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A combination of the cytokinesis-block micronucleus cytome assay and centromeric identification for evaluation of the genotoxicity of dicamba

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ABSTRACT

The purpose of this study was to further investigate the cytotoxic and genotoxic effects of dicamba and Banvel® employing the cytokinesis-block micronucleus cytome (CBMN-cyt) assay estimated by the analysis of the nuclear division index (NDI), the frequency of micronucleus (MN), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs). Besides, for mechanism of MN induction CREST anti-kinetochore antibody analysis was performed. The activities of both compounds were tested within the range of 50–500 μg/ml on Chinese hamster ovary (CHO-K1) cells. Overall, dicamba and Banvel® produced a NDI dose-dependent decrease but the response was statistically significant only in cultures treated with Banvel® at a 100–500 μg/ml concentration range. A dose-dependent induction of MN was observed after dicamba- and Banvel®-treatments within the 50–400 μg/ml and 50–500 μg/ml concentration-ranges, respectively. Induction of NPBs and NBUDs was significantly enhanced by both test compounds. The NPBs/MN ratio values found for dicamba and Banvel® were 0.04–0.11 and 0.05–0.18, respectively. Results clearly demonstrated that dicamba and Banvel® exerted both cyto- and genotoxic damage on CHO-K1 cells. Furthermore, the CBMN-cyt assay employed confirmed our previous investigations concerning the cellular and DNA damaging capabilities of dicamba and highlights that both clastogenic and aneugenic mechanisms are implicated in the MN induction.

1. Introduction

The massive liberation of chemical substances into the environment is a common feature worldwide. A large amount of these substances are herbicides, which accounted for the largest portion of world pesticide total consume reaching values higher than 5 billon pounds during 2000–2001 (Kiely et al., 2004).

Herbicides include a number of different types of inorganical and organical compounds that jeopardize the environment and human health. Exposure to some of these herbicides may lead to alterations in the genetic material thereby causing mutagenicity, carcinogenicity, teratogenicity, and immunotoxicity, among other side effects (Dearfield et al., 1999; IARC, 1977, 1999). The monitoring of the herbicides' genetic damaging capability and cytotoxic potential has been addressed applying a battery of both different endpoints *in vitro* and *in vivo* and target cellular systems from bacterial to human beings (Bull and Fletcher, 2006; EPA, 1974; IARC, 1991; Stevens and Sumner, 1991).

Auxinic herbicides were the first selective herbicides developed. Herbicides are classified as auxinic based on their growth-promoting effects observed in plant cell cultures, specific tissue systems (coleoptiles, roots), and in whole plants (González et al., 2007; Pipke et al., 1987). Generally, the auxinic herbicides are used to selectively control broadleaf weeds in grass crops such as cereal grains and turfgrass swards (Pipke et al., 1987; Reinbothe et al., 1996; Sterling and Hall, 1997). Although they continue to be a very important class of herbicides, their molecular mode of action has been recently characterized in *Arabidopsis thaliana* (Gleason et al., 2011). In plants, as it has been stated, these chemicals mimic the action of auxins mainly through manipulating the plant phytohormone responses (Gleason et al., 2011), but in mammals no mimic hormonal activity has been reported (Osterloh et al., 1983).

Among this family of herbicides, the 3,6-dichloro-2-methoxybenzoic acid, commonly known as dicamba, is a member of the benzoic acid family. It is a chlorinated benzoic acid-derivative compound registered in the United States as a post-emergent herbicide (EPA, 1983). It is produced in a variety of forms, including acid and different kinds of salts, e.g., dimethlylammonium salt, potassium salt, and sodium salt, among others (FAO, 2001). Dicamba is a selective systemic herbicide used against annual and perennial broad-leaved weeds and bush species (http://www.fao.org/ag/AGP/agpp/Pesticid/Specs/docs/Pdf/new/dicamba.pdf). Currently, there is a number of registered products containing

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dicamba; a majority of them for use in food producing crops (barley, rye, oat, triticale, wheat, grass pastures, grain sorghum, maize, rice, sugarcane, and potato). Other products are registered for use in non-food producing situations (mainly lawn, turf and recreational areas) (www.epa.gov/oppsrrd1/REDs/dicamba_red.pdf). This chlorobenzoic herbicide has also been widely employed throughout Argentina. According to Benbrook (2005), the use of dicamba has increased 157% since 2001 and the number of registered dicamba-containing commercial formulations has increased from 9 to 51 in the 1999–2009 decade (http://www.casafe.org). One of these formulations is Banvel®, a product applied to control broad leaves weeds resistant to 2,4-D or MCPA (http://www.syngenta.com.ar).

Although substantial information is available regarding dicamba's environmental and ecological impact (EPA, 1974, 1983; IARC, 1991), data regarding to its genotoxicity and cytotoxicity are scarce (For review, see Soloneski and Larramendy (2011) and references therein). Previously, we demonstrated that both dicamba and its Argentinean formulation Banvel® are DNA damaging agents since an enhancement of the frequency DNA-single strand breaks, sister chromatid exchanges, a delay in the cell-cycle progression, and a decrease of the mitotic activity were observed to occur on *in vitro* treated-mammalian cells (González et al., 2007, 2009).

In order to further elucidate the nature of the herbicide dicamba's and Banvel® cellular deleterious effects, in the current study we evaluated their potential to modify the mitotic status and to cause genomic instability employing the *in vitro* cytokinesis-block micronucleous cytome (CBMN-cyt) assay estimated by the analyses of the frequency of micronucleus (MN), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs) as well as the nuclear division index (NDI) on Chinese hamster ovary (CHO-K1) cells. Additionally, in order to differentiate between an aneugenic or clastogenic mechanism of MN induction, CREST anti-kinetochore antibody analysis was performed for centromere identification after herbicide treatment.

2. Materials and methods

2.1. Chemicals

Dicamba (3,6-dichloro-2-metoxybenzoic acid, CAS 1918-00-9), dimethyl sulfoxide (DMSO, CAS 67-68-5), cytochalasin B from *Dreschslera dematioidea* (Cyt-B, CAS 14930-96-2), were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Fluorescein isothiocyanate (FITC)-conjugated purified human anti-centromere proteins antibody was purchased from Antibodies Incorporated (Daves, Ca, USA). Banvel® (57.71%) was kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina).

2.2. Cell cultures and herbicide treatment for the cytokinesis-block micronucleous assay

CHO-K1 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 pre-cleaned 22 mm × 22 mm cover slips 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. Prior to use, dicamba was first dissolved in DMSO and then diluted in culture medium while Banvel® was directly diluted in culture medium. Both dicamba and Banvel® were diluted so that addition of 100 μl into cultures allowed reaching the required concentration described in Section 3 within the range of 50–500 μg/ml. 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 the cells 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) were run simultaneously with herbicide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Cultures were duplicated for each experimental point, in at least three independent experiments. The same batches of culture medium, serum and reagents were used throughout the study.

2.3. Slide preparations

At the end of the culture period, 500 μl acetic acid/methanol (1:6) were added to each dish for a 15 min prefixation period. Cells were then fixed with acetic acid/methanol (1:6) for 15 min. Afterwards, cells were stained with 3% Giemsa solution. The cover slips were air-dried and then placed down onto pre-cleaned slides using Depex mounting medium.

2.4. Examination of slides and assessment of 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 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 the total number of viable cells scored.

2.5. Micronucleus analysis

For the MN assay, at least 1000 binucleated (BN) cells were scored blind by one researcher at 600× magnification per experimental point from each experiment. The number of BN cells with none, one, two or three MN was determined in BN cytokinesis-block cells following the examination criteria reported by Fenech (2007). Briefly, the criteria employed in identifying MN were: diameter smaller than 1/3 of that of the main nuclei, non-refractibility, same staining intensity as or lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, MN boundary distinguishable from main nuclei boundary, and no more than 4 MN associated with the nuclei. Necrotic or apoptotic cells were not included in the scoring of BN cells according to criteria proposed by Fenech (2007).

2.6. Nucleoplasmic bridges and nuclear buds analysis

NPBs and NBUDs were scored blind by one researcher in 1000 BN cells at 600x magnification per experimental point from each experiment. Examination criteria followed those established elsewhere (Fenech, 2007). Briefly, continuous DNA-containing structures linking the nuclei were scored as NPBs when their width was smaller than 1/4th of the diameter of the nuclei and showed the same staining characteristics as the main nuclei. NBUDs were recognized as similar in appearance and staining intensity to MN but connected with the nucleus via a bridge that can be slightly narrower than the diameter of the bud or by a much thinner bridge. Additionally, the NPBs/MN ratio was calculated as proposed by Thomas et al. (2003). Accordingly, the total number of NPBs counted for a specific pesticide at a specific concentration was divided by the total number of MN counted for the same treatment. Then, whether an aneugenic effect prevails over a clastogenic activity, a minimum number of NPBs will be formed, and the NPBs/MN ratio will approach 0 while for severe clastogens the value increases over 0.70 (Thomas et al., 2003).

2.7. Micronucleus assay using CREST anti-kinetochore antibody analysis

Experiments were set up with cells cultured as explained in Section 2.2. Treatments with the test compounds were performed 24 h after plating with 50 μg/ml of both dicamba Banvel®. Results of previous experiments (see Section 2.2) showed that at this concentration of both dicamba and Banvel® is the lowest dose that significantly increased the frequency of MN without altering the NDI. Immediately after treatments, 3 μg/ml Cyt-B was incorporated into cultures and then the cells incubated at 37 °C in a 5% CO_2 atmosphere under a safety light for an additional 24 h period until harvesting. Negative (untreated cells and solvent vehicle-treated cells) and positive controls (0.04 μg/ml colchicine) were run simultaneously with pesticide-treated cultures. At the end of the culture period, cells were consecutively fixed with ice-cold (−20 °C) methanol (20 min) and acetone (10 min). Cells were then permeabilized with PBS containing 0.05% Tween 20 (3 × 5 min), followed by PBS containing 10% SDS and 0.1%Triton (1 × 5 min) and PBS containing 0.05% Tween 20 (3 × 5 min). Finally, cells were incubated with FITC-conjugated purified human anti-centromere antibody (1:50 in PBS containing 0.2% Tween 20) overnight in at 37 °C in a humidified box. Afterwards, cells were washed in PBS containing 0.1% Tween 20 (1 × 20 min) and counterstained with propidium iodide (1 μg/ml, 7 min), and finally mounted with an antifading medium (Vectaschield mounting medium H1000, Vector Laboratories, Burlingame, CA, USA). Slides were coded and scored at blind by one researcher. The frequency of cells with kinetochore-positive and kinetochore-negative micronucleus were determined in approximately 500 micronucleated interphases at each treatment concentration using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination and a DP71 Olympus integrated high-sensitivity monochrome charge-coupled device camera. A MN was classified as CREST-positive when at least one bright spot was clearly observed, while CREST-negative when no spots were observed within MN boundaries. Very week signals were not scored as a positive CREST signal. A positive CREST reaction reveals that the MN consists of one or more chromosomes and indicates primarily aneugenic effects whereas a negative CREST reaction indicates acentric fragments originated by a clastogenic effect (Miller and Adler, 1990; Olivero, 2008).

Table 1
Nucleoplasmic bridges (NPBs)/micronucleus (MN) ratios and nuclear division index (NDI) induced by dicamba and Banvel® in binucleated cytokinesis-blocked CHO-K1 cells.[a]

Concentration (μg/ml)	Dicamba[b]				Banvel®[b]			
	NPBs	MN	Ratio	NDI	NPBs	MN	Ratio	NDI
0	3.51	38.47	0.09	2.07	3.51	40.03	0.09	2.08
50	5.66	100.57***	0.06	2.00	7.10	96.36*	0.08	1.89
100	8.59	114.13***	0.08	1.99	10.45	108.11**	0.10	1.75*
200	8.56	137.11***	0.06	1.94	9.42	106.46**	0.09	1.48***
300	7.80	176.19***	0.04	1.95	17.85	101.91**	0.18	1.59***
400	18.98	165.05***	0.11	1.81	10.00	101.88**	0.10	1.52***
500	ND	6.30	113.96**	0.05	1.53***			

ND, not determined.
[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 values of NPBs or MN in 3000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments.
* $p < 0.05$; significant differences with respect to control values.
** $p < 0.01$; significant differences with respect to control values.
*** $p < 0.001$; significant differences with respect to control values.

2.8. Statistical analysis

Data were analyzed using the Statgraphics 5.1 Plus software. Analysis of variance (one-way ANOVA) was applied to compare data from the three independent experiments as total values of MN, NPBs and NBUDs/3000 BN cells between treated and control samples employing. Variables were tested for normality with Kolmogorov–Smirnov test and homogeneity of variances between groups was verified by Levene test. Pairwise comparisons between the different groups were made using the post hoc LSD test. Differences between NDI in CHO-K1-treated and control cells were evaluated by χ^2 test. The results obtained with the CREST analyses (% MN with positive signals in treated cultures versus % MN positive in control cultures) was analyzed by the χ^2 test. The relationship between the frequencies of MN, NPBs, and NBUDs and herbicide concentration data was evaluated by Spearman rank order linear correlation test. The chosen level of significance was 0.05, unless indicated otherwise.

3. Results

Since no difference of NDI, MN, NPBs and NBUDs frequencies were observed between untreated and negative controls (DMSO-treated cells) pooled data are presented for control values.

The NDI data from both dicamba- and Banvel®-treated BN cytokinesis-blocked CHO-K1 cells are presented in Fig. 1. Dicamba within the 50–400 μg/ml concentration range rendered a minor reduction in the NDI although not reaching statistical significance ($p > 0.05$) (Fig. 1, Table 1). The highest tested dicamba concentration produced severe cellular alterations that did not allow monitoring. Banvel® induced a significant NDI decrease in the 100–500 μg/ml concentration range ($p < 0.05$ and $p < 0.001$, for 100 and 200–500 μg/ml, respectively) (Fig. 1, Table 1). The delayed rate of NDI induced by both test compounds was due to a highly significant increased number of N_1 cells among all cells analyzed regardless of the herbicide concentration ($p < 0.001$) (data not shown). A correlation test showed that NDI decreased as a significant function of either the concentration of dicamba ($r = -0.96$, $p < 0.001$) or Banvel® ($r = -0.82$, $p < 0.01$) titrated into cultures. Furthermore, Fig. 1 shows the results of the analysis of herbicides-induced MN in BN cytokinesis-blocked cells. The frequency of MN was significantly increased when cells were exposed both to dicamba and Banvel® within the 50–400 ($p < 0.001$) and 50–500 μg/ml ($p < 0.05$ and $p < 0.01$, for 50 and 100–500 μg/ml, respectively) concentration range, respectively. A regression analyses showed a concentration-dependent increase in the frequency of herbicide-induced MN values for both dicamba ($r = 0.90$, $p < 0.001$) and Banvel® ($r = 0.62$, $p < 0.05$) (Fig. 1).

The frequencies of centromere-positive and centromere-negative MN induced by 50 μg/ml of both dicamba and Banvel® are depicted in Fig. 2. In negative controls, 270/548 (49.27%) out of the MN analyzed were centromere-positive whereas 278/548 (50.73%) were centromere-negative. When 0.04 μg/ml of colchicine was employed as positive control, an increased frequency of centromere-positive MN was observed (363/531; 68.36%) compared to negative control values ($p < 0.001$). On the other hand, 168/531 (31.64%) MN were CREST centromere-negative ($p < 0.001$). The frequency of centromere-positive MN in those dicamba- and Banvel®-treated cultures did not differ from that of negative control values ($p > 0.05$). After treatment with 50 μg/ml of dicamba or banvel®, 282/540 (52.26%) and 253/508 (49.80%) out of the MN showed centromere-positive signals, respectively (Figs. 2 and 3B–D).

The results of NPBs and NBUDs scored in BN cells are summarized in Fig. 4A and B, respectively. Overall, either dicamba and Banvel® induced an increase in the frequency of NPBs for all tested concentrations, but reaching statistical significance within the 100–400 μg/ml concentration range ($p < 0.05$ – $p < 0.001$) (Fig. 4A). The enhancement of NPBs showed a statistical association in regard to the herbicide concentration titrated into cultures for dicamba ($r = 0.85$, $p < 0.001$) but not for Banvel® ($r = 0.29$, $p > 0.05$). Both dicamba and Banvel® induced a significant enhancement of the frequency of NBUDs for all concentrations assessed ($p < 0.001$) (Fig. 4B). The NBUDs enhancement showed significant positive correlation with the concentration of both chemicals ($r = 0.90$, $p < 0.001$, and $r = 0.57$, $p < 0.05$ for dicamba and Banvel®, respectively).

The correlation matrix for the different frequencies of the DNA biomarkers (MN, NBUDs, NPBs) induced by dicamba and Banvel® is presented in Table 2. A correlation analysis revealed a significant dependence between the frequencies of MN, NPBs and NBUDs and the concentration of both compounds ($r = 0.60$–0.99; $p < 0.05$ – $p < 0.001$, and $r = 0.49$–0.92; $p < 0.05$ – $p < 0.001$, for dicamba and Banvel®, respectively).

The NBUDs/MN ratios for dicamba and Banvel® are shown in Table 1. Results revealed that ratios varied between 0.04 and 0.11 upon 50–400 μg/ml dicamba concentration range. In the case of Banvel®, the ratios varied from 0.05 up to 0.18 within the 50–500 μg/ml concentration range.

Table 2
Correlation matrix for DNA damage biomarkers of the CBMN assay induced by dicamba and Banvel®.[a]

	Dicamba			Banvel®		
		MN	NPBs		MN	NPBs
NBUDs	*r*	0.99	0.60	*r*	0.70	0.92
	p	0.001	0.05	*p*	0.01	0.001
NPBs	*r*	0.67		*r*	0.49	
	p	0.05		*p*	0.05	

r, Spearman correlation coeficient; *p*, probability.
[a] CHO-K1 cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later.

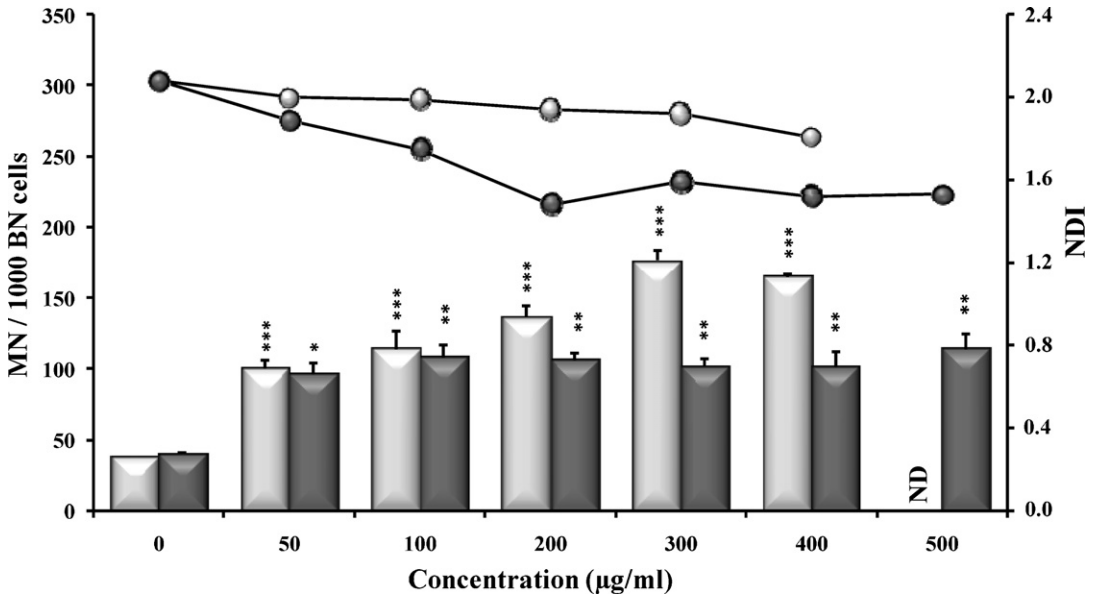

Fig. 1. Effect of dicamba (light grey) and its commercial formulation Banvel® (dark grey) on MN induction (bars) and NDI (circles) in binucleated cytokinesis-blocked CHO-K1 cells. CHO-K1 cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later. Results are presented as number of cells carrying 1, 2 or 3 MNs among 3000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments. ND, not determined. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, significant differences with respect to control values.

4. Discussion

The purpose of this study was to further investigate the effects of dicamba and one of its technical formulation as cellular and DNA damaging agents employing the CBNM-cyt assay and its combination with CREST anti-kinetochore antibody analysis for centromere identification to determine the origin of MN after herbicide treatment.

The CBMN-cyt assay has been validated as a sensitive method for measuring altered cellular viability preventing confounding effects caused by suboptimal cell division kinetics (Corvi et al., 2008; Fenech, 2006; Kirsch-Volders et al., 2011). In our study, besides the analysis of the mitotic status, the score of MN and two chromatin instabilities namely NPBs and NBUDs was included. Our results demonstrated that both dicamba and Banvel® produced a decrease in the NDI of CHO-K1 treated cells, but the response was statistically significant only in those Banvel®-treated cultures within the 100–500 μg/ml concentration range. Furthermore, either dicamba or its technical formulation significantly enhanced the MN, NPBs and NBUDs frequencies. These findings not only corroborate previous observations on the cellular toxicity of dicamba (González et al., 2006, 2007, 2009) but also highlight that both tests compounds inflict primary DNA damage.

Dicamba has previously been examined in a number of cyto- and genotoxicity assays. Recently, we have clearly evidenced its damaging activity in different mammalian cell targets (González et al., 2007, 2009). In the present report the mitotic status has been assessed by the NDI analysis. Surprisingly, the current observations were not consistent with our previous studies performed also on CHO-K1 cells. In the latter, cytotoxicity, when measured as cell-cycle kinetics, yielded a significant delay in the cell-cycle progression induced by 200 and 500 μg/ml of dicamba whereas Banvel® exerted a similar effect only at a 500 μg/ml concentration (González et al., 2007). These inconsistencies might be explained attaining to the methodologies applied. The cell-cycle progression analysis requires scoring a minimum of 100 metaphase cells whereas for the CBMN-cyt assay, and depending on the endpoint, 500 or 1000 BN cells have to be monitored. It has not escaped to our attention a possible intra-laboratory variation concerning cell viability in CHO-K1 cell cultures. In our 2007 study we reported no cell viability alteration using the ethidium bromide/acridine orange even when 500 μg/ml of dicamba and Banvel® were assessed (González et al., 2007). The same assay performed in the present investigation revealed a higher viability of those CHO-K1 cells than previously reported when treated with up to 200 μg/ml of dicamba and Banvel®. However, the viability in 500 μg/ml dicamba treated cultures decreased approximately 25% in regard to control values (data not shown), which limited, then, the number of BN cells available for monitoring. Such limitation is also corroborated by the decrease of N_2 cells induced by 400 μg/ml of dicamba found in the present study. According to the criteria proposed by Fenech (2007), BN cells, besides being numerically enough, must also exhibit viability features allowing the identification of nuclear and cytoplasmatic structures (Fenech, 2007). Cell cultures set up for the CBMN-cyt assay and treated with the highest concentration of dicamba showed a much altered cell morphology and thus did not render a sufficient BN cell number to analyze the MN, NPB, and NBUDs frequencies. Under the experimental conditions of the present report, the N_2 cell proportion and the NDI as biomarkers for mitotic status appeared to be more informative end points than cell-cycle progression for cytotoxicity assessment.

Since originally proposed in 1985 (Fenech and Morley, 1985), the MN assay in BN cells has evolved in a “cytome” that measures DNA damage events (Fenech, 2007; Kirsch-Volders et al., 2011). Our results revealed a marked genotoxic effect of dicamba measured by the MN, NPBs and NBUDs induction regardless of the concentration of the test compound assessed. Furthermore, a positive correlation between MN, NPBs and NBUDs was found and suggests a common mechanism initiated by dicamba-DNA induced breaks, an effect revealed previously in our previous study (González et al., 2007). Similar statistical associations have been reported in several investigations, e.g., MN/NBUDs in *in vivo* and *in vitro* human lymphocytes exposed to mitomycin C, colcemid or between smokers and non-smokers (Serrano-García and Montero-Montoya, 2001), MN/NBUDs/NPBs in human lymphocytes cultured under folic acid deficiency

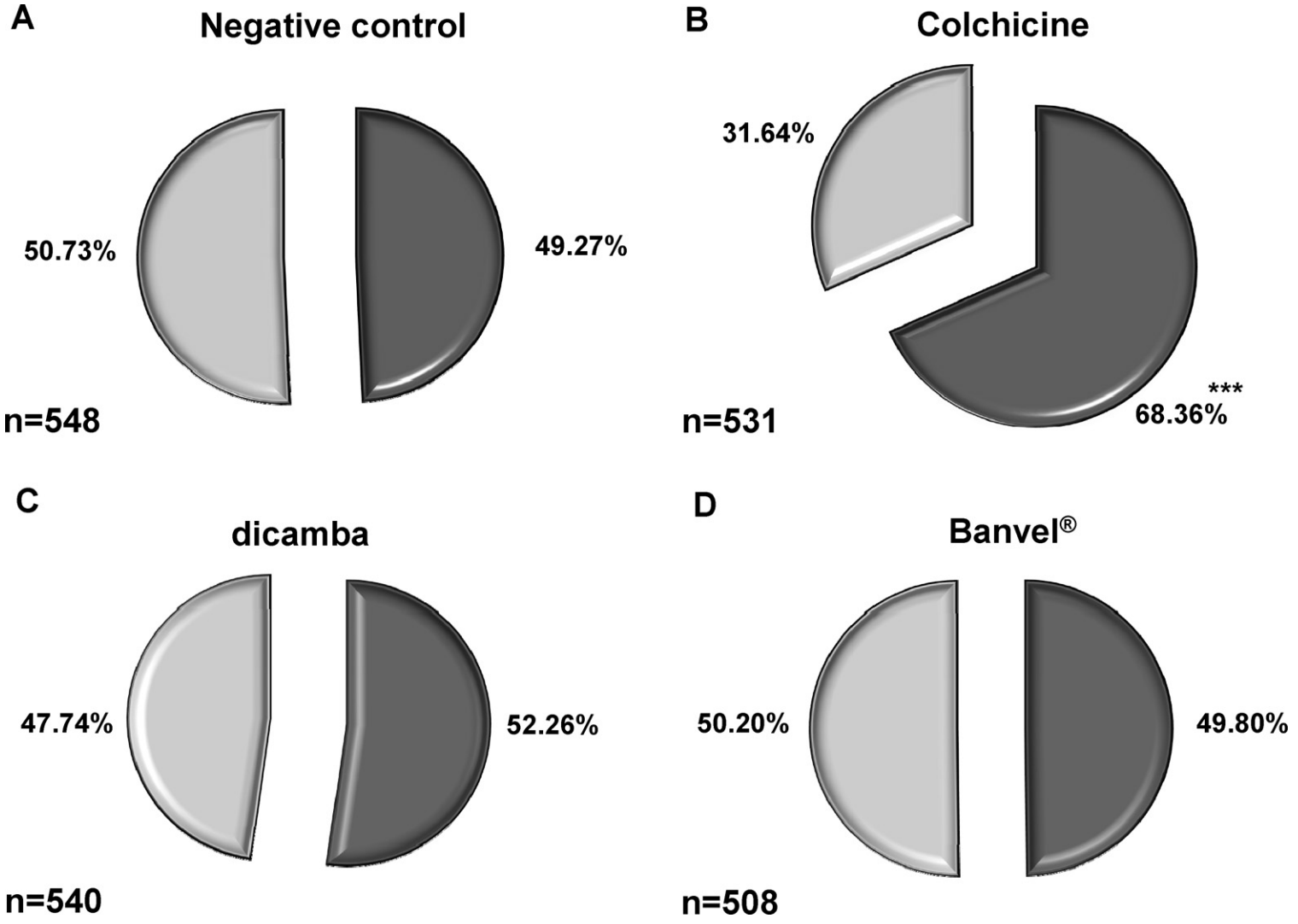

Fig. 2. CREST analysis of MN induced by 50 μg/ml of dicamba and Banvel®. Colchicine (0.04 μg/ml) was employed as positive control. Percentage of CREST-positive (dark grey) and CREST-negative (light grey) distribution. CHO-K1 cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later. Results are presented as number of MN in 500 binucleated cytokinesis-blocked cells of pooled data from three independent experiments. ***$p < 0.001$; significant differences with respect to control values.

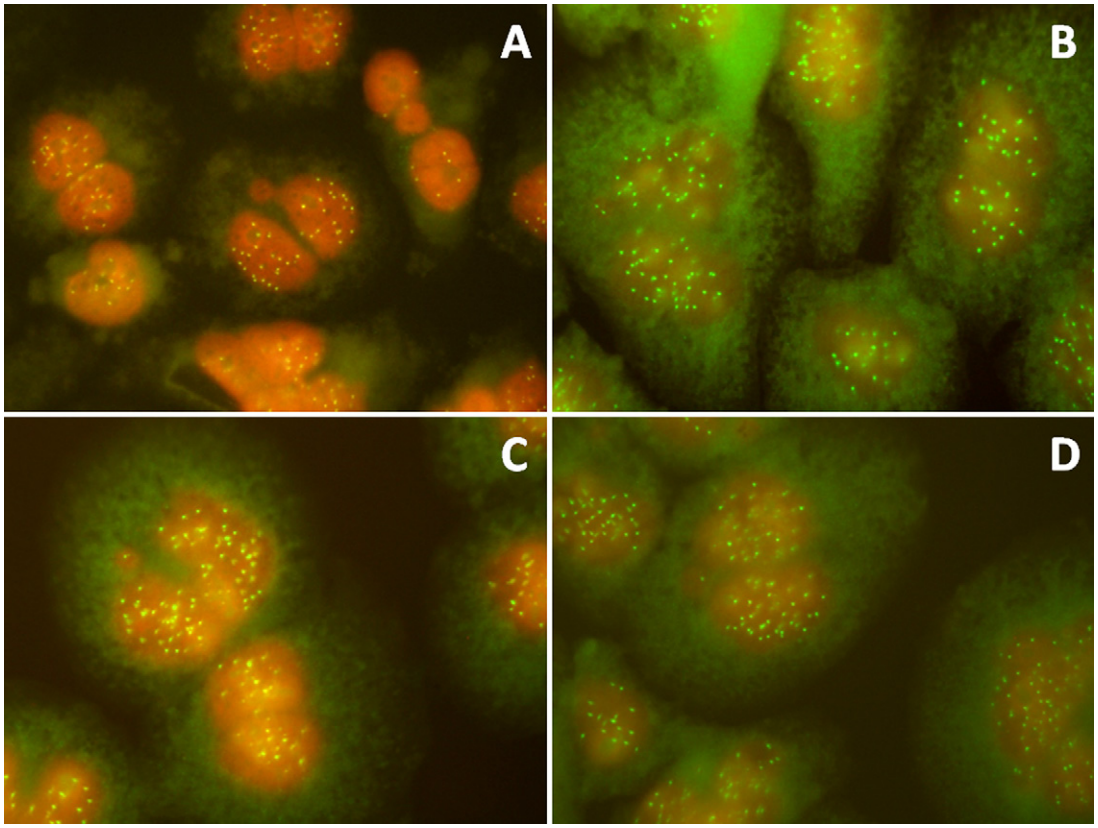

Fig. 3. Centromere-negative (A) and centromere-positive (B–D) micronucleated BN CHO-K1 cells after dicamba (A and B) and Banvel® (C and D) treatment. CHO-K1 cells were treated 24 h after seeding with 50 μg/ml of dicamba and Banvel® followed by cytochalasin B, and harvested 24 h later. After fixation, cells were incubated with FITC-conjugated purified human anti-kinetochore antibody and counterstained with propidium iodide.

(Fenech and Crott, 2002), under oxidative stress (Umegaki and Fenech, 2000), or exposed to pesticides (Mladinic et al., 2009).

MN may result from a large spectrum of genetic mechanisms due to exposure to clastogens or aneugens (i.e. acentric chromosome/chromatid fragments resulting from DNA breakage events, NPBs formation/breakage, gene amplification via breakage-fusion-bridge cycles) (Kirsch-Volders et al., 2011). The use of methodologies such as DNA probes also allows discerning the origin of MN and NPBs (Thomas et al., 2003) Fenech (2007). Furthermore, the use of the NPBs/MN ratio proposed by Thomas et al. (2003) could allow us to discern between both possibilities on the origin of dicamba- and Banvel®-induced MN. This ratio assumes that NPBs predominantly originate from complex chromosome rearrangements that occur only as the result of clastogenic effect, while MN could be induced by clastogenic as well as aneugenic activity. If the aneugenic effect prevails over clastogenic, the minimum number of NPBs will be formed, and the NPBs/MN ratio will approach 0, while for severe clastogens the value increases over 0.70. The ratios found for dicamba and Banvel® reached values of 0.04–0.11 and 0.05–0.18, respectively. Then an aneugenic effect for both test compounds could be suggested. However, this possibility was investigated when the possible origin of the MN induction was further analyzed when using CREST antibody. The CBMN-cyt assay in combination with CREST antibody that specifically stain kinetochore proteins has been widely employed (Benameur et al., 2011; Dorn et al., 2008; Parry and Parry, 2006). It distinguishes MN containing one or several whole chromosomes, which are positively labeled (centromere positive MN, due to aneugenic effect), or acentric chromosome fragments, which are unlabeled due to the absence of centromere (centromere negative MN, due to clastogenic effect). To further extend our investigation on the nature of the genotoxic capability of dicamba, we assessed the occurrence of centromeric signals in the MN by the CREST technique. Our analysis revealed an equivalent percentual distribution of 52.20% and 47.74% of CREST-positive and CREST-negative dicamba-induced MN, respectively. In regard to the nature of the genotoxic capability of the commercial formulation our results led to similar outcomes: 49.80% and 50.20% of CREST-positive and CREST-negative MN, respectively, and NPBs/MN ratio values in a 0.05–0.18 range. In the context of the present results, we could demonstrate that dicamba, and to the same extend Banvel®, induced MN through a clastogenic as well an aneugenic effect, at least on CHO-K1 cells.

In good agreement with the putative origin of dicamba-induced MN by an aneugenic effect, several investigations on the effect of dicamba on cellular microtubular structures of *Tradescantia* sp. and *Nicotiana tabacum* have been reported. Mohammed and Ma, 1999 report that this benzoic derivative induces MN in *Tradescantia*. These authors suggest, in accordance with Wuu and Grant (1966, 1967), that dicamba interferes with the normal spindle function. Similarly, Ovidi et al. (2001), an Italian commercial formulation (Banvel 21S, 0.35 mg dicamba/ml) produced partial alterations in the microtubular apparatus of *N. tabacum*'s generative cells. It must be also taken in consideration that plants and animals present differential capability for herbicides' activation (Plewa et al., 1984), which has been attributed to the generation of different genotoxic metabolites and their responses to the spindle disruption (Dimitrov et al., 2006). To the best of our knowledge, no studies dealing with the

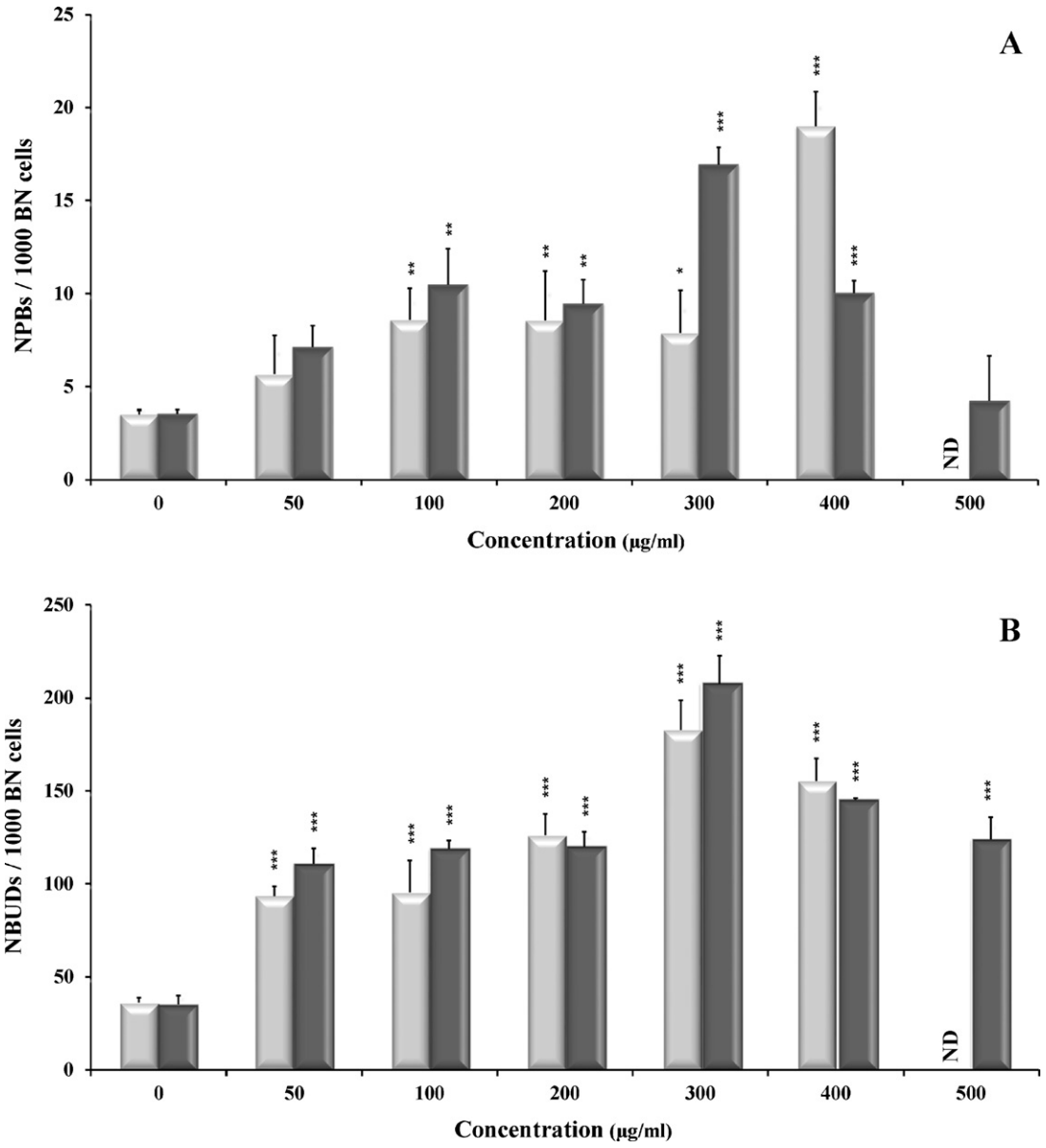

Fig. 4. Effect of dicamba (light grey bars) and its commercial formulation Banvel® (dark grey bars) on NPBs (A) and NBUDs (B) induction in binucleated cytokinesis-blocked CHO-K1 cells. CHO-K1 cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later. Results are presented as mean NPBs or NBUDs per 3000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments. ND, not determined. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values.

effect of dicamba on mitotic spindle in animal cells have been reported.

We employed the commercial mixture Banvel®, the form in which it is applied in agriculture and introduced into the environment. Furthermore, as transgenic plants resistant to dicamba have already been developed (Behrens et al., 2007) and there are a growing number of glyphosate resistant weeds, the use of alternative herbicides containing dicamba will likely gain increased usage (Gleason et al., 2011). In previous studies we employed several endpoints to assess the cyto- and genotoxic capacities of this technical formulation. In particular, DNA damage measured by the sister chromatid exchange (SCE) frequency rendered significantly higher SCE values when compared to dicamba's values (González et al., 2009). This situation was again noticed by us in the current study. Although the commercial formulation contains 57.71% of dicamba, the DNA lesions exerted by Banvel® were of similar level than that induced by dicamba alone, and even greater depending on the endpoint, e.g. induction of NBUDs. Therefore these data strongly suggest that the excipients contained in the field formulation resulted in an increase of cyto- and genotoxicity beyond dicamba itself. This in agreement with investigations addressing comparatively the toxicity of pure compounds and their commercial formulations (Brand and Mueller, 2002; González et al., 2006, 2007; Peixoto et al., 2008; Sanchez et al., 2006; Soloneski et al., 2002, 2008; Zeljezic et al., 2006). Furthermore, we have suggested that dicamba damaging mechanism is mediated by oxidation i.e. reactive oxygen species delivery whereas Banvel® exerts its deleterious effect by a different mechanism rather than only through reactive oxygen species generation (González et al., 2009).

5. Conclusions

Four conclusions can be drawn from the present report. First, regardless of the mechanism of damage, the CBNM-cyt assay

has proved to be an effective tool to investigate cellular and nuclear alterations induced by dicamba and its commercial formultation Banvel®. Secondly, the CBNM-cyt methodology and its combination with CREST anti-kinetochore antibody analysis for centromere identification allowed us to reveal that dicamba and Banvel® induced MN through both a clastogenic and an aneugenic mechanisms. Third, although previous investigations assessing the damaging potential of dicamba have revealed conflicting or even opposite outcomes, the results of the present study clearly demonstrated that this compound induces genomic instability and alters the mitotic status in CHO-K1 cells. Four and last, the current results corroborated previous findings indicating that Banvel® exerts both cyto- and genotoxic effects, even in a greater extent than the pure compound. The present outcomes regarding the commercial formulation highlight that pesticide mixtures (active plus inert ingredients) have effects that cannot be accurately predicted by using data about active ingredients alone.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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