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

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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.

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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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