Mutat Res Gen Tox En 829–830 (2018) 26–32

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

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

The metabolism of imidacloprid by aldehyde oxidase contributes to its clastogenic effect in New Zealand rabbits

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ARTICLE INFO

Keywords:
Neonicotinoids
Aldehyde Oxidase
DNA damage
Oxidative stress
Telomerase

ABSTRACT

Imidacloprid (IMI) is a systemic, chloro-nicotinyl insecticide classified in Regulation N° 1272/2008 of the European Commision as "harmful if swallowed and very toxic to aquatic life, with long-lasting effects". IMI is metabolized *in vitro* both by aldehyde oxidase (AOX) (reduction) and by cytochrome P450s enzymes (CYPs). In the present study, the AOX inhibitor sodium tungstate dihydrate (ST) was used to elucidate the relative contribution of CYP 450 and AOX metabolic pathways on IMI metabolism, in male rabbits exposed to IMI for two months. To evaluate the inhibition effectiveness, various metabolite concentrations in the IMI and IMI + ST exposed groups were monitored. DNA damage was also evaluated in micronucleus (MN) and single cell electrophoresis (SCGC) assays in both groups, along with oxidative stress (OS) with the inflammatory status of the exposed animals, in order to clarify which metabolic pathway is more detrimental in this experimental setting. A significant increase in the frequency of binucleated cells with MN (BNMN, 105%) and micronuclei (MN, 142%) was observed after exposure to IMI ($p < 0.001$). The increase in the ST co-exposed animals was less pronounced (BNMN 75%, MN 95%). The Cytokinesis Block Proliferation Index (CBPI) showed no significant difference between controls and exposed animals at any time of exposure ($p > 0.05$), which indicates no cytotoxic effect. Similarly, comet results show that the IMI group exhibited the highest achieved tail intensity, which reached 70.7% over the control groups, whereas in the IMI + ST groups the increase remained at 48.5%. No differences were observed between all groups for oxidative-stress biomarkers. The results indicate that the AOX metabolic pathway plays a more important role in the systemic toxicity of IMI.

1. Introduction

Imidacloprid (IMI) (EC 428-040-8) is a systemic, chloro-nicotinyl insecticide in the chloro-nicotinyl nitro-guanidine chemical group [1,2]. In the European level, it is recognized as biocidal-active substance that is approved under the Regulation N° 528/2012/EC. It is classified in Annex VI of the Regulation No. 1272/2008/EC as harmful if swallowed (H302) and very toxic to aquatic life, with long-lasting effects (H400, H401).

IMI is quickly absorbed by the oral route and rapidly distributed in

Abbreviations: IMI, imidacloprid; IMI + NH, desnitro-imidacloprid; CBMN, cytokinesis block micronucleus assay; BNMN, binucleated cells with micronucleus; CBPI, cytokinesis block proliferation index; MN, micronuclei; SCGE, single cell gel electrophoresis; OS, oxidative stress; TA, telomerase activity; OECD, Organization for Economic Co-operation and Development; AOX, aldehyde oxidase; CYP, cytochrome p450; ST, sodium tungstate dihydrate; ECHA, European Food Safety Authority; PBMC, peripheral blood mononuclear cell; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; Hb, hemoglobin; GSH, reduced glutathione; CAT, catalase; Crbnls, protein carbonyls; EDTA, ethylenediamine tetraacetic acid; LMA, low melting agarose; EtBr, ethidium bromide; TCA, trichloroacetic acid; Tris–HCL, tris(hydroxymethyl)aminomethane hydrochloride; DNPH, 2,4 dinitrophenylhydrazine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ETH, ethirimol; LOD, limit of detection; LOQ, limit of quantitation; SD, standard deviation; ANOVA, analysis of variance

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https://doi.org/10.1016/j.mrgentox.2018.03.002
Received 13 October 2017; Received in revised form 8 March 2018; Accepted 9 March 2018
Available online 19 March 2018
1383-5718/

most organs and tissues. In rats, the oral absorption is estimated to be 92–99%. IMI degrades to many metabolites formed *via* multiple pathways. Common or structurally similar metabolites have been found in rats, goats and hens. Based on structural considerations, the following metabolites are of toxicological significance to humans: 6-chloronicotinic acid (6-CNA), imidazolidine 4- and 5-hydroxy compounds, olefinic imidacloprid, desnitro-imidacloprid (IMI-NH) and the nitrosoimine compound. IMI metabolites are primarily excreted in the urine as glutathione and glycine conjugates of mercaptonicotinic acid and hippuric acid [3]. IMI-NH is of particular interest, due to its nicotinic-type action that prefers mammalian *versus* insect nicotinic acetylcholine receptors (nAChRs) [4,5], and therefore it binds very strongly to mammalian nerve receptors but not to insect nerve receptors. IMI-NH is not toxic to insects, but it is about four to five times more toxic than IMI to mice [6,7].

In vitro studies have indicated the importance of cytochrome P450s (CYPs) in IMI oxidation and aldehyde oxidase (AOX) in IMI reduction. Currently, the most frequently used insecticides are neonicotinoids that are metabolized *in vitro* by AOX on reduction of the nitro-imino group and by CYPs *via* oxidation reactions. Similarly, *in vitro* reduction of the nitro-imino group of IMI by AOX leads to the desnitro-imidacloprid metabolite (IMI-NH), while oxidation reactions by CYP lead to other primary metabolites, such as 6-CNA.

AOX metabolizes many xenobiotics *in vitro*, but its *in vivo* importance is usually not clear compared to that of cytochrome P450 (CYP) and other detoxification systems. Swenson and Casida [8] established the relative importance of AOX and CYP *in vivo* using the mouse model.

Sodium tungstate dihydrate (ST) is a substance that does not have yet a registration number according to Article 2 of the REACH Regulation (EC) No. 1907/2006 but is classified with warning statements such as 'Category 4', 'Acute toxicity', 'Oral', 'harmful if swallowed' by Regulation No. 1272/2008/EC. It has been shown to reduce AOX activity *in vivo* in mammals by replacing molybdenum at the active center, rendering it inactive [9–11].

The aim of the present study is to elucidate which IMI metabolic pathway, the AOX or the CYP, could be more detrimental in a systemic way. To accomplish this, concentrations of AOX and CYP metabolites, along with the major metabolite of IMI, 6-CNA, were monitored in various matrixes such as hair, urine and blood of New Zealand male rabbits exposed either to IMI alone or co-exposed to IMI and ST, in order to inhibit AOX activity. Various parameters including DNA damage (micronuclei test, comet assay), oxidative stress (total antioxidant activity-TAC) and systemic inflammation, were measured. The study hypothesis is summarized in Table 1 and can be found in the Supplementary information section.

2. Materials and methods

2.1. Animals and administration protocol

Nine 3-month-old New Zealand white male rabbits were used in this study. The animals were housed in individual metal cages at the laboratory animal house facilities of the School of Medicine, University of Crete, Heraklion, under a 12 h dark/light cycle and a steady ambient temperature between 20 and 23°C. Commercial rabbit pellets and drinking water were provided *ad libitum*. The animals were acclimatized under these conditions for approximately 2 weeks. IMI was provided gratis by Vapco (Jordan) and ethirimol (ETH), which is the internal standard used, while 6-CNA, IMI-NH and ST were purchased from Sigma–Aldrich, Chemie GmbH. Animals were divided into 3 groups, consisting of 3 animals per group, as shown in Table 2 (see Supplementary information section).

Since oral LD_{50} values of IMI for rabbits have not yet been estimated, based on the known oral values for rats [12], dermal values for rabbits, and similar dosage schemes developed according to Kavvalakis [13], an exposure dose was decided with an oral dose of 30 mg/kg bw per day. The administered doses for ST were estimated based on the available literature, bearing in mind that a dose of 0.7 mg/mL/water was found to inhibit AOX metabolism in mice [8]. All doses were administered orally, diluted in 500 mL water, three times per week. All efforts were made to minimize any possible suffering. During the study period, all rabbits were regularly observed and their health condition was closely monitored. No adverse signs were observed throughout the experiment concerning food and water consumption, skin and eye conditions, excretion of urine and feces. The animals were exposed for 2.5 months and then sacrificed by veterinarians at approximately the age of 6 months by administering first a sedative injection of Xylapan (20 mg/mL xylazine hydrochloride) and Narketan (100 mg/mL ketamine hydrochloride) with a ratio of 2/1, and then an injection of Dolethal (200 mg/mL pentobarbitone sodium), which is a euthanasia agent. The present study was approved by the Veterinary Administration Office of Heraklion (Crete, Greece), the Animal Investigation Committee of the University of Crete (Heraklion, Crete, Greece) and conformed to the National and European Union Directions for the care and treatment of laboratory animals. After euthanasia, blood samples were collected into heparinized vials (Collection Test Tube 13 × 75 mm with Lithium Heparin × 4 mL, Sterile, FL Medical-Vacumed) to be used for the MN assay, comet assay and for the metabolite detection and in vials containing EDTA (Vacuette Blood Collection Tubes, spray dried K3EDTA × 3 mL, Greiner bio-one) to be used for the OS and TA assay, and all sampled vials were then stored at 2–8 °C, until further analysis.

2.2. Micronucleus assay (MN) in rabbit lymphocytes

The MN test is an official regulatory 'tool' in the European Legislation (B.12, Regulation 440/2008/EC) validated by OECD (*OECD TG 474*, 1997).

Whole blood (0.5 mL) was initially added to 6.5 mL Ham's F-10 medium, 1.5 mL fetal calf serum, and 0.3 mL phytohemagglutinin (this is done to stimulate cell division). Cultures were then incubated at a temperature of 37 °C for a period of 72 h. Six μg/mL of cytochalasin-B was added 44 h after culture initiation. Cells were collected by centrifugation 72 h after the incubation process. A mild hypotonic solution of Ham's F-10 medium and milli-Q water (1:1, v/v) was then added to the cell solution and left for 3 min at room temperature. Cells were fixed with a methanol: acetic acid solution (5:1, v/v) placed on microscope slides and stained with Giemsa [14,15]. These slides were then placed under a Nikon Eclipse E200 microscope where the binucleated cells (BN) and micronuclei (MN) could be clearly viewed. One thousand BN with intact cytoplasm were scored per slide for each sample, in order to calculate the frequency of MN. Standard criteria were used for scoring the MN [16].

The Cytokinesis Block Proliferation Index (CBPI) is calculated, by counting 2000 cells and based on a specific equation, to determine additional possible cytotoxic effects [17].

2.3. Comet assay in rabbit lymphocytes

The comet assay is another fast and efficient method for obtaining details regarding DNA damage and possible repair procedures in individual cells [18]. It is widely accepted in the field of *in vivo* research experiments, besides human and environmental studies, up until today and is an official test validated by OECD (*OECD TG 489*, 2014). The slide preparation for the alkaline comet assay was performed conventionally, as described by Singh [19], with some slight modifications. All microscopic slides were covered with 0.65% normal melting agarose (NMA) prepared in PBS (Ca^{2+}- and Mg^{2+}- free). Isolated lymphocytes were mixed with 100 μL of LMA at a temperature of 37 °C to form a cell suspension. Slides were maintained at 4 °C for 10 min to solidify the cell suspension layer. Coverslips were removed laterally and the slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM