D. Early apoptotic (white bar sections), late apoptotic (light gray bar sections), live (dark gray bar sections), and necrotic cells (black bar sections) among all cells from pirimicarb- and Aficida®-treated cell cultures are shown.

Our results obtained from the cytotoxicity assays indicate that there are differences between the two bioassays employed concerning their sensitivity to the carbamates assayed. This observation can be explained by the nature of each bioassay. The NR uptake assay is a colorimetric bioassay measuring the uptake of dye by lysosomal functionality, whereas the MTT assay is mainly based on the enzymatic conversion of MTT by the succinic dehydrogenase in the mitochondria. It is accepted that a reduction in lysosomal activity reflects enhanced instability of the cell membranes (Borenfreund and Puerner, 1985; Molinari et al., 2009; Nikoloff et al., 2012). As shown in CHO-K1 cells, the NR uptake reveals higher cytotoxicity following carbamate exposure than that observed when the MTT bioassay is employed for an equivalent concentration. Previous reports demonstrated that the NR uptake assay also showed greater sensitivity than the MTT bioassay when other *in vitro* test systems were employed (Putnam et al., 2002; Repetto et al., 2008). According to our current results, it can be claimed that this effect may be due to direct alterations in the lysosomal functionality of mammalian cell membranes, increasing the susceptibility of cells to toxicants like carbamates. It was reported previously by us that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed, which highlights the importance of using more than one bioassay to determine cell viability in *in vitro* studies

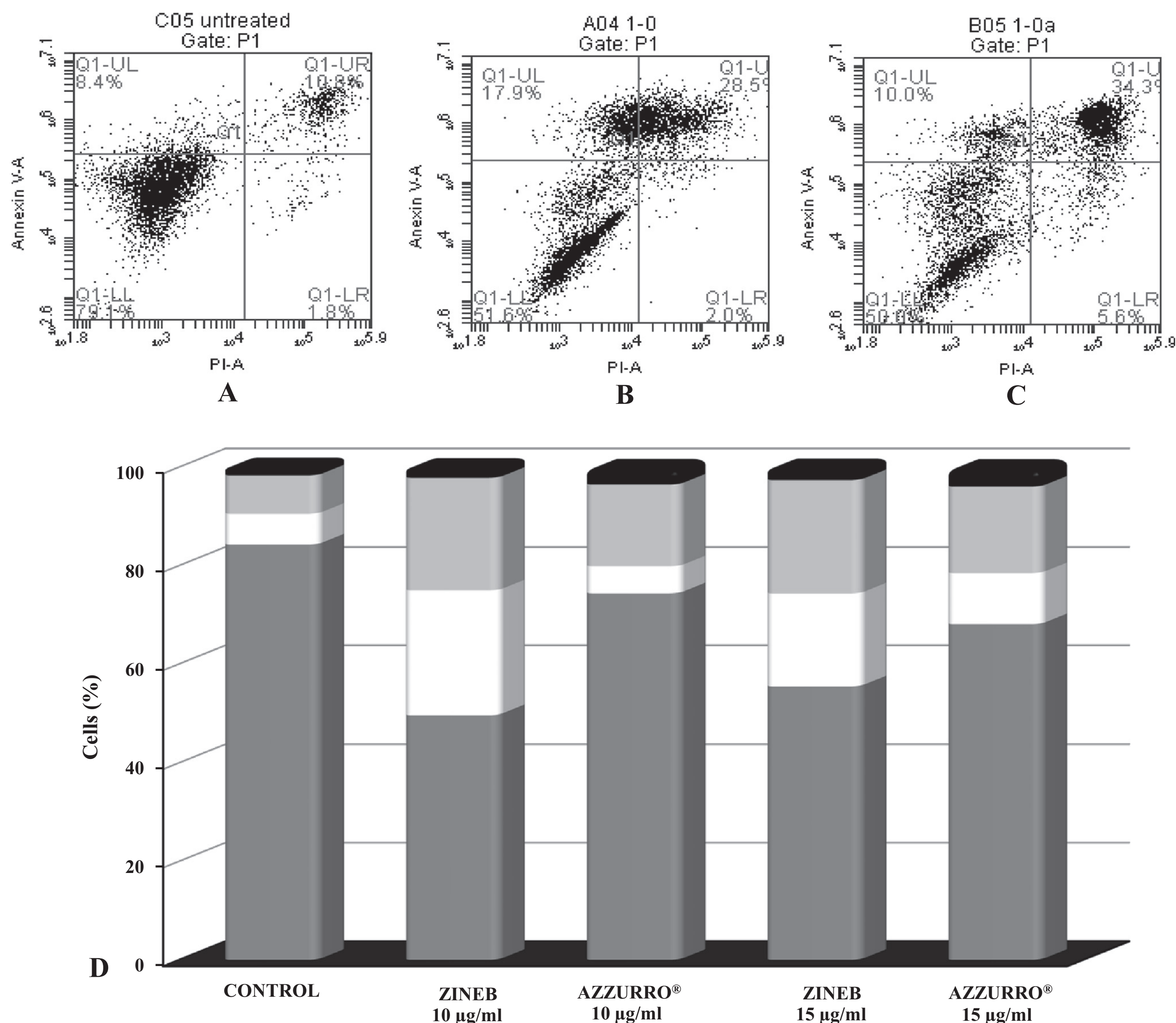

Fig. 4. Zineb and the zineb-based commercial formulation Azzurro® induce apoptosis in CHO-K1 cells. Cells were treated with test compounds and processed 24 h later with the Annexin V/PI staining for quantification of the incidence of apoptosis cells by flow cytometry. A–C, representative dot plot analyses of control (A), 10 µg/ml zineb-treated (B), and 15 µg/ml zineb-treated (C) cultures. The cell populations were discriminated in each quadrant as viable cells in the lower left (LL; Annexin V negative/PI negative), early apoptotic cells in the upper left (UL; Annexin V positive/PI negative), late apoptotic cells in the upper right (UR; Annexin V positive/PI positive), and necrotic cells in the lower right quadrant (LR; Annexin V negative/PI positive). The percentages of cells from four independent experiments after flow cytometry analyses are shown in D. Early apoptotic (white bar sections), late apoptotic (light gray bar sections), live (dark gray bar sections), and necrotic cells (black bar sections) among all cells from zineb- and Azzurro®-treated cell cultures are shown.

(Molinari et al., 2009; Nikoloff et al., 2012, 2014; Soloneski and Larramendy, 2010). Our results are consistent with this assumption.

Detection of apoptosis is of great importance in many areas of biological and medical research because a wide variety of physiological and pathological processes are involved (McGahon et al., 1995). The present study demonstrates that the carbamates we assayed and their commercial formulations induce the formation of DNA fragmentation and increase apoptotic frequency in exposed CHO-K1 cells. The results indicate that only the higher pirimicarb concentration is able to increase the frequency of late apoptotic CHO-K1 cells. Aficida®-exposed cultures showed a significant increase in both early and late apoptotic cells. Our data show for the first time apoptosis induction when treating CHO-K1 cells with both pirimicarb and the pirimicarb-based formulation Aficida®. This is consistent with the MN induction observed in 100 and 200 µg/ml Aficida®-exposed CHO-K1 cells where not only the maximum level of early and late apoptosis occurred after 24 h of treatment, but also the least MN induction for Aficida®. Thus, it could be suggested that a considerable number of damaged cells undergo apoptosis rather than MN formation, although the precise mechanism of the genotoxic potential of pirimicarb has not yet been fully elucidated. A recent study demonstrated that pirimicarb affects the glucose, fatty acid, and protein metabolism as well as the energy metabolism and oxidative balance of the mouse liver (Wang et al., 2014). In summary, the apoptosis induction reported here could be correlated with the stronger oxidative stress demonstrated in mouse hepatic cells after pirimicarb exposure.

In this study, we also assayed whether zineb and Azzurro® can induce apoptosis at concentrations above the threshold for MN induction. A statistically significant increase in both early and late apoptotic cells for both compounds was observed when the

fungicide was used at both 10 and 15 µg/ml. After zineb exposure, apoptotic cells increased by approximately 50% relative to control values, whereas a 25% increase of apoptotic cells was found after Azzurro® exposure. Our results on the effect of zineb on apoptosis induction are in agreement with those of Astiz et al. (2012). They demonstrated previously that the administration of low doses of zineb to rats provokes a severe reactive oxygen species increase in specific brain regions, enhancing the activation of programmed cell death via the calpain system (Grosicka-Maciąg et al., 2013) Several reports have suggested that interphase microtubules seem to play an important role in signal transduction (Pietenpol and Stewart, 2002). Alterations in microtubule polymerization may impair the cellular ability to activate the apoptosis mechanism (Pietenpol and Stewart, 2002). Interestingly, in a previous study, we demonstrated that zineb and Azzurro® could modify the mitotic spindle apparatus in exposed CHO-K1 cells (Soloneski et al., 2003a), limiting cell proliferation throughout the cellular cycle. Thus, the elimination of micronucleated cells does occur, probably by activation of an apoptosis pathway and decreasing cell survival long enough to contribute to MN formation. In our present study, it could be suggested that MN-bearing cells had severe DNA damage that might trigger apoptotic death after zineb exposure.

Finally, it is worth mentioning, as stated previously, that Aficida® was more cytotoxic, through the activation of a differential apoptotic pathway, than the same concentration of the active ingredient pirimicarb. Our results reveal an approximately 25% increase in apoptotic cell frequency (early and late) after 200 µg/ml Aficida® treatment compared to that induced by pirimicarb. As a commercial formulation, Aficida® contains 50% of inert and other ingredients, theoretically not possessing cytotoxic properties. The differential toxicity of pesticide commercial formulations and their active ingredients now appears to be a general feature of pesticide toxicology. Several studies describe that the toxicity of nonactive ingredients present in pesticide formulations could be more toxic than the pesticide active ingredient itself, and could affect the overall toxicity of the product (González et al., 2006; Molinari et al., 2009; Soloneski et al., 2001; Soloneski and Larramendy, 2010; Zeljezić et al., 2006). In agreement, the U.S. EPA (Mesnage et al., 2014; USEPA, 2010) has recently modified the regulations for naming "other ingredients" present within a technical formulation in addition to the active ingredient, and therefore adjuvants should be considered putative toxic "active" compounds. Our current observations could suggest that the apoptotic effect induced by Aficida® is most probably due to the presence of xenobiotic(s) included in the excipient of the carbamate-based formulation, with either intrinsic toxic effects or the capacity to exacerbate the toxicity of the pure agrochemicals, or both. Additional evidence is required to support the latter possibility.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

This study was supported by Grants from the National University of La Plata (Grants 11/N699 and 11/N746) and the National Council for Scientific and Technological Research (CONICET, PIP N° 0344) from Argentina.

References

Alavanja, M.C.R., Hoppin, J., Kamel, F., 2005. Health effects of chronic pesticide exposure: cancer and neurotoxicity. Annu. Rev. Public Health 25, 155–197.

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B., Lakra, W.S., 2009. Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 47, 650–656.

Anderson, D., Richardson, C.R., Howard, C.A., Bradbrook, C., Salt, M.J., 1980. Pirimicarb: A Cytogenetic Study in the Rat. World Health Organization.

Astiz, M., de Alaniz, M.J.T., Marra, C.A., 2012. The oxidative damage and inflammation caused by pesticides are reverted by lipoic acid in rat brain. Neurochem. Int. 61, 1231–1241.

Bolognesi, C., 2003. Genotoxicity of pesticides: a review of human biomonitoring studies. Mutat. Res. 543, 251–272.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.

Bradley, M.O., Bhuysn, B., Francis, M.C., Langenbach, R., Peterson, A., Huberman, E., 1981. Mutagenesis by chemical agents in V79 Chinese hamster cells: a review and analysis of the literature. A report of the Gene-Tox program. Mutat. Res. 87, 81–142.

Circu, M.L., Aw, T.Y., 2010. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic. Biol. Med. 15, 749–762.

Ciscato, C.H., Gebara, A.B., Spinosa, H.S., 2002. Pesticide residues in cow milk consumed in São Paulo City (Brazil). J. Environ. Sci. Health B 37, 323–330.

Eastmond, D.A., Tucker, J.D., 1989. Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. Environ. Mol. Mutagen. 13, 34–43.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Enninga, I.C., 1986. Evaluation of the mutagenic activity of zineb in the Ames Salmonella/microsome test. Unpublished report dated. December 29, 1986 from Pennwalt Holland B.V.

Fenech, M., 2000. The *in vitro* micronucleus technique. Mutat. Res. 455, 81–95.

Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. Nat. Protoc. 2, 1084–1104.

Fenech, M., 2008. The micronucleus assay determination of chromosomal level DNA damage. Methods Mol. Biol. 410, 185–216.

Fenner-Crisp, P.E., 2001. Risk-assessment and risk management: the regulatory process. In: Krieger, R.I., Krieger, W.C. (Eds.), Handbook of Pesticide Toxicology, second ed., San Diego, pp. 681–690.

Ferguson, L.R., Denny, W.A., 2007. Genotoxicity of non-covalent interactions: DNA intercalators. Mutat. Res. 623, 14–23.

González, M., Soloneski, S., Reigosa, M.A., Larramendy, M.L., 2003. Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. IV. DNA damage and repair kinetic assessed by single cell gel electrophoresis (SCGE) assay on Chinese hamster ovary (CHO) cells. Mutat. Res. 534, 145–154.

González, N.V., Soloneski, S., Larramendy, M.L., 2006. Genotoxicity analysis of the phenoxy herbicide dicamba in mammalian cells *in vitro*. Toxicol. In Vitro 20, 1481–1487.

González, N.V., Soloneski, S., Larramendy, M.L., 2007. The chlorophenoxy herbicide dicamba and its commercial formulation Banvel induce genotoxicity in Chinese hamster ovary cells. Mutat. Res. 634, 60–68.

Grosicka-Maciąg, E., Szumiło, M., Czeczot, H., Kurpios-Piec, D., Skrzycki, M., Rahden-Staroń, I., 2013. Modulation of antioxidant defense system by the dithiocarbamate fungicides maneb and zineb in Chinese hamster V79 cells and the role of N-acetyl-L-cysteine. Food Chem. Toxicol. 60, 130–134.

Hayes, A.W., 2007. Principles and Methods of Toxicology, fifth ed. CRC Press.

Hour, T.C., Chen, L., Lin, J.K., 1998. Comparative investigation on the mutagenicities of organophosphate, phthalimide, pyrethroid and carbamate insecticides by the Ames and lactam tests. Mutagenesis 13, 157–166.

Huntingdon Research Centre, H.R., 1985. Reverse mutation/*Salmonella typhimurium*. Report No. FMT4/85394. Unpublished report dated August 23, 1985 from Huntingdon Research Centre, England.

IARC, 1976. Some Carbamates, Thiocarbamates and Carbazides. International Agency for Research on Cancer, Lyon.

IARC, 2003. Monographs on the evaluation of carcinogenic risk to human. International Agency for Research on Cancer, vols. 5–53. IARC Press, Lyon.

ICH, 2011. Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2 (R1). In: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Jones, K., Howard, C.A., 1989. Pirimicarb (technical): an evaluation in the mouse micronucleus test. Unpublished report No. CTL/P/2641 from Central Toxicology Laboratory, Zeneca. Submitted to WHO by Syngenta Crop Protection AG. Conducte according to OECD 474 (1983). GLP compliant.

Kirsch-Volders, M., Vanhauwaert, A., Eichenlaub-Ritter, U., Decordier, I., 2003. Indirect mechanisms of genotoxicity. Toxicol. Lett. 140–141, 63–74.

Knasmüller, S., Mersch-Sundermann, V., Kevekordes, S., Darroudi, F., Huber, W.W., Hoelzl, C., Bichler, J., Majer, B.J., 2004. Use of human-derived liver cell lines for

the detection of environmental and dietary genotoxicants; current state of knowledge. Toxicol. In Vitro 20, 315–328.
Kulling, S., Metzler, M., 1997. Induction of micronuclei, DNA strand breaks and HPRT mutations in cultured Chinese hamster V79 cells by the phytoestrogen coumoestrol. Food Chem. Toxicol. 35, 605–613.
Larramendy, M.L., Soloneski, S., 2011. Integrated Pest Management and Pest Control. Current and Future Tactics European Union, INTECH, Rijeka, Croatia, p. 668.
Lee, B.W., Oh, S.H., Chung, J.H., Moon, C.K., Lee, B.H., 2004. *N*-nitroso metabolite of carbofuran induces apoptosis in CHL cells by cytochrome c-mediated activation of caspases. Toxicology 201, 51–58.
Mazzotti, F., Sabbioni, E., Ghiani, M., Cocco, B., Ceccatelli, R., Fortaner, S., 2001. *In vitro* assessment of cytotoxicity and carcinogenic potential of chemicals: evaluation of the cytotoxicity induced by 58 metal compounds in the Balb/3T3 cell line. Altern. Lab. Anim. 29, 601–611.
McGahon, A.J., Martín, S.J., Bissonnette, R.P., Mahboubi, A., Shi, Y., Mogil, R.J., Nishioka, W.K., Green, D.K., 1995. The end of the (cell) line: methods for the study of apoptosis *in vitro*. Methods Cell Biol. 46, 153–185.
Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, E., 2014. Major pesticides are more toxic to human cells than their declared active principles. Biomed. Res. Int., 1–8 http://dx.doi.org/10.1155/2014/179691, ID: 179691.
Miki, I., Ishihara, N., Otoshi, M., Kase, H., 1993. Simple colorimetric cell-cell adhesion assay using MTT-stained leukemia cells. J. Immunol. Methods 164, 255–261.
Molinari, G., Soloneski, S., Reigosa, M.A., Larramendy, M.L., 2009. *In vitro* genotoxic and citotoxic effects of ivermectin and its formulation ivomec®on Chinese hamster ovary (CHO_{K1}) cells. J. Hazard. Mater. 165, 1074–1082.
Nag, S.K., Raikwar, M.K., 2011. Persistent organochlorine pesticide residues in animal feed. Environ. Monit. Assess. 174, 327–335.
Nikoloff, N., Soloneski, S., Larramendy, M., 2012. Genotoxic and cytotoxic evaluation of the herbicide flurochloridone on Chinese hamster ovary (CHO-K1) cells. Toxicol. In Vitro 26, 157–163.
Nikoloff, N., Larramendy, M.L., Soloneski, S., 2014. Comparative evaluation *in vitro* of the herbicide flurochloridone by cytokinesis-block micronucleus cytome and comet assays. Environ. Toxicol. 29, 884–892.
OECD, 2007. *In Vitro* micronucleus test, OECD Guidelines for the Testing of Chemicals. Organization for Economic Co-operation and Development, OECD, Paris.
OECD, 2010. Guideline for the Testing of Chemicals. *In Vitro* Mammalian Cell Micronucleus Test (#487). Organization for Economic Co-operation and Development, OECD, Paris.
Pietenpol, J.A., Stewart, Z.A., 2002. Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicol. In Vitro 181–182, 475–481.
Pilinskaia, M.A., 1974. Results of cytogenetic examination of persons occupationally contacting with the fungicide zineb. Genetika 10, 140–146.
Pilinskaia, M.A., 1982. Cytogenetic effect of the pesticide pirimor in a lymphocyte culture of human peripheral blood *in vivo* and *in vitro*. Tsitologiia Genetika 16, 38–42.
Putnam, K.P., Bombick, D., Doolittle, D.J., 2002. Evaluation of eight *in vitro* assays for assessing the cytotoxicity of cigarette smoke condensate. Toxicol. In Vitro 16, 599–607.
Repetto, G., del Peso, A., Zurita, J.L., 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat. Protoc. 3, 1125–1131.
Shahabuddin, M., Gopal, M., Vijayalaxmi, K.K., 2005. The genotoxicity of DNA intercalating drug 8-methoxy pyrimido [4′,5′:4,5]thieno(2,3-b)quinoline-4(3H)-one. Drug Chem. Toxicol. 28, 345–357.
Soloneski, S., Larramendy, M., 2010. Sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary (CHO-K1) cells treated with insecticide pirimicarb. J. Hazard. Mater. 174, 410–415.
Soloneski, S., González, M., Piaggio, E., Apezteguía, M., Reigosa, M.A., Larramendy, M.L., 2001. Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. I. Genotoxic evaluation on cultured human lymphocytes exposed *in vitro*. Mutagenesis 16, 487–493.
Soloneski, S., González, M., Piaggio, E., Reigosa, M.A., Larramendy, M.L., 2002a. Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. III. Genotoxic evaluation on Chinese hamster ovary (CHO) cells. Mutat. Res. 514, 201–212.
Soloneski, S., Reigosa, M.A., Larramendy, M.L., 2002b. Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. II. Clastogenesis on immunophenotyped human lymphocytes assessed by the micronucleus test. Environ. Mol. Mutagen. 40, 57–62.
Soloneski, S., A, R.M., Larramendy, M.L., 2003a. Effect of the dithiocarbamate pesticide zineb and its commercial formulation, the azzurro. V. Abnormalities induced in the spindle apparatus of transformed and non-transformed mammalian cell lines. Mutat. Res. 536, 121–129.
Soloneski, S., Reigosa, M.A., Larramendy, M.L., 2003b. Vitamin E prevents ethylene bis(dithiocarbamate) pesticide zineb-induced sister chromatid exchange in Chinese hamster ovary cells. Mutagenesis 18, 505–510.
Ündeger, Ü., Basaran, N., 2005. Effects of pesticides on human peripheral lymphocytes *in vitro*: induction of DNA damage. Arch. Toxicol. 79, 169–176.
USEPA, 1974a. Compendium of Registered Pesticides. US Government Printing Office, Washington, DC.
USEPA, 1974b. Pesticide Fact Sheet: Pirimicarb. US Government Printing Office, Washington, DC.
USEPA, 1996. Pesticide Fact Sheet: Zineb. US Government Printing Office, Washington, DC.
USEPA, 2004. ECOTOX: Ecotoxicology Database. USEPA/ORD/NHEERL, Mid-Continent Ecology Division. <http://www.epa.gov/ecotox/ecotox_home.htm>.
USEPA, 2006. Carbofuran I.R.E.D. Facts. U.S. Environmental Protection Agency, Office of Pesticide Programs, U.S. Government Printing Office, Washington, DC.
USEPA, 2010. Inert Ingredients Eligible for FIFRA 25(b) Pesticide Products Last Updated December 20, 2010 U.S. Environmental Protection Agency, Office of Pesticide Programs.
Vera Candioti, J., Natale, G.S., Soloneski, S., Ronco, A.E., Larramendy, M.L., 2010a. Sublethal and lethal effects on *Rhinella arenarum* (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida®. Chemosphere 78, 249–255.
Vera Candioti, J., Soloneski, S., Larramendy, M.L., 2010b. Genotoxic and cytotoxic effects of the formulated insecticide aficida on *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces: Poeciliidae). Mutat. Res. 703, 180–186.
Wang, P., Wang, H.-P., Xu, M., Liang, Y., Sun, Y., Yang, L., Li, L., Li, W., Wu, Y., 2014. Combined subchronic toxicity of dichlorvos with malathion or pirimicarb in mice liver and serum: a metabonomic study. Food Chem. Toxicol (in press).
Wildgoose, J., Howard, C.A., Richardson, C.R., Randall, V., 1987. Pirimicarb: a cytogenetic study in human lymphocytes *in vitro*. Central Toxicology Laboratory. Report No. CTL/P/1655, GLP, Unpublished.
Zeljezić, D., Garaj-Vrhovac, V., Perkovic, P., 2006. Evaluation of DNA damage induced by atrazine and atrazine-based herbicide in human lymphocytes *in vitro* using a comet and DNA diffusion assay. Toxicol. In Vitro 20, 923–935.
Zhang, G., Hu, X., Pan, J., 2011. Spectroscopic studies of the interaction between pirimicarb and calf thymus DNA. Spectrochim. Acta, Part A 78, 687–694.