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Genotoxicity and cytotoxicity assessment of new ethyl-carbamates with ixodicidal activity

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ABSTRACT

The mammalian erythrocyte micronucleus test was used on the peripheral blood of Wistar rats exposed to two new ethyl-carbamates: ethyl-4-bromophenyl-carbamate (LQM 919) and ethyl-4-chlorophenyl-carbamate (LQM 996) to analyze their genotoxic potential. The mitotic index and cell proliferation kinetics in human lymphocyte cultures in the presence of these ethyl-carbamates were used to evaluate cytotoxicity and cytostaticity respectively. Exposure to greater acute doses (300 mg/kg) and to all of the subchronic doses (12.5, 25 and 50 mg/kg daily for 90 days) of these ethyl-carbamates induced an increased frequency ($p < 0.05$) of micro-nucleated polychromatic erythrocytes (MN-PCE) compared with rats not exposed to the ethyl-carbamates. Increases in MN-PCE was higher in males than in females exposed to LQM 996 50 mg/Kg ($p < 0.05$). All observed changes in rats return 21 days after suspending ethyl-carbamate exposure. The highest concentration (0.3 mM) of both ethyl-carbamates in lymphocyte cultures increased the percentage of cells in first division metaphase and decreased the percentage of cells in third division metaphase, indicating an increase in cell cycle length or a possible cell cycle arrest in metaphase (cytostatic effect). The results of this study show that the evaluated ethyl-carbamates may induce genotoxic damage in rats and alterations in the human lymphocyte cell cycle.

1. Introduction

Ticks are blood-sucking arthropods that parasitize domestic animals, wildlife animals and humans and are the most important ectoparasites of cattle in the tropical and subtropical areas of Central and South America, Africa and Australia [1]. *Rhipicephalus microplus* is the most important tick species to investigate in these areas because it produces large economic losses for livestock. Anemia, reduced growth, lower reproductive performance, lower meat and milk production, decreased quality of fur, paralysis and transmission of diseases such as babesiosis and anaplasmosis are all effects of tick infestation [2,3]. The most commonly used strategy for controlling ticks has been the use of chemical ixodicides. Nevertheless, the high selection pressure caused by their exaggerated use has promoted resistance to primary commercial ixodicides [4]. This resistance has compelled the development of new pharmaceutical alternatives for the control of ticks, particularly the development of new molecules for which ticks have not developed resistance. In this context, two new carbamates, ethyl-4-bromophenyl-carbamate (LQM 919) and ethyl-4-chlorophenyl-carbamate (LQM 996), have been synthesized at Universidad Nacional Autónoma de México (UNAM).

These carbamates negatively affect reproduction of *R. microplus*, both in susceptible strains and in those resistant to the commercial ixodicides used in Mexico [5,6]. The carbamates cause alterations to the reproductive organs, vitellogenesis and viability of ovarian cells of *R. microplus* and cause harm suggestive of apoptosis. These effects were found to be independent of acetylcholinesterase inhibition

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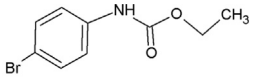
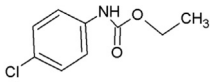
Table 1
Chemical structures and molecular weights of the evaluated carbamates.

Carbamate identification code	Chemical structure	Molecular weight (g/mol)
LQM 919	 Ethyl (4-bromophenyl) carbamate	244
LQM 996	 Ethyl (4-chlorophenyl) carbamate	199.63

[7]. Likewise, acute and subchronic toxicity studies in rats have shown that these carbamates produce morphological and functional alterations in hepatic and renal cells, as well as oxidative stress [8,9]. The above suggests a potential risk for the use of these products on animals for human consumption and for the persons that apply the products. Therefore, the lack of data from genotoxicity testing may be a safety concern. The *in vivo* mammalian micronucleus test is widely used to identify chemicals with genotoxic potential. This test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents (OECD, 1997). The purpose of this study was to provide data about of the genotoxic, cytotoxic and cytostatic potential of LQM 919 and LQM 996 compounds. We evaluated the genotoxic effects of these compounds *in vivo* using rat erythrocyte micronucleus test and the *in vitro* cytostatic effect on proliferation of human peripheral blood mononuclear cell stimulated with phytohemagglutinin.

2. Materials and methods

2.1. Carbamates

The carbamates used in this study were designed and synthesized at the Medicinal Chemistry Laboratory at FES-Cuautitlán-UNAM using a benzimidazole molecule as the structural base. The carbamates were synthesized by reacting aryl- and alkylamines with sodium hydride and benzene diethylcarbonate, followed by column chromatography purification; next, the products were recrystallized. The carbamates were structurally characterized through interpretations of their spectra, hydrogen and carbon-13 nuclear magnetic resonance and mass spectrometry [10]. The chemical structures, nomenclature, molecular weights and identification codes of the carbamates are shown in Table 1.

2.2. Animals

Clinically healthy seven- to eight-week-old male and female Wistar rats obtained from the Production and Experimentation Unit of Laboratory Animals (UPEAL-CINVESTAV, México D.F, Mexico) weighing between 175 and 200 g were used. All rats were kept in groups of three to five individuals. The environmental temperature was maintained at $22 \pm 2\,^{\circ}C$ with a relative humidity between 30 and 70% and a 12 h light-dark cycle. They were fed with commercial feed and water *ad libitum*. This study was approved by the Internal Committee for the Care of Experimental Animals of the Postgraduate Program of Animal Production and Health of UNAM in Mexico.

2.3. Evaluation of genotoxicity in rats with acute exposure

Thirty-five male Wistar rats were distributed into seven groups (n = 5). Rats in groups 1 and 2 orally received 50 and 300 mg/kg, respectively, of LQM 919 dissolved in 1 mL of dimethylsulfoxide (DMSO) and this solution mixed in 2 mL of corn oil. Rats in groups 3 and 4 received 50 and 300 mg/kg, respectively, of LQM 996 dissolved in 1 mL of DMSO and mixed in 2 mL of corn oil. The doses were selected based on our previous studies of acute oral toxicity [8].

Rats in group 5 received DMSO dissolved in corn oil and rats in group 6 only received corn oil (negative control groups), whereas rats in group 7 were used as a positive control. The volume administrated to each group were the same. The rats of groups 1 through 6 were administered the treatment in a single dose using oral gavage. Rats in the positive control group received a single intraperitoneal injection of 20 mg/kg of cyclophosphamide in 0.9% saline solution [11]. Two peripheral blood samples 100 μL each were collected into vials containing heparin from the caudal vein; the first sample was obtained before carbamate exposure and was used to the analysis of the basal frequencies of micronuclei in the Wistar rats (0.1 ± 0.22) the second sample was taken 48 h after carbamate exposure. Genotoxicity was evaluated by the mammalian erythrocytes micronucleus test in the samples collected.

2.4. Evaluation of genotoxicity in rats with subchronic exposure

Eighty-two Wistar rats were distributed into nine groups, and the characteristics and treatments performed in these groups are shown in Table 2. The doses were selected based on our previous studies of acute oral toxicity [8]. The rats were exposed daily to the carbamates LQM 919 or LQM 996 dissolved previously in DMSO. The concentration of each carbamate were adjusted weekly in drinking water according to animal weight and water consumption to maintain constant dose exposure during 90 days. The final concentration of DMSO in drinking water was 0.4%. After exposure time, peripheral blood samples from the caudal vein were collected into vials containing heparin. An additional satellite group was included; blood samples from the satellite group were obtained 21 days after the carbamate exposure was suspended. The genotoxicity was evaluated by the mammalian erythrocyte micronucleus test in the samples collected.

2.5. Mammalian erythrocyte micronucleus test

The micronucleus assay was performed as previously described by Salazar et al. [12]. Peripheral blood smears were air-dried and fixed in absolute methanol. The slides were stained with acridine orange (Sigma–Aldrich) and were coded before blind scoring. The frequency of micronuclei was evaluated in mature erythrocytes (normochromatic) and reticulocytes (polychromatic). For each rat, 2000 normochromatic erythrocytes (NCE) and 2000 poly-