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


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ARTICLE INFO

Keywords: Telomerase
Oxidative stress
DNA damage
Aldehyde Oxidase
Neonicotinoids

ABSTRACT

Imidacloprid (IMI) is a systemic, chloro-nicotinyl insecticide classified in Regulation N° 1272/2008 of the European Commission 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 (SCGE) 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...
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 mercaptanocotic acid and hippuric acid [3]. IMI-NH is of particular interest, due to its nictinic-type action that prefers mammalian versus insect nictinic 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. IMI degrades to many metabolites formed 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.

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 matrices 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 capacity-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<sub>50</sub> 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 Kavalakis [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 Do-lthal (200 mg/mL pentobarbionate 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<sup>2+</sup> and Mg<sup>2+</sup> - 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...
Na<sub>2</sub>EDTA, 10 mM Tris, pH = 10) including 1% Triton X and 10% DMSO. Slides were left in the lysing solution, at 4°C, for at least 1 h. Following the lysing step, the slides were placed in a horizontal electrophoresis tank filled with cold freshly prepared electrophoresis solution (1 mM Na<sub>2</sub>EDTA and 300 mM NaOH, pH = 13) at 4°C for 20 min to prepare the DNA for electrophoresis by loosening the tightly double-helix structure. After the electrophoresis, the slides were removed from the tank and neutralized by immersing in Tris buffer (0.4 M Tris, pH = 7.5) for 15 min, then refreshing the buffer every 5 min. Finally, the slide scoring was done using double slides prepared for each subject and a hundred cells were analyzed per subject at ×400 magnification, under an Olympus fluorescent microscope equipped with an excitation filter of 546 nm wavelength and a barrier filter of 590 nm. Comet Assay IV image analysis system (Perceptive Instruments) was used blindly by a one slide reader to score DNA damage, shown as tail intensity.

### 2.4. Assessment of oxidative stress markers

Sampled blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes for measuring: total antioxidant activity (TAC), thiobarbituric acid reactive substances (TBARS), protein carbonyls (CARB), reduced glutathione (GSH) levels and catalase activity. Blood samples were centrifuged immediately at 1370 × g for 10 min at 4°C and the plasma was collected and used for measuring the oxidative stress indicators. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020 × g for 15 min at 4°C and then the erythrocyte lysate was collected for the measurement of catalase activity. A portion of erythrocyte lysate (500 μL) was treated with 5% trichloroacetic acid (TCA) (1:1 v/v), vortexed vigorously, and centrifuged at 28,000 × g for 5 min at 4°C. The supernatants were then removed, treated again with 5% TCA (1:3.1 v/v) and centrifuged once again at 28,000g for 5 min at 4°C. The clear supernatants were transferred to Eppendorf tubes and were used for the estimation of GSH. Plasma and erythrocyte lysate were stored at −80°C prior to biochemical analyses. For TBARS determination, a slightly modified assay of Keles [20] was applied, protein carbonyls were determined based on the method of Patsoukis [21], GSH was measured according to Reddy [22], catalase activity was determined based on the method of Aebi [23], and determination of TAC was based on the method of Janaszewska and Bartosz [24].

### 2.5. Telomerase activity

Telomerase activity in PBMCs was measured using a commercial telomerase PCR–ELISA (Roche Diagnostics Corp., Indianapolis, IN, USA), based on the telomeric repeat amplification protocol [25]. The method for the isolation of PBMCs is described elsewhere [26].

### 2.6. Rabbit hair and urine sampling

Hair and urine was collected initially prior to exposure and then once every month. In order to assess the accumulation of IMI and IMI-NH metabolites, the hair specimens were collected from the back of each animal just below the head. This pattern remained the same throughout the experiment. Collected hair samples were sealed in aluminium foil, labeled and stored in A4 paper envelopes in a dry dark place, at room temperature until analysis. Urine samples were collected in falcons (Tube 15 mL, 120 × 17 mm, PP tube, HD-PE cap, SARSTEDT) and stored at a temperature of −20°C until analysis.

### 2.7. Hair sample preparation procedures

For the removal of the external contaminants a procedure used in previous studies [27,28], was employed with slight modifications. Briefly, hair specimens were washed once in 5 mL of water (for 10 min) and twice in 5 mL of methanol (for 1 min) at room temperature. Washed hair specimens were dried in the oven. The methanol wash was tested for IMI and 6-CNA to confirm that external contamination was eliminated. Subsequently, an amount of 50 mg of hair was cut in 2–3 mm pieces, weighed and finally transferred in a glass test-tube with a screw top. Ten μL of ethirimol (ETH) were added (10 μg/mL) as internal standard. In order to isolate the analytes from the hair samples, the hair was incubated with 2 mL of methanol at room temperature in an ultrasonic bath for 3 h. The temperature of the bath during the ultrasonic extraction did not exceed 50°C. The methanol extract was filtered through 0.2 μm membrane filter (EconoFilter, Agilent Technology) to a glass test-tube. The same procedure was repeated once more and finally the total 4 mL of methanol were evaporated to dryness under a gentle nitrogen stream at room temperature. The residue was dissolved in 100 μL of methanol and 10 μL were injected to the LC–MS system.

### 2.8. Urine and serum sample preparation procedures

To 0.5 mL of urine, 10 μL of ETHE solution (10 μg/mL in methanol) were added. The specimen was further diluted with 1.5 mL of water (HPLC grade) and 2 mL of dichloromethane were added. The mixture was shaken mechanically for 10 min, and centrifuged at 4000 rpm for 5 min, after which the organic layer was collected. The aqueous phase was acidified with 10 μL of HCl 6 M and then 2 mL of dichloromethane were added again. The extraction procedure was repeated once more. Both organic layers (total 4 mL) were mixed and evaporated to dryness under a gentle nitrogen stream at 25°C. The residue was reconstituted in 100 μL of methanol and 10 μL were injected into LC–MS.

### 2.9. Liquid chromatography and mass spectrometry conditions

Liquid chromatography was carried out using the Shimadzu Prominence LC system consisting of a binary LC pump, a vacuum degasser, an auto sampler and a column oven. A gradient of 0.1% formic acid in water (solvent A) and methanol (solvent B) were selected for routine use: starting at 20% of solvent B, 100% B (15.0 min linear ramp) and finally 20% B (5 min). Total mobile phase pumped at 0.6 mL/min through a Discovery C18 HPLC column (25 cm × 4.6 mm, 5-μm) thermostated at 30°C. A 10 μL volume of each sample was injected in the mobile phase flow. A mass spectrometer (LCMS-2010 EV Shimadzu), comprising an atmospheric pressure chemical ionization (APCI) interface and a single quadrupole mass filter, was used to detect and quantify IMI and IMI-NH metabolites in column effluent (see Fig. 1, Supplementary information section). Interface, curved desolvation system (CDL) and heat block temperatures were 400°C, 200°C and 200°C, respectively. The detector voltage was 1.5 kV and the nebulizing gas flow 2.5 L/min. Drying gas was set at 0.02 MPa. Ion signals were acquired in time-selected ion monitoring (SIM) mode: with ions m/z = 256.10, 211.85, 174.95 for IMI; 157.85, 189.90, 96.00 for 6-CNA; 211.05, 213.00 for IMI-NH; and 210.10 for ETHE; used as internal standard (see Table 1). The mass-spectrometry operating conditions were tuned according to the manufacturer procedure. Data acquisition and processing were performed using LC–MS Solution software (Shimadzu, version 3.40.307). Stock and working solutions of the parent compound of IMI and IMI-NH metabolites, IMI-NH and 6-CNA at a concentration of 1 mg/mL were prepared in methanol. Working solutions of mixed analytes were prepared weekly by dilutions in methanol in the concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1 and 10 μg/mL. All working solutions were stored at −20°C. The analytical parameters of the developed LC–APCI(+)-MS base method for the determination and quantification of IMI, 6-CNA and IMI-NH in hair, urine and blood samples are shown in Table 1.

### 3. Statistical methods

Statistical analysis of the MN data was performed with the G-test for independence on 2 × 2 tables. The chi-squared test was used for the
4. Results

4.1. Metabolite concentrations in urine, hair and blood serum

Levels of IMI and its metabolites, IMI-NH and 6-CNA, were measured after the first and second month of administration in all groups (CON, IMI, IMI-NH) in urine and hair samples, and in blood samples at the end of the administration scheme (2 months). The results are summarized in Table 2. Overall mean IMI levels in urine significantly increased (F = 12.77, df = 1, p = .023) through time. The above described pattern was not observed for IMI levels measured in hair samples. IMI-NH levels in urine were shown to be affected by ST (F = 109.99, df = 1, p < .001) and decreased through time (F = 140.28, df = 1, p < 0.001; 1st month p = .024; 2nd month p = .019). IMI-NH blood levels were not affected by the addition of ST. All measured levels in hair were below the limit of detection (LOD). The 6-CNA metabolite of IMI was not measured in hair samples due to LOD limitations. In blood samples there was no significant effect from the addition of ST after 2 months of exposure (p = .086). Urine levels of 6-CNA were not affected.

Based on urine excretion, it is quite clear that from the addition of ST, the concentration of the IMI-NH metabolites in the IMI-ST group is reduced by 91% compared to the IMI group one month after exposure and the same pattern was observed at the end of the administration scheme. Although excretion of IMI-NH decreases with time, the efficiency of ST as an inhibitor of IMI-NH metabolites remained the same, 91% and 86%.

4.2. Metabolite excretions in urine, hair and blood serum

Calculating the excretion rate of urine can be accomplished by taking into account parameters such as daily urine excretion, detection concentration of the requested metabolite and the total administered amount of the substance, in this case, IMI. For blood, the same pattern was followed, but the daily amount was considered stable due to controlled sampling procedures giving approximately 6 mL of blood. The excretion rate of IMI, IMI-NH and 6-CNA in hair is not used as a calculation method of excretion and can only be applied for accumulation rates.

The excretion rate of IMI as a parent compound in urine without the addition of ST during the first month was 148% and the second month 182%. With the concomitant administration of ST, urine excretions were 288% and 239%. Regarding IMI-NH as a metabolite of AOX metabolism, without the addition of ST during the first month it was found to be 505% and for the second month 89.3%. With the concomitant administration of ST, urine excretions were 44.3% and 12.2%. Finally, the excretion rate of 6-CNA, the major metabolite of IMI, was found to be 27.5% the first month and 12.9% the second month. Under the influence of ST, urine excretions were 19.8% and 35.1%, respectively (Fig. 2, see Supplementary information section). In blood samples, however, due to sampling only of the final month, excretion rate of IMI without the influence of ST was 0.68% and under the influence of ST, 3.3%. Regarding IMI-NH, without the influence of ST, the excretion rate was 0.04% and under the influence of ST, 0%. Finally, 6-CNA, was
found to be 0.02% and under the influence of ST, 0.09%.

### 4.3. Micronucleus assay

Table 3 Results of MN assay in cultures of rabbit lymphocytes showing BN, BNMN, MN and CBPI, from the exposure of IMI and IMI + ST.

<table>
<thead>
<tr>
<th>Exposed group</th>
<th>BN cells scored</th>
<th>BNMN ± s.e.</th>
<th>G</th>
<th>p</th>
<th>MN ± s.e.</th>
<th>G</th>
<th>p</th>
<th>CBPI ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline values</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>3000</td>
<td>6.33 ± 0.82</td>
<td>6.33 ± 1.25</td>
<td>1.54 ± 0.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMI</td>
<td>3000</td>
<td>7.00 ± 0.82</td>
<td>0.069</td>
<td>0.793</td>
<td>151</td>
<td>0.697</td>
<td>1.53 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>IMI + ST</td>
<td>3000</td>
<td>6.33 ± 0.47</td>
<td>0.000</td>
<td>1.000</td>
<td>7.33 ± 0.94</td>
<td>0.151</td>
<td>0.697</td>
<td>1.49 ± 0.02</td>
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<tr>
<td><strong>Two months of exposure</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>CON</td>
<td>3000</td>
<td>7.00 ± 0.82</td>
<td>7.33 ± 0.47</td>
<td>1.46 ± 0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMI</td>
<td>3000</td>
<td>14.33 ± 0.47</td>
<td>5.927</td>
<td>0.005</td>
<td>15.33 ± 0.47</td>
<td>6.687</td>
<td>0.010</td>
<td>1.47 ± 0.02</td>
</tr>
<tr>
<td>IMI + ST</td>
<td>3000</td>
<td>11.00 ± 0.82</td>
<td>1.960</td>
<td>0.162</td>
<td>12.33 ± 0.82</td>
<td>2.85</td>
<td>0.091</td>
<td>1.45 ± 0.01</td>
</tr>
</tbody>
</table>

BN: Binucleated cells (for each rabbit 1000 BN cells were scored, three rabbits/group result in a total of 3000 BN cells scored), BNMN: Binucleated cells with micronuclei, MN: micronuclei, CBPI: Cytokinesis Block Proliferation Index, s.e.: standard error, p: statistical parameter, *p < 0.05, comparison made with the control group, G. 2POi ln(Oi/Ei), where Oi is the observed frequency in a cell, Ei is the expected frequency under the null hypothesis, ln denotes the natural logarithm and the sum is taken over all non-empty cells.

The frequency of BNMN was increased compared to the controls after the 2-month exposure (Table 3) by 126% for IMI and 73% for IMI + ST, whereas regarding the frequency of MN, the increase reached 142% and 95%, respectively.

The CBPI showed no statistically significant difference between controls and exposed animals at any time of exposure (p > 0.05), which indicates that there is no overall cytotoxic effect.

### 4.4. Comet assay

An increase in tail intensity (Fig. 3, see Supplementary information section) was observed in both exposure groups after 2 months compared to controls (70.7% IMI group, 48.5% IMI + ST groups), although not statistically significant (Fig. 1).

### 4.5. Oxidative stress markers

The levels of oxidative stress markers for all studied groups are presented in Table 4. TAC and glutathione remained practically unchanged and unaffected by IMI exposure. A nearly significant increase (23% Crbnls) in protein oxidation accompanied by a decrease in lipid peroxidation (9% TBARS) was observed in the IMI group. Catalase activity was also non-significantly decreased (15%). This redox disturbance was not observed in the presence of ST (p = .125).

### 4.6. Telomerase activity

In Fig. 2, telomere activity (%) does not differ significantly among exposed groups (ANOVA p = .068, Kruskal-Wallis p = .051). Nevertheless, the IMI + ST group showed the higher activity (37% increase in comparison to IMI group), probably indicating higher systemic inflammation levels.

### 5. Discussion

In the present study, we investigated whether the AOX metabolic reduction of IMI is systemically more detrimental than the CYP oxidative metabolism thereof. Systemic damage was assessed by measuring genotoxic and cytotoxic effects, by assessing oxidative status of the...
animals, as well as the systemic inflammation state.

From the results, we can additionally note that the best matrix to observe IMI concentrations is via hair samples and for IMI-NH via urine samples, where we monitored the 24 h urine production of the animals from the first week during the first month of administration and the same pattern was followed also for the second month. In order to calculate the excretion rate of IMI, IMI-NH and 6-CNA, the amount of urine excreted by an animal depends on many factors, including food and water consumption, activity and environmental temperature. A 24-h urine volume of an adult rabbit, with free access to food and water, varies within a wide range (20–350 mL/kg bw), with an average excretion rate of about 130 mL/kg bw [29]. In our current study, the excretion rate fell between these levels, with a urine excretion rate of 330 mL/day. The reduced metabolites of IMI (IMI-NH) are excreted first, while IMI as the parent compound, was excreted in urine and blood with increased concentrations by the end of the experiment. The oxidized IMI metabolites (6-CNA) are excreted steadily over the monitored 2-month period. The rate of excretion of IMI in urine samples increased slightly at the end of the 2-month period; while IMI-NH after the second month was reduced dramatically in the urine, obviously from the AOX inhibitory effect of ST, and in the hair samples because hair as a matrix cannot show the excretion rate of a substance but only the rate of accumulation.

However, the AOX-generated IMI metabolites are not all regarded detoxification products. IMI-NH is assumed to be a possible contributor to the nicotinic effects of IMI. IMI-NH can become over 300 times more potent than IMI at the mammalian nAChR and when referring to mouse in vivo effects of IMI, IMI-NH can become a clastogenic effect in exposed rabbits and that this effect is attenuated by co-administration of the AOX inhibitor ST. This highlights the AOX-mediated metabolism of IMI to IMI-NH as a prominent pathway to the genotoxicity of IMI and IMI + ST groups. IMI, besides this study in rabbits, has also been studied in loaches for which IMI had a genetic toxic effect on erythrocytes [36], and on the Argentinian frog for which signs of genotoxicity were also present [37]. Furthermore, the genotoxicity of IMI was found to have a potential adverse effect in human peripheral blood samples [38], but studying the relation of the metabolic activation and composition of a commercial product containing IMI on human peripheral blood samples did not show genotoxicity at a specific given dose [39].

The oxidative as well as the inflammatory status of mammals exposed to neonicotinoid insecticides, such as IMI, have been studied often. It is believed that insecticides affect the vital immune mechanisms and can induce various inflammatory conditions [40]. Oxidative stress has been also studied in rabbits following long-term exposure to diazinon and propoxur, showing a discrete and concentration-dependent effect in the liver and kidney [41].

Evaluation of the possible genotoxic and cytotoxic effects in human peripheral blood lymphocytes exposed in vitro to thiacloprid and clothianidin showed that at high concentrations they significantly reduced human lymphocyte viability and eventually caused cell death [42]. Possible cytotoxic and DNA damage effects of common neonicotinoid insecticides acetamiprid, clothianidin, IMI, thiacloprid and thiamethoxam in hepatocellular carcinoma (HepG2) and neuroblastoma (SH-SY5Y) cells showed changes in DNA damage at the high concentrations of these neonicotinoids after a 24-h exposure by using the alkaline comet assay in HepG2 and SH-SY5Y cells [43].

Neonicotinoids not only control insect pests but also, independently, alter plant growth and responses to stress. IMI, thiacloprid, acetamiprid, thiamethoxam and clothianidin, but not nitenpyram and di-n-octetufuran, induced foliar lesions and peroxidative damage in soybean (Glycine max) seedlings assayed with the 3,3-diaminobenzidine stain [44].

6. Conclusions

The results of the present study clearly demonstrate that IMI induces a clastogenic effect in exposed rabbits and that this effect is attenuated by co-administration of the AOX inhibitor ST. This highlights the AOX-mediated metabolism of IMI to IMI-NH as a prominent pathway to the mutagenic effects of this pesticide, in detriment to the CYPs-mediated metabolism.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

The authors would like to thank Dr. Stagko Dimitrio for his continuous support on the oxidative stress analysis as well as Aristidis K. Tsamoukas for helping through the animal procedures.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mrgentox.2018.03.002.
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