

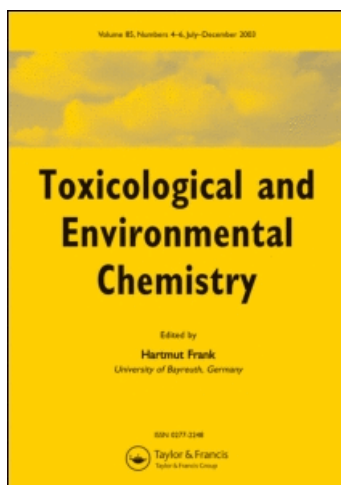
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Toxicological & Environmental Chemistry

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713653210>

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Online publication date: 19 August 2010

To cite this Article Molinari, Gabriela , Soloneski, Sonia , Reigosa, Miguel Antonio and Larramendy, Marcelo Luis(2010) 'Genotoxic and cytotoxic *in vitro* evaluation of ivermectin and its formulation ivomec® on *Aedes albopictus* larvae (CCL-126™) cells', *Toxicological & Environmental Chemistry*, 92: 8, 1577 — 1593

To link to this Article: DOI: 10.1080/02772241003625284

URL: <http://dx.doi.org/10.1080/02772241003625284>

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Genotoxic and cytotoxic *in vitro* evaluation of ivermectin and its formulation ivomec® on *Aedes albopictus* larvae (CCL-126™) cells

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(Received 5 January 2010; final version received 3 February 2010)

Genotoxic effects of ivermectin (IVM) and its commercial formulation ivomec® (IVM 1.0%) were studied on *Aedes albopictus* larvae (CCL-126™) cells by sister chromatid exchange (SCE) and single cell gel electrophoresis (SCGE) while cytotoxicity was determined by cell-cycle progression (CCP), proliferative rate index (PRI), mitotic index (MI), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and neutral red (NR) endpoints within a 1–250 µg mL⁻¹ concentration range. While IVM and ivomec® did not markedly affect SCE frequencies, these agents induced DNA-strand breaks enhancing both slightly damaged and damaged cells at 25–50 and 5–50 µg mL⁻¹ IVM and ivomec®, respectively. Both compounds exerted a delay in CCP and reduction of PRI at 10 µg mL⁻¹. Cytotoxicity was observed at concentrations higher than 25 µg mL⁻¹. A marked reduction of about 98% and 94% of MI compared to controls was noted with 25 µg mL⁻¹ of IVM and ivomec®, respectively. NR and MTT assays revealed that both compounds induced a cell growth inhibition within the 1–250 µg mL⁻¹ concentration range. Data indicated that IVM and ivomec® exert both genotoxicity and cytotoxicity in insect cells *in vitro*, at least in *A. albopictus* larvae CCL-126™ cells.

Keywords: comet assay; SCE assay; cell-cycle kinetics; MTT assay; neutral red assay; mosquito cells; cell growth inhibition

Introduction

Ivermectin (IVM) is an antibiotic originally isolated from the mycelia of a new species of actinomycetes, *Streptomyces avermectinius* sp. nov. (www.kitasato.or.jp). IVM is a member of a family of 16-membered macrocyclic lactones, named avermectins A_{1a}–B_{2b} (Omura and Crump 2004; Omura 2008) in which those members of the A series, with a 5-methoxy group, are markedly less active than those of the B series, possessing a 5-hydroxy group. Afterwards, the antibiotic was refined, and after reduction of the 22,23-olefin of the most active avermectins, namely B_{1a} and B_{1b}, a 22,23-di-hydro B₁ complex (mixture of 80% B_{1a} and 20% B_{1b}) was developed. The compound was then marketed as a veterinary drug under the generic and non-proprietary name IVM, proving to be both the most potent and safest avermectin derivative (Chabala et al. 1980).

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IVM demonstrated activity against not only to endoparasites but also to ectoparasites, being the first so-called “endectocide” agent and becoming an effective, potent systemic antiparasitic agent (Chabala et al. 1980; Kita et al. 2007). Thus, IVM has been recognized globally as one of the most important endectocide drugs in animal and human health (www.drugs.com). While IVM is effective against a wide range of nematodes, arachnids, and insects, this drug is not useful against flat helminthes, protozoa, bacteria, and fungi (www.inchem.org). IVM is characterized by possessing a unique mode of action indicating a lack of cross-resistance with other existing compounds. IVM produces parasite paralysis by ingestion or contact and eventually death of the invertebrate and several sublethal metabolic disorders (Campbell and Benz 1984). The drug inhibits the neurotransmission, interfering then with neuromuscular synapses. Basically, there is substantial evidence that IVM among vertebrates enhances the release of the neurotransmitter γ -amino butyric acid (GABA) from the neurons simultaneously enhancing the binding of GABA to its postsynaptic receptors, leading to the consequent opening of chloride channels and decreased cellular functions. In invertebrates, IVM targets the glutamate-gated chloride channels located at neuromuscular junctions, independently of GABA since GABA channels are ubiquitous from mammals, thereby paralyzing pharyngeal and somatic muscles (Campbell and Benz 1984; Omura 2002). Moreover, McCavera et al. (2007) recently suggested that ligand-gated chloride channels, including both the glutamate-(GluCl) and GABA-gated channels, are the targets of the macrocyclic lactones family in laboratory models, such as *Caenorhabditis elegans* and *Drosophila melanogaster*.

The Environmental Protection Agency (EPA) (www.epa.gov) has not reached any conclusion about the status of this compound in terms of its putative effects on humans and other species. However, a unique member of the avermectins family, the abamectins has been classified by the EPA as a class II toxicity pesticide with moderately toxic effect for organisms (www.epa.gov). Since both human cancer epidemiology and positive rodent cancer bioassays data have not been reported, the International Agency on Research of Cancer (IARC) has not listed this antibiotic as carcinogen so far (www.iarc.fr).

Cytogenetic studies on established insect cell lines are limited with regard to those performed on mammalian cell lines, and most investigations provide information on morphological and chromosomal features (Bolzán et al. 1998, 2000). However, several studies on other cytogenetic aspects of cultured insect cells have been reported, including the effects of mutagens and carcinogens estimated using different endpoints for genotoxicity and cytotoxicity. Furthermore, it was demonstrated that among insects, mosquito cells cultures provide a useful *in vitro* system for studying deleterious effects induced by xenobiotics (Mukherjee et al. 1986; Bolzán et al. 1998, 2000). Investigations demonstrated that DNA damage induced by different chemicals was found to be less extensive and repaired more efficiently in *Aedes albopictus* larvae cell lines compared to Chinese hamster ovary (CHO-K1) cells (Bianchi and López-Larrazza 1991; López-Larrazza et al. 2006). It has been proposed that either variation in chromatin structure or different levels of glutathione (GSH) might play a prominent role in the high resistance to many DNA damaging agents by mosquito cells compared to mammalian cells (Bianchi and López-Larrazza 1991; Wei et al. 1993).

To the best of our knowledge, no studies on the geno- and cytotoxic effects exerted by IVM have been reported both for humans and for the major vector organisms in which the compound is employed worldwide to prevent parasitosis in their host.

Thus far, Molinari et al. (2009) evaluated the *in vitro* genotoxic and cytotoxic effects of the IVM and one of its commercial formulation ivomec® on CHO-K1 cells employing several cytogenetic endpoints. Results showed that while IVM and ivomec® did not modify sister chromatid exchange (SCE) frequencies, these compounds induced single DNA-strand breaks as evidenced by the comet assay. Furthermore, both compounds exerted a delay in cell-cycle progression (CCP), cytotoxicity, alteration in the mitotic activity, and cell growth inhibition.

Knowing the differential mechanism of action of IVM on mammal and invertebrate cells, the aim of this study was to determine if mosquito cells are sensitive to the antiparasitic action of IVM. Several endpoints were employed for both genotoxicity including the SCE and single cell gel electrophoresis (SCGE) and for cytotoxicity CCP, mitotic index (MI), proliferative rate index (PRI), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and neutral red (NR) to analyze the effects exerted on *A. albopictus* larvae CCL-126™ cells by IVM and ivomec® (IVM 1%).

Materials and methods

Chemicals

IVM (22,23-dihydroavermectin B1, CAS 70288-86-7) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ivomec® (IVM 1.0%) was kindly provided by Merial Argentina S.A. (Buenos Aires, Argentina). Colchicine (CAS 64-86-8), ethanol (CAS 64-17-5), dimethyl sulfoxide (DMSO, CAS 67-68-5), neutral red dye (NRD, CAS 553-24-2), 5-bromo-2'-deoxyuridine (BrdU, CAS 59-14-3), and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, CAS 57360-69-7) were purchased from Sigma Chemical Co. Bleomycin (BLM) (Bleomycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina).

Cell cultures and pesticide treatment for SCE and CCP assays

Aedes albopictus larvae (CCL-126™, American Type Culture Collection, Manassas, VA, USA) cells were grown in Mitsuhashi and Marmorosch culture medium (Sigma Chem. Co.) supplemented with 20% fetal calf serum (FCS) (Gibco Grand Island, NY, USA), 100 units mL⁻¹ penicillin (Gibco) and 100 µg mL⁻¹ streptomycin (Gibco) at 28°C in a 5% CO₂ atmosphere. Experiments were set up with cultures in the log phase of growth. The cells were seeded in T25 flasks at a density of 10⁶ cells per flask. Treatments with the test compounds were performed 24 h after plating. Prior to use, IVM was first dissolved in DMSO and then diluted in culture medium while ivomec® was directly diluted in culture medium. Both test compounds were diluted so that addition of 100 µL into cultures reaching the required concentration specified in Results section within the range 0–250 µg mL⁻¹. The final solvent concentration was <1% for all treatments in the different experiments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (1 µg mL⁻¹ BLM-1 h pulse treatment) were run simultaneously with pesticide-treated cultures. BLM was selected due to its capacity of introduce both single- and double-strand breaks into DNA and to induce SCE in different *in vitro* mammalian cell systems (Larramendy et al. 1989; Bolzán and Bianchi 2004; Molinari et al. 2009; Sánchez et al. 2009). None of the treatments produced significant pH changes in the

culture medium. Thereafter, $10\text{ }\mu\text{g mL}^{-1}$ BrdU was incorporated into cultures and then the cells incubated at 28°C in a 5% CO_2 atmosphere under a safety light for an additional 24 h period until harvesting. Cultures were duplicated for each experimental point, in at least three independent experiments. The same batches of culture medium, sera, and reagents were used throughout the study.

Chromosome preparations

During the last 5 h of culture, the cells were treated with $0.2\text{ }\mu\text{g mL}^{-1}$ colchicine. Cells were detached, collected by centrifugation, hypotonically shocked (0.075M KCl, RT, 20 min), and fixed in methanol/acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique.

FPG method for sister chromatid differentiation

Chromosome spreads were stained using the fluorescence plus giemsa (FPG) technique for sister chromatid differentiation as previously described elsewhere (Larramendy and Knuutila 1990). Slides were coded and scored blind by one researcher.

Cell-cycle kinetics and MI

A minimum of 100 metaphase cells per sample were scored to determine percentage of cells that had undergone one (M_1), two (M_2), and three (M_3) mitoses. The PRI was calculated for each experimental point as previously described (Lamberti et al. 1983). The MI was determined by scoring 1000 cells from each experimental point and expressed as number of mitoses among 1000 nuclei. Changes in the MI were expressed as a factor (f) of the mean MI from treated cultures (MI_t) over the mean MI from controls (MI_c) ($f = \text{MI}_t/\text{MI}_c$) (Miller and Adler 1989).

SCE analysis

A total of 25 well-spread diploid M_2 cells metaphases were scored per experimental point from each treatment. The data are expressed as the mean number of SCE per cell $\pm$ S.E. from three independent experiments.

NR assay

The NR assay is based on the initial protocol described by Borenfreund and Puerner (1985). Briefly, 2.5×10^4 CCL-126TM cells mL^{-1} was cultured in Mitsuhashi and Marmorosch culture medium on 96-well microplates for 24 h. Then, the culture medium was removed and the cells were treated with IVM and ivomec[®] within a range $0\text{--}250\text{ }\mu\text{g mL}^{-1}$ for 24 h. A 0.5% ethanol-treated and 1% DMSO-treated cells were used as positive and negative controls, respectively. Following exposure with tested compounds, cells were incubated for an additional 2 h period in the presence of $100\text{ }\mu\text{g mL}^{-1}$ NR dissolved in serum free medium. The absorbance at 540 nm was recorded using the microplate spectrophotometer system (7530 Microplate

reader, Cambridge Technology Inc., USA). Results were expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel.

MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

The methodology was performed following the technique described in detail by Kosmider et al. (2004). A 2.5×10^4 CCL-126TM cells mL⁻¹ were cultured in Mitsunashi and Marmorosch medium culture for 24 h on 96-well microplates. Afterwards, the culture medium was removed and the cells were treated with IVM and ivomec[®] within a range of 0–250 µg mL⁻¹ for 24 h. A 0.5% ethanol-treated and 1% DMSO-treated cells were used as positive and negative controls, respectively. Following exposure with tested compounds, 100 µL of MTT (2 mg mL⁻¹) were added for an additional 2 h period. Then, the formazan crystals were dissolved in 100 µL of DMSO. Absorbance at 550 nm was measured with a spectrophotometer (7530 Microplate reader, Cambridge Technology Inc. USA). Results were expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel.

Cell cultures and pesticide treatment for SCGE

Prior to drug treatment, nearly confluent CCL-126TM cells were detached, centrifuged, and then resuspended in complete culture medium. Then, aliquots containing 2×10^5 cells mL⁻¹ were incubated for 80 min at 28°C in a 5% CO₂ atmosphere in culture medium containing the test compounds. Both IVM and ivomec[®] were used at a final concentration between 5 and 50 µg mL⁻¹. The final solvent concentration was <1% for all the treatments in all experiments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (10 µg mL⁻¹ BLM) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Thereafter, the cells were washed twice with insecticide-free complete culture medium. The SCGE and cell viability assays were performed immediately after the 80 min treatment. Cultures were duplicated for each experimental point, during at least three independent experiments. The same batches of cultures medium, sera, and reagents were used throughout the study.

Cell viability assay

At the end of the culture period, cell viability was determined using the ethidium bromide/acridine orange assay as described elsewhere (González et al. 2009). Briefly, one aliquot of 5 µL of a 1:1 freshly prepared mixture of ethidium bromide (100 µg mL⁻¹) and acridine orange (100 µg mL⁻¹) was mixed with 50 µL of the cell suspension under study. Afterwards, the cells were analyzed using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. At least 500 cells were counted per experimental point from each experiment, and the results expressed as percentage of viable cells among all cells.

SCGE assay

The SCGE assay was performed following the alkaline procedure described by Molinari et al. (2009). Briefly, two solutions containing 0.5% normal melting agarose (NMA) and 0.5% low-melting agarose (LMA) solution in Ca^{2+} - Mg^{2+} -free PBS were performed. Seventy-five μL NMA were transferred onto 100% ethanol pre-cleaned slide, spread evenly, and placed at 28°C for 20–30 min. Afterwards, 95 μL of LMA together with 7×10^3 cells (20 μL cell suspension + 75 μL LMA) was applied, covered with a coverslip and placed at 4°C for 15 min. After this layer had solidified, a third layer of 75 μL LMA was added and slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Tris pH 10, 1% Triton X-100, 10% DMSO) and then lysed at darkness for 2 h period (4°C). When this period concluded, slides were placed in a horizontal electrophoresis buffer (1 mM Na_2EDTA , 300 mM NaOH, pH 13, 20 min, 4°C) to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 30 min at 0.8 V cm^{-1} . Finally, the slides were neutralized with a solution comprising 0.4 M Tris-HCl, pH 7.5, stained with 4-6-diamino-2-phenylindole (DAPI) (Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Slides were coded and scored blind by one cytogenetist. Analysis of the slides was performed under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The cellular nucleus diameters of the nucleus plus migrated DNA were individually measured using a calibration scale with an X100 fluorescence objective from 50 randomly selected cells per experimental point for each experiment. Cells were visually graded into three categories as suggested elsewhere depending on DNA damage level as undamaged, slightly damaged, and damaged, respectively (González et al. 2003) and expressed as mean values of cells $\pm$ S.E. from each cellular category of three independent experiments.

Statistical analysis

The two-tailed Student's *t*-test was used to compare SCE frequencies, MTT and NR data between treated and control groups while a χ^2 -test was employed for CCP data. The non-parametric Kruskal–Wallis and Tukey tests were used to compare the effect of a pesticide over control cells in comet assay experiments while individual comparisons between pairs of data were performed using the Mann–Whitney test. The level of significance chosen was 0.05 unless otherwise indicated.

Results

Since no difference of SCE, CCP, PRI, MI, MI factor, cell viability, and SCGE values were observed between untreated and negative controls (untreated and DMSO-treated cells), pooled data are presented for these control values.

Figure 1 summarizes the results of the analysis of IVM- and ivomec[®]-induced SCE as well as the BLM-treated (positive control) in CCL-126TM cells. As expected, results revealed statistically significant differences between negative (untreated and DMSO-treated cells) and positive controls. No marked enhancement of SCE frequencies were induced when cells were incubated with $1\text{--}10 \mu\text{g mL}^{-1}$ of IVM and ivomec[®], respectively. When $25 \mu\text{g mL}^{-1}$ of either IVM or ivomec[®] was employed,

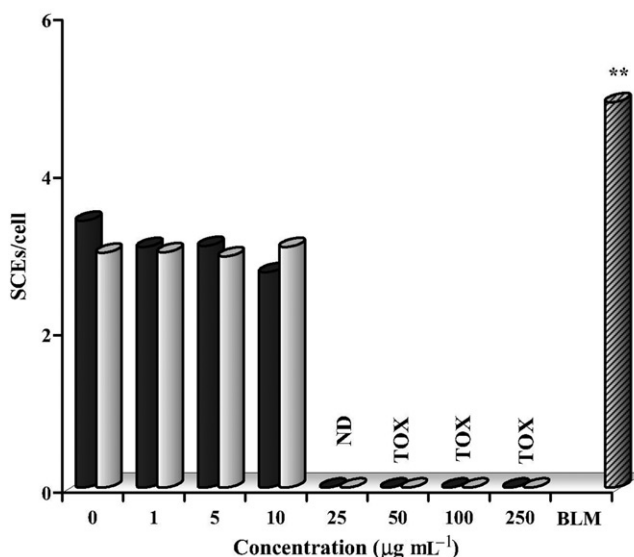


Figure 1. Effect of *in vitro* treatment with IVM (black columns) and ivomec[®] (white columns) on SCE frequency from *A. albopictus* larvae CCL-126TM cells. From each experiment, cultures were harvested after 24 h treatment and the frequency of SCE were determined in 25 M₂ mitoses for each experimental point. For each test compound, data are expressed as mean SCE per cell $\pm$ S.E. from three independent experiments (*y*-axis) and plotted against the antibiotic concentration (0–250 $\mu\text{g mL}^{-1}$ concentration range; *x*-axis). Bleomycin (BLM, 1.0 $\mu\text{g mL}^{-1}$) was used as positive control (stripped column).

Notes: ND – not determined and TOX – toxic. ** $p < 0.01$.

no SCE values were detected due to a low frequency of M₂ mitoses present in treated cultures. For both IVM and ivomec[®], cytotoxicity was observed in those cultures incubated within the 50–250 $\mu\text{g mL}^{-1}$ concentration range, indicated by a complete cellular death found in the cultures (Figure 1).

Table 1 presents the results of the SCGE assay performed on CCL-126TM cells after a 80 min pulse-treatment of IVM- and ivomec[®] in a 5–50 $\mu\text{g mL}^{-1}$ concentrations. For both chemicals, total cell count, and cellular viability remained unchanged from control values regardless of the treatment over the employed concentration range. BLM treatment (positive control) induced a significant increase in the frequency of both slightly damaged and damaged cells simultaneously with a decrease in the frequency of undamaged cells (Table 1). IVM treatment induced significant DNA damage as evidenced by increased frequency of both slightly damaged and damaged cells, concomitant with a significant decrease in the proportion of undamaged cells in the 25–50 $\mu\text{g mL}^{-1}$ concentration range but not at 5 $\mu\text{g mL}^{-1}$ (Table 1, Figure 2). A correlation test demonstrated that the frequency of either slightly damaged ($r=0.97$) or damaged cells ($r=0.94$) rose significantly in a concentration-dependent manner (Table 1). Overall, image analysis revealed a positive relationship between both IVM concentration and DNA damage, estimated by cell length (Figure 2a, $r=0.65$) and cell width (Figure 2c, $r=0.51$) as well as combined cell length and cell width within the 5–50 $\mu\text{g mL}^{-1}$ concentration range.

Table 1. Frequencies of undamaged, slightly damaged, and damaged cells in control and IVM- and ivomec®-treated *A. albopictus* cells detected by the alkaline SCGE.^a

	Concentrations ($\mu\text{g mL}^{-1}$)	Percentage of cells ^b			Viability
		Undamaged	Slightly damaged	Damaged	
BLM ^c	0.0	90.6 $\pm$ 1.7	9.3 $\pm$ 1.7	0.0 $\pm$ 0.0	99.6 $\pm$ 0.3
	10.0	4.0 $\pm$ 1.15***	67.3 $\pm$ 2.4***	28.6 $\pm$ 1.3***	87.6 $\pm$ 4.7
IVM	5.0	82.6 $\pm$ 3.7	17.3 $\pm$ 3.7	0.0 $\pm$ 0.0	98.0 $\pm$ 0.4
	25.0	54.0 $\pm$ 1.5***	40.6 $\pm$ 2.2***	5.3 $\pm$ 0.6*	95.5 $\pm$ 1.9
Ivomec®	50.0	16.6 $\pm$ 4.3***	50.6 $\pm$ 2.0***	32.6 $\pm$ 4.6***	90.0 $\pm$ 3.8
	5.0	40.3 $\pm$ 1.3***	57.6 $\pm$ 1.8***	2.0 $\pm$ 0.6	98.8 $\pm$ 0.2
	25.0	46.0 $\pm$ 1.0***	52.6 $\pm$ 1.6***	1.3 $\pm$ 0.6	97.5 $\pm$ 1.2
	50.0	26.0 $\pm$ 2.2***	62.0 $\pm$ 2.9***	10.6 $\pm$ 0.8**	90.8 $\pm$ 2.2

Notes: ^aCells were treated with the test compounds and harvested at the 80 min thereafter, and processed following procedure for SCGE after viability counting.

^bUndamaged cells, diameter ≤ 30 mm; slightly damaged cells, diameter 31–45 mm; damaged cells width and length > 45 mm; results are expressed as mean values of 150 cells $\pm$ S.E. of three independent experiments (50 cells/experiment).

^cBleomycin (BLM, 10.0 $\mu\text{g mL}^{-1}$) was used as positive control.

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Ivomec® treatment induced DNA damage as evidenced by a significant increase in the frequency of slightly damaged associated with a significant decrease in the proportion of undamaged cells at 5–50 $\mu\text{g mL}^{-1}$. Further, a significant rise in the proportion of damaged cells was only induced when 50 $\mu\text{g mL}^{-1}$ was present. A correlation test demonstrated that the frequency of either slightly damaged ($r = 0.65$) or undamaged cells ($r = -0.74$) increased and decreased as an independent function of the concentration of ivomec® (Table 1). On the other hand, only the frequency of ivomec®-induced damaged cells was found to rise in a concentration-dependent manner at 5–50 $\mu\text{g mL}^{-1}$ concentrations ($r = 0.90$). Overall, image analysis revealed a positive relationship between both ivomec® concentration and DNA damage, estimated by cell length (Figure 2b, $r = 0.43$) and cell width (Figure 2d, $r = 0.29$) as well as combined cell length and cell width within the 5–50 $\mu\text{g mL}^{-1}$ concentration range.

The results of the CCP analysis in CCL-126TM cells treated with different concentrations of IVM and ivomec® and the positive control (BLM) are presented in Figure 3 while the concomitant PRI values are summarized in Table 2. BLM treatment (positive control) induced a significant inhibition of the CCP and a significant reduction of the PRI. A significant delay in CCP was observed in both 10 $\mu\text{g mL}^{-1}$ IVM- and ivomec®. Only a significant reduction of the PRI was noted when 10 $\mu\text{g mL}^{-1}$ of IVM was employed. When 25 $\mu\text{g mL}^{-1}$ of either IVM or ivomec® was used, no CCP data were detected due to a low frequency of M₂ mitoses present in treated cultures. Finally, IVM or ivomec® exerted a cytotoxic effect on CCL-126TM cells within the 50–250 $\mu\text{g mL}^{-1}$ concentration range (Figure 3, Table 2).

The MI data for IVM- and ivomec®-treated cultures are presented in Table 2. BLM (positive control) treatment produced a quantitative depression of MI.

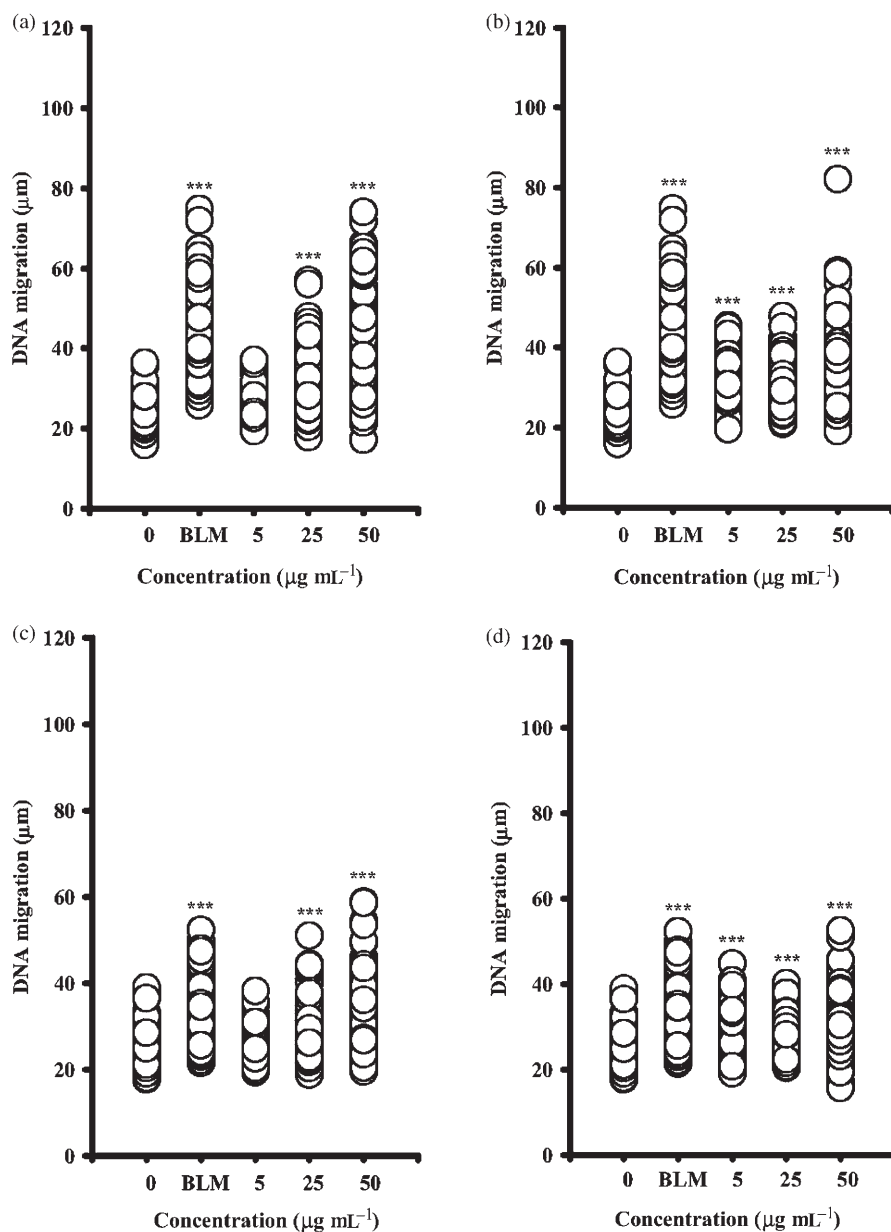


Figure 2. Effect of IVM ((a) and (c)) and ivomec® ((b) and (d)) on the length of the comet tails ((a) and (b)) and on the diameter ((c) and (d)) at the estimated trailing edge of comet tails in *A. albopictus* larvae CCL-126TM cells. Cultures were harvested at 80 min from antibiotic treatment and processed following procedure for SCGE. Electrophoresis was performed at 4°C for 20 min (pH 13, 0.8 V cm⁻¹), and cells stained with DAPI. DNA migration (μm) was determined in 150 cells for each experimental point. For each antibiotic, pool data from three independent experiments are reported as DNA migration (y-axis) and plotted against the antibiotic concentration (0–50 μg mL⁻¹ concentration range; x-axis). Notes: Bleomycin (BLM, 10 μg mL⁻¹) was used as positive control. ****p* < 0.001.

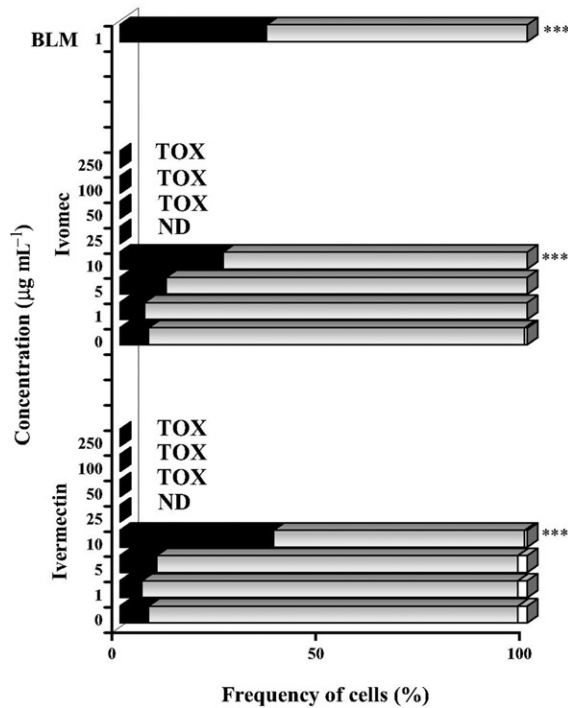


Figure 3. Effect of *in vitro* treatment with IVM and ivomec[®] on CCP from *A. albopictus* larvae CCL-126TM cells. Cultures were harvested after 24 h treatment and the frequency of first (M₁, black bar areas), second (M₂, grey bar areas), and third (M₃, white bar areas) were determined in 200 mitoses for each experimental point. For each test compound, pooled data from three independent experiments are expressed as mean percentages of M₁, M₂, and M₃ (*x*-axis) and plotted against the antibiotic concentration (0–250 µg mL^{−1} concentration range; *y*-axis).

Notes: Bleomycin (BLM, 1 µg mL^{−1}) was used as positive control (stripped column). ****p* < 0.001.

Table 2. PRI, MI, and MI factor (*f*) in control and IVM- and ivomec[®]-treated *A. albopictus* cells after 24 h of treatment.

Concentration (µg mL ^{−1})	IVM			Ivomec [®]		
	PRI ^a	MI ^a	<i>f</i> ^a	PRI ^a	MI ^a	<i>f</i> ^a
0.0	1.95 ± 0.03	91.00 ± 9.00	1.00 ± 0.00	1.94 ± 0.04	105.33 ± 4.18	1.00 ± 0.00
BLM ^b	1.64 ± 0.18*	89.67 ± 8.88	1.03 ± 0.11	1.64 ± 0.18*	89.67 ± 8.88	0.91 ± 0.14
1.0	1.97 ± 0.02	88.00 ± 5.57	1.04 ± 0.21	1.94 ± 0.02	105.00 ± 7.77	1.15 ± 0.36
5.0	1.93 ± 0.01	100.67 ± 5.21	1.19 ± 0.22	1.85 ± 0.06	106.33 ± 7.31	1.14 ± 0.28
10.0	1.63 ± 0.15*	77.66 ± 5.70	0.91 ± 0.17	1.75 ± 0.11	91.00 ± 3.00	0.98 ± 0.26
25.0	ND	2.33 ± 1.45***	0.03 ± 0.01	ND	6.33 ± 1.20***	0.06 ± 0.02
50.0	TOX	TOX	TOX	TOX	TOX	TOX
100.0	TOX	TOX	TOX	TOX	TOX	TOX
250.0	TOX	TOX	TOX	TOX	TOX	TOX

Notes: ND – not determined (insufficient M₂); TOX– toxic.

^aResults are presented as mean values ± S.E. of three independent experiments.

^bBleomycin (BLM, 1.0 µg mL^{−1}) was used as positive control.

p* < 0.05 and **p* < 0.001.

For both chemicals, no statistical alteration in the mitotic activity of CCL-126TM cells was noted within the 1–10 $\mu\text{g mL}^{-1}$ concentration range. A marked reduction of about 98% and 94% was found in cells at 25 $\mu\text{g mL}^{-1}$ IVM- and ivomec[®] with respect to the mitotic activity of control cultures. At this concentration, the mitotic cell population of IVM- and ivomec[®]-treated cultures decreased significantly (Table 2).

A loss of lysosomal activity, indicated by a significant decrease in uptake of NR, was observed when CCL-126TM cells were exposed to 1–250 $\mu\text{g mL}^{-1}$ IVM and ivomec[®] (Figure 4). Similar loss of lysosomal activity was induced by IVM or equivalent concentration of ivomec[®] throughout the 0.25–250 $\mu\text{g mL}^{-1}$ concentration range. A regression test showed that lysosomal activity markedly fell as a function of the antibiotic concentrations in IVM- and ivomec[®]-treated cultures ($r = -0.75$; $r = -0.57$), respectively (Figure 4). Alteration in the energetic cell metabolism induced by the compounds measuring the ability of the mitochondrial succinic dehydrogenase was further analyzed by the MTT assay. The results presented in Figure 5 show a marked decrease in the MTT dye reduction to an insoluble violet formazan product at 10–250 $\mu\text{g mL}^{-1}$ IVM. Similar results were found with the 1–250 $\mu\text{g mL}^{-1}$ ivomec[®] concentrations. Figure 5 also shows that 1–10 $\mu\text{g mL}^{-1}$ IVM induced less alteration in cellular metabolism than the equivalent concentration of ivomec[®]. A regression test showed that mitochondrial activity fell significantly as a function of the antibiotic concentrations in IVM and ivomec[®].

Discussion

The study was conducted using eight endpoints for both genotoxicity including SCE frequency and SCGE assays and cytotoxicity measuring CCP, MI, proliferative rate index, cell viability, NR, and MTT bioassays. Overall, the results demonstrated that

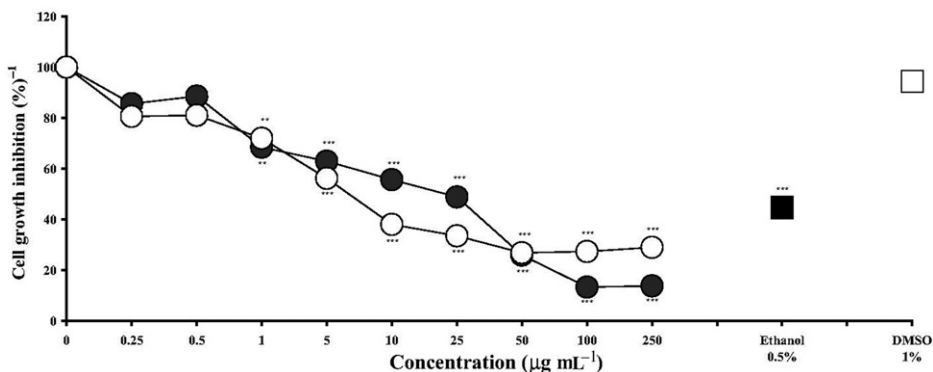


Figure 4. Lysosomal activity assessed with the NR assay in IVM- (black circles) and ivomec[®]-treated (white circles) *A. albopictus* larvae CCL-126TM cells. Cultures were incubated for 2 h with NR after 24 h treatment. Results are expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel (y -axis) and plotted against the antibiotic concentration (0–250 $\mu\text{g mL}^{-1}$ concentration range; x -axis). A 0.5% ethanol-treated (black squares) and 1% DMSO-treated (white squares) cells were used as positive and negative controls, respectively.

Note: ** $p < 0.01$; *** $p < 0.001$.

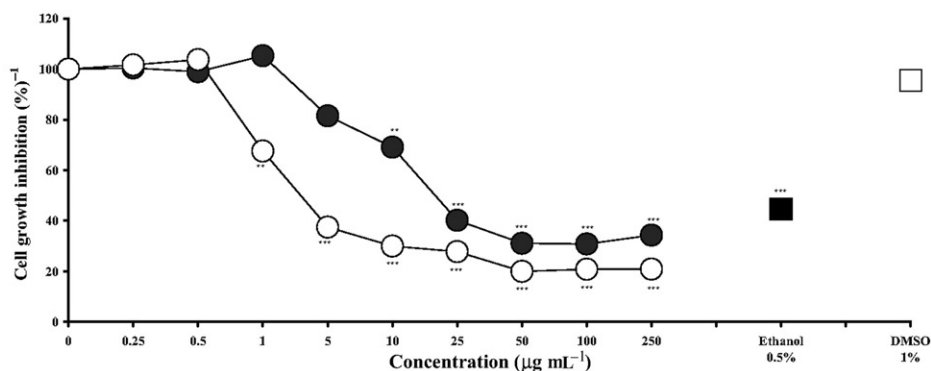


Figure 5. Cellular metabolism inhibition evaluated by MTT assay in IVM- (black circles) and ivomec®-treated (white circles) *A. albopictus* larvae CCL-126™ cells. Cultures were incubated for 2 h with MTT after 24 h of the pesticide treatment. Results are expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel (y -axis) and plotted against the antibiotic concentrations (0–250 $\mu\text{g mL}^{-1}$ concentration range; x -axis). A 0.5% ethanol-treated (black squares) and 1% DMSO-treated (white squares) cells were used as positive and negative controls, respectively. Note: ** $p < 0.01$; *** $p < 0.001$.

while both IVM and ivomec® were not able to markedly enhance the frequency of SCE in cells incubated with up to 10 $\mu\text{g mL}^{-1}$, higher concentrations of 25–250 $\mu\text{g mL}^{-1}$ induced cellular toxicity by delaying the CCP, decreasing both the proliferative rate and MI, inhibiting cellular growth, increasing lysosomal damage as well as decreasing general cellular metabolism. However, it is worth mentioning that both chemicals were able to produce primary lesions in cellular DNA as evidenced by the comet assay.

SCE formation is closely dependent upon DNA replication, and although the damage can be induced at any stage of the cell-cycle, the lesions responsible for SCE persist to at least the first onset of DNA replication (Wilson and Thompson 2007). The SCE assay has been widely adopted to assess the genotoxic potential of both mutagenic and carcinogenic agents (Wilson and Thompson 2007). Our current observations in CCL-126™ cells indicated that IVM as well as IVM-containing technical formulation ivomec® were not able to produce DNA lesions leading to SCE formation. The results were obtained after a continuous antibiotic 24 h treatment which covers the time required for CCL-126™ cells to perform two cell-cycles which is confirmed by the frequency of second mitoses observed at this harvesting. Nevertheless, other investigators demonstrated the highest incidence of second mitosis in *A. albopictus* larvae cells after 48 h incorporation of the BrdU, the period when harvesting is usually performed (Mukherjee et al. 1986; Bianchi et al. 1988; Wei et al. 1993; Bolzán et al. 2000). It is conceivable that differences among cell lines, culture media, and/or culture conditions may be responsible for this discrepancy.

When analyzing the basal level of SCE in *A. albopictus* cells, as well as in other insect cell lines, the existence of cells with zero background of SCE is not a rare event, although it does not exist in mammalian cells (Wei et al. 1993). This phenomenon could be explained on the basis of the different DNA content

of the diploid cells under analysis. Kato (1977) suggested that the basal level of SCE of a given cell type is proportional to the number of DNA bp in the diploid genome. Nevertheless, Adams et al. (1979) indicated that in *A. albopictus* cells SCE frequency value is comparable or at least equivalent to that of most mammalian cells. Accordingly, it has been suggested that the low DNA content of *A. albopictus* cells is not sufficient to explain the low basal level of SCE on such cells (Wei et al. 1993). Our present observations as well as previously reported data indicate that the basal level of SCE in mosquito cell lines is approximately 0.33-fold less than that of CHO-K1 cells (Mukherjee et al. 1986; Bianchi et al. 1988; Bolzán et al. 2000). Other reasons rather than cellular DNA content could also explain this observation. Mosquito cells might be more resistant than CHO-K1 cells to BrdU allowing a discernible differentiation of sister chromatids to be achieved in some cells without a concomitant induction of SCE (Gatti et al. 1979). It is known that the SCE frequency is directly proportional to the concentration of BrdU in the first but not in the second cell-cycle, and that SCE occur primarily at the second S-period as a consequence of the replication of the BrdU-substituted DNA chain (Wilson and Thompson 2007). Further, differences in the cellular concentration of GSH, between CCL-126TM and CHO-K1 cells might be also included as putative factor for the observed basal frequencies of SCE. Glutathione, an intracellular radical scavenger, is one of the most important and versatile resistance mechanisms in the protection of cellular macromolecules, including DNA, against reactive xenobiotics (Watson et al. 2004; García-Rubio et al. 2005). Wei et al. (1993) demonstrated that treatment with BrdU increased GSH concentration in *A. albopictus* cells but decreased its steady-state in CHO-K1 cells. Furthermore, a higher cellular GSH content was suggested to be the basis by which BudR-substituted chromatin of mosquito cells is more resistant to DNA degradation by xenobiotics than that of CHO-K1 cells (Bianchi and López-Larraz 1991). Finally, another possibility could arise from differences in the intrinsic nature of the DNA between mosquito and CHO-K1 cells characterized by containing heavily methylated and heavily demethylated DNA, respectively (Orian et al. 2009). Bianchi et al. (1988) found that no SCE induction occurred in CHO-K1 and *A. albopictus* larvae (LRBEH) cells when the DNA enters replication symmetrically methylated, as usually takes place in vertebrate cells, or symmetrically demethylated, as in the case for mosquito cells. At present, there is no clear experimental data that could allow us to determine which of the aforementioned possibilities might be responsible for this phenomenon.

Previously Molinari et al. (2009) demonstrated that IVM was not able to increase the frequency of SCE in CHO-K1 cells, supporting our current observations. Furthermore, in agreement with the plausible explanations it was suggested elsewhere for there is a lack of SCE induction in IVM- and ivomecTM-treated hamster cells (Molinari et al. 2009). These findings highlight that the SCE endpoint was unable to reveal a genotoxic effect induced by the pure antiparasiticide and its commercial formulation ivomec[®] on insect cells, whether lesions leading to SCE induction were produced in cellular DNA. On the other hand, it was possible to demonstrate the ability of IVM and ivomec[®] to induce DNA damage revealed by the comet assay which directly indicates that both compounds are capable of producing single DNA-strand breaks in the genome of mosquito CCL-126TM cells. In agreement with this observation, Molinari et al. (2009) recently reported the ability of both IVM and ivomec[®] to induce DNA-strand breaks on CHO-K1 cells as

evidenced by the SCGE assay. It should be mentioned, however, the time period between test compound-treatment and cell harvesting greatly varies under different culture conditions required for the endpoint employed, namely 24 h and 80 min for SCE analysis and comet assay, respectively. Whether antiparasiticide-induced DNA-strand breaks are (1) not responsible for the induction of SCE or (2) rapidly repair after injury and then are only detected with the comet assay but not long enough to induce SCE is still an open question. Further IVM- and ivomec[®]-induced DNA damage and repair experiments are currently under development in order to elucidate this putative explanation. Thus far, there is no information on the capability of IVM and ivomec[®] to produce DNA-strand breaks qualitatively, and quantitatively analyzed by the comet assay endpoint performed in insect cells. Our present observations allow us to report for the first time the analysis of the both antiparasiticide-induced *in vitro* DNA damage in insect cells, at least in *A. albopictus* larvae CCL-126TM cells. A short 80 min pulse-treatment of 25–50 µg mL⁻¹ of IVM as well as 5–50 µg mL⁻¹ of ivomec[®], induced a significant level of single DNA-strand breaks. These findings together with our previous results on CHO-K1 cells (Molinari et al. 2009) agree with previous observations highlighting a genotoxic activity in rats exposed *in vivo* to the most active form of the avermectin (avermectin B_{1a}), the abamectin, in which single strand-DNA break induction was noted in hepatocytes (EPA 1989).

Our current findings demonstrated that either IVM or ivomec[®] failed to accumulate mitotic cells and delayed the average cell generation time in CCL-126TM cells. CCL-126TM cultures regardless whether they were exposed to 10 µg mL⁻¹ IVM- or ivomec[®] exhibited a significant delay in CCP. Furthermore, both IVM- and ivomec[®]-incubated cells showed a significant decrease at 25–250 µg mL⁻¹ in the MI as well as PRI which are considered by several investigators as two informative and reliable monitoring bioassays when these endpoints are employed for cytotoxicity and cellular proliferation kinetics (Carballo et al. 2006; González et al. 2009; Molinari et al. 2009). These findings agree with previous documented observations indicating that one of the major cellular responses *in vivo* and *in vitro* after exposure to chemicals, ionizing radiation, and/or other agents with clastogenic capacity may be involved in the inhibition and/or the delay of the cell-cycle kinetics (Soloneski et al. 2001, 2002, 2007; González et al. 2006).

Our findings reveal the ability of both IVM and ivomec[®] to increase cellular growth inhibition in CCL-126TM cell line as demonstrated by the NR and the MTT bioassays. By using the same assays, similar observations were recently reported in CHO-K1 cells with IVM and ivomec[®] treatment (Molinari et al. 2009). Molinari et al. (2009) together with our current observations could allow one to assume that the cytotoxicity effects of the antiparasiticide or its metabolites may be implicated in alterations in cellular proliferation kinetics either in mammalian or insect cells. Rodriguez and Mattei (1988) reported that pig kidney IB-RS-2 cells showed a concentration-dependent growth inhibition and a decrease in protein synthesis and glucose utilization when cultured in the presence of different levels of IVM whereas DNA and RNA synthesis remained unchanged (Mattei and Rodriguez 1994). Mattei and Rodriguez (1994) also demonstrated a decrease in the activity of lactate dehydrogenase in IVM-treated pig kidney IB-RS-2 cells. It is known that lactate dehydrogenase activity is inhibited by several xenobiotics including some carbamate pesticides like carbofuran and its major metabolite diethyldithiocarbamate (Gupta et al. 1991).

Our observations showed the ability of the active ingredient IVM and one of the Argentinean IVM-containing formulation, as the ivomec[®], to induce both genotoxicity and cytotoxicity *in vitro*, at least under the experimental conditions. Furthermore, these findings constitute the first evidence of the genotoxic and cytotoxic properties that the antiparasiticide IVM possess on mosquito cells treated *in vitro*. These observations verify and extend our recently reported results on CHO-K1 cells after *in vitro* treatment with both IVM and ivomec[®] (Molinari et al. 2009). Data indicate that the anthelmintic IVM as well as ivomec[®] exert cellular genotoxicity and cytotoxicity in *in vitro* conditions, and that effect occurs not only in mammalian cells but also in other cell systems, such as CCL-126TM cells.

Acknowledgments

We thank M.I. Urrutia Ing. for technical assistance. This study was supported by grants the National University of La Plata (11/N493 and 11/N564), and the National Agency of Scientific and Technological Promotion (PICT 2004 no. 26116) from Argentina.

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