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Bioactive Constituents, Metabolites, and Functions

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The Metabolism Distribution and Effect of Thiamethoxam After Oral Exposure in Mongolian racerunner (*Eremias argus*)

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ABSTRACT: Systematically evaluation of the metabolism, distribution and effect of thiamethoxam in mongolian racerunner (*Eremias argus*) were carried out after oral exposure. The HPLC equipped with Q Exactive focus was used for identification and concentration analysis of thiamethoxam and its metabolites. Percutaneous and urine excretions were the primary ways for the elimination of thiamethoxam and its metabolites, and the limiting factor was urine output. Demethylation thiamethoxam and clothianidin were the main metabolites of thiamethoxam in lizard. The CYP3A4, CYP3A7 and CYP2C9 played a crucial role in the metabolism process. Aldehyde oxidase only dominated the nitro-reduction process of demethylation thiamethoxam and clothianidin. Glutathione S-transferase might be related to the clearance process of thiamethoxam and its metabolites. The findings indicated that thiamethoxam might pose potential carcinogenic and hepatic injury risk to lizards. The results enrich and supplement the knowledge of the environmental fate of thiamethoxam in reptiles.

Key words: *Eremias argus*, Thiamethoxam, Metabolism, Distribution

INTRODUCTION

Because of the excellent properties of low application rate, broad spectrum, high efficiency, and quick absorption and translocation in plants, neonicotinoids have become the fastest growing insecticides in the world[1-3]. Compared to mammals, neonicotinoids show a higher degree of specificity for insect nicotinic acetylcholine receptors (nACRs) and are therefore considered to be an environmentally friendly insecticide[4,5]. Thiamethoxam (CAS name: tetrahydro-3-methyl-N-nitro-4H-1,3,5-oxadiazin-4-imine, TMX) contains a 2-chloro-5-(chloromethyl)- thiazole (Figure 1), which exhibits high insecticidal contact, stomach and systemic activity[6,7]. TMX is widely used to prevent and control commercially important pest insects, such as aphids, whiteflies, thrips, golden needles, leaf miners and some *Lepidoptera* and *Coleoptera* species[8,9]. As a common seed coating insecticide, TMX is recommended to provide continuous protection from insect herbivory throughout the entire growing season[10,11]. However, only 2-20% of the pesticide is absorbed by the target crop and the rest is left in the soil environment[12]. TMX could persist in the soil with the half-life exceeding 350d[13]. Unreasonable and repeated use of TMX results in residues in soil and other environmental media[14,15], which might pose potential risks to organisms in the soil as well as higher animals.

Although neonicotinoids are considered highly selective for insect nAChRs, the

N-desnitro and descyano metabolites of neonicotinoids might be more toxic to mammalian nAChRs than that of insects [16-18]. The metabolites of TMX may have greater environmental risks than TMX. For example, TMX is considered to be one of the major neonicotinoid pesticides that may cause a significant decline in bee populations[13,19], and its metabolite clothianidin (CLO) might aggravate this toxic effect[20,21]. The demethylated metabolite of TMX had been shown to have carcinogenic effects in mice[22]. The metabolite of TMX is one of the important factors causing the toxic effect of TMX. Therefore it is crucial to understand the metabolic process of TMX and the form of metabolites in vivo to further assess their toxicological risk. The studies of the metabolism of TMX were mainly concentrated in mammals, such as mice, rats and rabbits[22-24]. However, the metabolism and distribution of TMX in reptiles has rarely been reported.

As an anthropogenic pollutant, pesticides are considered to be an important cause of population decline in reptiles[25,26]. Inhalation, food intake, and skin penetration are the primary routes for reptiles exposed to pesticides directly[27]. As an important animal in agro-ecosystem[28], soil-living habits and regular swallowing of soil[29] make lizards exposed to pesticides at great risks. Because of the repeated use and long persistence in soil, TMX could be transferred into cultivated soil in large amounts, which threatens lizards living in farmland soil. Although the metabolic behavior of TMX in mammals has been reported, the metabolism of exogenous substances in living

organisms is species-dependent due to the subtle differences in the structure of metabolic enzymes between species. To our knowledge, as a species exposed directly to soil pesticide residues, lizards have rarely been used as experimental animal in the metabolic studies of TMX.

To complement this research area, Chinese lizards (*Eremias argus*) were used to evaluate the metabolism, distribution and effect of TMX on reptiles in this study. *E. argus* is a kind of small species commonly found in the north of the Yangtze River. It is widely distributed in China's important agricultural areas such as the North China Plain and Northeast China Region[30,31]. In these areas, the widespread use of pesticides has become a direct threat to the survival of lizards.

To better describe the actual environmental behavior and ecological risks of TMX in lizards from a comprehensive perspective, this study was performed to obtain the biological fate of TMX in lizard blood and to evaluate the metabolism and distribution of TMX and its metabolites in various tissues. The changes in the mRNA expressions of the metabolic enzymes in liver, kidney, and brain were used to identify enzymes that play a major role in TMX metabolism which were verified by subsequent enzyme inhibitor experiments. This study obtained basic data on the metabolism, distribution, and effects of TMX in lizards, and provided research guidance for the subsequent toxic effects of TMX and its metabolites in reptiles.

MATERIALS AND METHODS

Reagents

Thiamethoxam (TMX, 98.2% purity) and clothianidin (CLO, 99.0% purity) were provided by Institute for the Control of Agrochemicals, Ministry of Agriculture. All solvents of methanol, ethanol, acetone, acetonitrile, n-hexane, and isopropanol were HPLC grade and purchased from Dikma (Beijing, China). Nootkatone, sulfaphenazolum, quinidine, omeprazole, 2,4-Dinitrochlorobenzene, ketoconazole and estrogen (analytical grade) were purchase from Sigma-Aldrich (Beijing, China).

Animals and husbandry

The sexually mature *E. argus* (3-3.5 g) were collected from our breeding colony in Changping district, Beijing, China. Lizards were reared in 5 ×1.2 × 0.4 m solid bottom indoor aquarium covered with 10 cm fallen leaves and mollisol. The temperature and humidity were kept at 25-30 ^{o}C and 30-60%, respectively. Daylight lamps (100 W) were set to a 14:10-h light: dark photoperiod to provide enough light and maintain the setting temperature. Lizards were fed with mealworms (*Tenebrio molitor*) twice a day. The water was sprayed every other day and the excreta and residues were cleaned twice a week.

Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). The animal care and use procedures were approved by Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

Exposure experiment and sampling

TMX was first dissolved in the ethanol then dispersed in corn oil. The content of ethanol should be less than 10%. The testing dose was 20mg/kg $^{-bw}$. The corn oil-ethanol lactescence were continually mixed on the magnetic stirring apparatus before dosing. The microinjector was used to deliver a volume of 15-30μL corn oil or corn oil-ethanol lactescence into the oral cavity of each lizard according to the body weight.

After oral administration, lizards were euthanized at 1, 3, 6, 10, 12, 16 and 24h. Three lizards were selected randomly at each sampling point. The blood, brain, heart, lungs, stomach, intestine, liver, kidney, skin, fat and gonad of each lizard were collected for concentration analysis of TMX and its metabolites. The brain, kidney and liver were collected, weighed, and frozen at -80 ^{o}C with RNA store at 12 and 24h. The dosing lizards were housed in 30 ×30 ×20 cm glass cage with a water dish. The experimental conditions were the same as the rearing condition. A diet for one lizard was consisted of two mealworms per day.

120

121 **Chromatographic separation and concentration analysis**

122 The blood and tissue samples were used for concentration analysis of TMX and its
123 metabolites. Whole blood (50μL) was transferred to a 2-mL polypropylene centrifuge
124 tube and 2 mL acetonitrile was added. Tissue homogenization matrices (0.05-0.1 g)
125 were transferred into a 10mL polypropylene centrifuge tube and 10 mL acetonitrile
126 was added. The mixture was mixed and vortexed for 3 min, exposed to ultrasonic
127 vibration for 20 min, then centrifuged at 10,000 r/min for 5 min. The supernatants
128 were collected. The sample was re-extracted in the same manner and the supernatants
129 were combined. The supernatants were evaporated to near dryness at 30 °C using a
130 vacuum rotary evaporator and dried under a gentle stream of nitrogen. The residue
131 was re-dissolved in 1 mL of acetonitrile and passed through a 0.22μm filter (Nylon 66)
132 into a sample vial for HPLC-QE analysis.

133

134 The TMX and its metabolites were separated by HPLC (Ultimate 3000, Thermo
135 Scientific, USA) equipped with Acquity HSS T3 column (Waters, 2.1mm id ×10 cm
136 long). The mobile phase was a mixture of 80% acetanitrile and 20% water with a flow
137 rate of 0.25 mL/min. The column temperature was set at 35 ^{o}C and the injection
138 volume was 10 μL. The scanning mode was positive-ionization mode.

139

A Q Exactive Focus (Thermo Scientific, USA) with a heat electrospray ionization, a quaternary pump, an autosampler, an online vacuum degasser, and a thermostatted column compartment was employed for analysis. The optimized parameters of MS are: spray voltage: +3500 or -3000V; sheath gas pressure: 35 arb; aux gas pressure: 5 arb; sweep gas pressure: 0 arb; capillary temperature: 320 $^{\circ}$C; auxiliary gas heater temperature: 300 $^{\circ}$C; S-lens RF level: 50 V, scan mode: (1) full MS: resolution: 70 000; automatic gain control target: $1.0e^6$; maximum injection time: 50 ms; scan range: 100-1000 *m/z*; (2) dd-MS^2/dd-SIM: resolution: 35000; automatic gain control target: $1.0e^6$; maximum injection time: 50 ms; loop count: 5; isolation window: 3.0 *m/z*; NCE/stepped: 20 30 40; dynamic exclusion: 10s. Nitrogen was used for spray stabilization and as the collision gas in the C-trap. The details of quantitative and quantitative ionization of TMX and its metabolites were shown in Table S1.

Isolation of RNA, cDNA synthesis and real-time PCR

Trizol reagent (Life Technology, Beijing, China) was used to isolate total RNA from lizard liver, brain and kidney. Remove traces of DNA by incubation with DNAse-I (Ambion). The RNA was dissolved in RNase-free water and stored at -80 ^{o}C. Reverse transcription reaction mixtures contained 4μL of DNTP, 2μL of Oligo $(dT)_{15}$ primers and 22μL of total RNA. The mixture was heated at 70 ^{o}C for 5 min and quickly cooled down on ice. Added 8 μL of 5×6 buffer, 2μL of M-mlv, and 40 units RNAsin (an RNase inhibitor) in a total volume of 41μL after cooling. The mixture was

161 incubated at 42 ^{o}C for 50 min and then heated to 95 ^{o}C for 5 min to inactivate the
162 reverse transcription reaction.
163
164 Genes of P450 enzymes family (*cyp 1a1, cyp 2c8, cyp 2d3, cyp 2d6, cyp 3a4, cyp*
165 *3a7*), Glutathione S-transferase (GST) family (*gstt, gsta, gstm, gstp*) and Aldehyde
166 oxidase (*aox*) were selected in this study. Primers were designed using NCBI
167 Primer-Blast (Table S2). The SYBR GREEN PCR kit (Tiangen Biotech, Beijing,
168 China) was used in Real-time PCR performed in the MX3005P realtime quantitative
169 polymerase chain reaction system (Stratagene, USA). The thermal cycle settings were:
170 5min at 95 ^{o}C, 40 cycles of 30s at 95 ^{o}C, 40s at 54 ^{o}C and 40s 72 ^{o}C. The MxPro
171 software was used in sample analysis. According to our previous results, the *β-actin*
172 gene was considered to be the most stable reference gene[32]. We sequenced the
173 Real-time PCR products and performed sequence alignments to verify product
174 specificity.
175
176 **Inhibition of enzyme activity**
177 The enzyme inhibitors nootkatone, sulfaphenazolum, quinidine, omeprazole,
178 ketoconazole, 2,4-dinitrochlorobenzene, and estrogen were dissolved in Me_2SO and
179 configured as 1mmol /L solution. Fresh liver was quickly collected after each lizard
180 (background clean) was euthanized. The blood on the surface of liver was washed
181 away with 8.5% salt water. The liver was then placed in 50mL centrifuge tube, adding

30mL potassium phosphate buffer (pH=7.4) for tissue homogenate. Incubation mixtures were prepared as follow: 1mL liver tissue homogenate, 10μL TMX solution (100 ppm, ACN), 5μL enzyme inhibitor solution. The ACN and Me_2SO ratio in incubations did not exceed 1.5%. Considering the lizard is hypothermic animal, incubations were aerobic at 25 ^{o}C for 1h. At the end of the reaction, 2 mL of ice-acetonitrile was added to terminate the reaction. After mixing and shaking, the mixtures were centrifuged at 10,000 rpm for 5 min. The supernatants were filtered through a 0.22μm filter (Nylon 66) into a sample vial for HPLC-QE analysis.

Data analysis

Pharmacokinetic parameters were calculated in the degradation of TMX. The degradation of TMX in blood appeared to follow a pseudo-first-order kinetic reaction. The relation between concentrations of TMX (C) and the sampling time (t) was expressed as follow:

$$\lg C = -\frac{k \cdot t}{2.303} + b \qquad (1)$$

The half-life period $t_{1/2}$ of TMX was calculated as follow:

$$t_{1/2} = \frac{0.693}{k} \qquad (2)$$

The SPSS 16.0 was used for correlation and significance analysis. All the other values in the text were presented as mean±SD.

RESULTS

Chromatography and Identification

The standard HPLC/QE conditions provide good detection of TMX and its metabolite CLO (Figure. S1). Linear calibration curves were obtained over the concentration range of 0.004-1mg/L for TMX (y=48185.9x+615486, R^2=0.9976), CLO (y=14901.2x+214429, R^2=0.9977). Six replicate recovery evaluations were performed at three fortified concentrations. The concentration levels were 0.01, 0.1, and 1mg/kg (mg/L for blood) for TMX and CLO. Recoveries of each chemical ranged from 83 to 97 percent. The precision of the assay for each chemical ranged from 2 to 6 percent (RSD). LODs were 0.003 mg/kg (mg/L) in lizard tissues (blood) for each chemical. Based on tissue and blood concentration at minimal fortified level, the LOQs were considered to be 0.01 mg/kg (mg/L). The HPLC/QE method described in this study was validated for the detection of TMX and CLO in lizards.

The Q Exactive focus has a resolution of more than 10, 000, 000 full width half maximum, with higher resolution polarity switching and higher resolution[33]. Higher quality resolution provides better selectivity for qualitative analysis of complex matrices[34]. The selection of qualitative and quantitative ions for TMX-dm, TMX-dm-NO, TMX-NH_2, CLO-dm, CLO-NO, and CLO-NH_2 (structural formula seen Figure. 1) was generally followed the method described by Dick et al. [23] and made some modifications. In the MRM spectra of TMX and CLO (standards, dissolved in acetonitrile), we speculated that the formulas of fragment ion peaks at

m/z 131.9669 should be $C_4H_3ClNS^+$, which is consistent with the results of mass bank. This fragment is the chlorine-substituted thiazole ring part of TMX and CLO (Figure. S2). In fact, the metabolites that concerned in our study all contain this chlorine-substituted thiazole ring, which could produce an ion peak of 131.9669 in mass spectrometry. After we used the m/z of each metabolite as the parent ion for MRM mode scans on treated lizard samples, we observed the fragment ion peak at m/z 131.9669 in all scans (data not shown). Therefore, the fragment ion of 131.9669 was used as the quantitative ion for relative quantitative analysis of each metabolite.

Metabolism in lizard blood

After single oral administration, the blood concentration-time curve of TMX was studied (Figure 2). The concentration of TMX first increased with time, reached the highest value at 3 h, and then decreased over time. The decline pattern was in accordance with the first order kinetics. According to equation 2, the $t_{1/2}$ of TMX was 2.70 h. The elimination of TMX in lizard blood was fast.

Tissue distribution

The residues of TMX and its metabolites were detected in tissue and blood samples at 1, 3, 6, 12, 24h (shown in Figure.3). The concentration of TMX in blood rapidly reached the maximum value at 3h after oral administration, while the highest residual concentration in tissues was detected 6h over oral administration. TMX was absorbed

245 quickly into the blood, and then allocated to each tissue according to the
246 two-compartment model. In all tissues, the highest TMX and CLO concentrations
247 were detected in gonad at 6h after administration. The high residue in gonad indicated
248 that it might have potential reproductive toxicity risk to lizards. The TMX residues in
249 heart and lung were also very high probably due to ample blood exchange in these
250 organs. The lowest residues of TMX and its metabolites were detected in fat. TMX
251 and its metabolites had good water solubility; therefore did not accumulate easily in
252 fat. The residues of TMX and its metabolites were also extremely low in brain. The
253 blood-brain barrier hindered the transmission of TMX and its metabolites to the brain.
254 The residues of TMX in skin were maintained at a high level. Although the
255 concentration of TMX in skin decreased with time, the concentrations of its
256 metabolites remained at a relatively stable concentration after 6h. As a common
257 molting animal, lizards can get rid of pollutants through molting. Therefore, the high
258 pollutant residues in the skin provided a possibility to remove pollutants through
259 molting.
260
261 Liver and kidney were considered as the main metabolism organs. The residues of
262 TMX were not high in liver and kidney in our study that reflected the balance of
263 metabolism and absorption. It was worth noting that highest residuals of CLO and
264 TMX-dm were detected in kidney. As water-soluble compounds, CLO and TMX-dm
265 were easily excreted through urine. *Eremias argus* adapted to the dry life. Low urine

volume was the main limiting factor for the residual of TMX metabolites in the kidneys of lizards.

Quantification of mRNA by real time PCR

P450s, aldehyde oxidase (AOX) and glutathione S-transferase (GST) are studied as important neonicotinoid pesticides metabolism related enzyme systems[24]. The expression of the P450s gene family (*cyp1a1, cyp2c8, cyp2d3, cyp2d6, cyp3a4, cyp3a7*), GST gene family (*gstt, gsta, gstm, gstp*) and *aox* in the lizard liver, brain and kidney was determined at 12h after oral administration (Figure. 4). In the liver, the expressions of *cyp3a4* and *aox* were up regulated significantly. Meanwhile, the expression of *gsta* and *gstm* showed obvious inhibition ($p<0.05$). In the brain, in addition to the slightly increased expression of *cyp3a7*, other enzyme genes in P450s family showed no obvious change ($p>0.05$). In GST family, the expressions of *gstt* and *gsta* increased significantly, while the expressions of *gstm* and *gstp* didn't change obviously ($p>0.05$). In kidney, the mRNA expressions of GST family didn't show obvious change. We observed the up-regulated expression of *cyp3a7*, while other P450 genes and *aox* were inhibited to varying degrees. These results indicated that CYP 3A4 and AOX played a leading role in TMX metabolism in liver, while CYP 3A7 played a leading role in kidney. CYP 3A is of great significance in the metabolic process of TMX.

Effects of Enzyme inhibitors

Changes in concentration of TMX and its primary and secondary metabolites after addition of enzyme inhibitors were shown in Figure. 5. In sulfaphenazolum and ketoconazole groups, the production of all metabolites was inhibited and this phenomenon was more pronounced in sulfaphenazolum group. This result indicated that in addition to CYP 3A4, CYP 2C9 might also play a key role in the metabolism of TMX. The formation of TMX-DM-NH_2 was inhibited in estrogen group while the formation of CLO-NH_2 and CLO-NO did not change significantly. The results show that AOX has a greater effect on the nitro reduction of TMX-DM than CLO.

DISCUSSION

N-demethylation, N-nitro reduction and cleavage of the oxadiazine are considered to be the major metabolic reactions after TMX entry into organisms[35]. However, as a neonicotinoid with tertiary nitrogen, TMX is a poor substrate for nitro reduction[23]. Therefore, CLO (formed by cleavage of the oxadiazine) and TMX-dm (formed by demethylation) are the main metabolites of TMX. CLO is widely detected in metabolic tests of TMX in mammals, plants, and insects[35-38]. P450s family is thought to play an important role in TMX metabolism towards CLO. Results from human recombinant CYP450 enzymes in vitro experiments showed that CYP 3A4, 2C19 and 2B6 participated in this metabolic process, and the role of CYP 3A4 is much more important than 2C19 and 2B6[39]. This result is consistent with the strong expression of

308 *cyp3a4* in the liver of our study. It has been reported that C or N-de-hydrocarbon and
309 C-hydroxylation are the common ways of CYP3A4 metabolism[40]. Meanwhile, we
310 detected strong expression of *cyp3a7* in kidney. The CYP 3A family may be
311 associated with the epoxide reduction in the metabolism process of TMX to CLO.
312 The enzyme inhibitor test showed that CYP 2C9 was also involved in the metabolism
313 of TMX. The CYP 2C9 and 2C19 genes share 91% homology, and their catalytic
314 substrates are approximately the same[41]. The content of CYP 2C9 in animals exceeds
315 that of CYP 2C19[42]. The CYP 2C9 and 2C19 have different effects on TMX
316 metabolism in different species.
317
318 As a demethylated metabolite of TMX, TMX-dm is considered as a potential
319 carcinogen and is the main cause of TMX's carcinogenicity[22,43]. The residual
320 concentration of TMX-dm varied significantly in metabolic studies of different
321 species. For example, studies had shown that TMX could be converted to TMX-dm
322 and cause a significant increase in liver cancer in mice. However, the residue of
323 TMX-dm was very low and did not show carcinogenic effect in rat. Meanwhile,
324 almost no TMX-dm was detected in human microsome assays[44]. It is reported that
325 CYP 2C19 is the key enzyme for the conversion of TMX to TMX-dm[39]. However, the
326 production of TMX-dm varied in different spices suggested that the conversion of
327 TMX to TMX-dm might be a complicated process not only dominated by one
328 metabolic enzyme. The difference in the structure and activity of metabolic enzymes

between species is the main reason for this result. In our study, TMX-dm residues were detected in all organs except brain and fat, with the highest residue concentration detected in kidney. This might pose a potential carcinogenic risk to the lizard.

The electronegative N-nitro tip (=N-NO_2) is considered as a characteristic structure that can selectively bind to the insect nAChR[45,46.] However, nitro-reduction of nitroneonicotinoids to form the positively charged aminoguanidine and guanidine metabolites is an activation of mammalian neurotoxicity[17,23]. Although some P450 enzymes such as CYP 1A2, CYP 2B6, CYP 2D6 and CYP 2E1 were reported to be involved in this process, AOX was still considered to be the main enzyme affecting this process[47,48]. When specific electron donors are present, AOX could reduce nitroguanidine neonicotinoids to form nitroso metabolites by two electrons and to form aminoguanidines by six electrons[49]. As we discussed above, TMX is not a suitable nitro-reduction substrate, whereas TMX-dm and CLO showed opposite properties. It had been reported that AOX of rabbit liver reduced TMX-dm to TMX-dm-NO and TMX-dm-NH_2, CLO to CLO-NO and CLO -NH_2 [24]. The results of our study confirmed this conclusion. The upregulation of AOX mRNA expression in the liver facilitated further nitro reduction of TMX-dm and CLO. It should be noted that the expression of AOX in the kidney is not active, and the inhibition of AOX did not have a significant effect on the production of the metabolites in the enzyme

inhibitor test. Because of rapid excretion, low residual concentrations of metabolites in lizard do not stimulate the strong expression of AOX in our study.

GST is a type of phase II isoenzyme that involved in eliminating exogenous contaminants through the coupling of glutathione (GSH) and electrophilic substrates to generate soluble compounds[50]. The substitutions of chlorine on thiazole and pyridine of neonicotinoid could be partially to be replaced by GSH, yielding ultimately to the N-acetylcysteine, S-substituted-cysteinyl and S-methyl derivatives[35,39]. It is reported that RNA interference of GST genes increases the susceptibility of insects to TMX[51]. GST might be related to the clearance process of TMX and its metabolites. Meanwhile, GST is an important indicator of the body's oxidative stress response[52]. Changes in GST-related regulatory genes in the kidney were not significant, but expressions of *gsta* and *gstm* were severely inhibited in the liver. This result indicated that TMX might cause oxidative stress in the liver. Oral exposure to TMX may cause hepatic injury in lizards.

Kevin et.al reported the metabolism of TMX in mice[24]. The TMX concentrations in mice liver and blood declined quickly that consistent with our results. They found TMX was metabolically active in the mice brain and high residual concentration of TMX-dm was detected. However, the concentrations of TMX and its metabolites in lizard brain were very low in our study. TMX and its metabolites hardly entered the

371 brain through the blood-brain barrier in lizard. TMX-dm was tested to have a longer
372 persistence than TMX and CLO in mice liver. However, this phenomenon was not
373 observed in lizard liver. These results indicated that the metabolism of TMX in tissues
374 was species-dependent. TMX, TMX-dm, and CLO could be excreted in urine
375 (19-27%) and feces (0.9-1.5%) in mice within 24 hours. This was consistent with our
376 results. Low residue concentrations of TMX were detected in lizard kidney. TMX and
377 its metabolites were highly water-soluble. Therefore, excretion through the urine was
378 their main route of elimination. Metabolism and exclusion were the clearances of
379 TMX that occured in the kidney. However, the residues of TMX-dm and CLO were
380 very high in lizard kidney. *Eremias argus* is a type of animal that prefers arid
381 environments. Low urine output was the main cause of TMX-dm and CLO residues in
382 the kidneys. The residuals of TMX-dm in lizard tissues might pose a potential
383 carcinogenic risk to the lizard.
384

ABBREVIATIONS USED

TMX, thiamethoxam; CLO, clothianidin; CLO-dm, desmethylclothianidin; dm, desmethyl; TMX-dm, desmethylthiamethoxam; AOX, aldehyde oxidase; GST, glutathione S-transferase; GSH, glutathione.

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394 **SUPPORTING INFORMATION DESCRIPTION**

395 Supporting information contains two figures and two tables. The captions are listed as
396 below.

397

398 Figure. S1. Representative HPLC-MS chromatogram of the expectations of 1 mg/L
399 for (A) TMX, (B) CLO

400

401 Figure. S2. The structure of fragment ion peak at m/z 131.9669

402

403 Table S1 The quantitative and quantitative ionizations of TMX and its metabolites

404

405 Table S2 Primers used for PCR and the quantification of the mRNA expression by
406 real-time PCR.

407

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571 FIGURE CAPTIONS

572

573 Figure. 1. Structure of the thiamethoxam and its metabolites

574

575 Figure. 2. The TMX concentration-time curve in blood after single oral administration
576 (log C=-0.1116t+1.6567).

577

578 Figure. 3. Tissue distribution of TMX and its metabolites CLO and TMX-dm at 1, 3,
579 6, 12, 24h. The relative concentrations of TMX-dm in tissues were calculated base on
580 the relative concentration of TMX-dm in the liver at 3h as 100.

581

582 Figure. 4. Relative gene levels of *cyp1a1, cyp2c8, cyp2d3, cyp2d6, cyp3a4, cyp3a7,*
583 *gstt, gsta, gstm, gstp, aox* in the liver, kidney and brain at 12h. The result was
584 evaluated as the relative ratio of the expression level of each mRNA to that of β-actin.
585 Two bars labeled above the error line indicate a significant difference on gene
586 expression at the same time point between the control group and treatment groups at a
587 significant level of $p<0.05$ (one-way ANOVA).

588

589 Figure. 5. Changes in the concentration of TMX metabolites after enzyme inhibitor
590 addition. The result was evaluated as the relative ratio of each TMX metabolite
591 concentration level of each enzyme inhibitor group to that of control group.

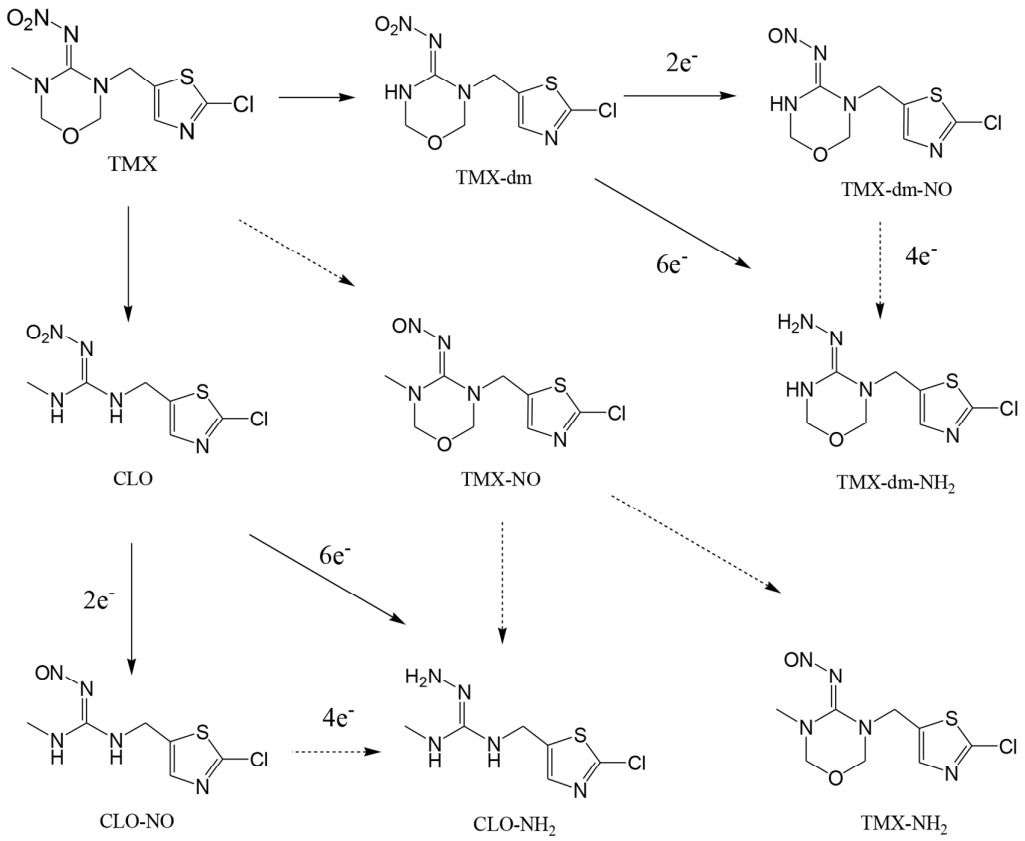

TMX
TMX-dm
TMX-dm-NO
CLO
TMX-NO
TMX-dm-NH2
CLO-NO
CLO-NH2
TMX-NH2
2e-
6e-
4e-
6e-
2e-
4e-

Figure. 1

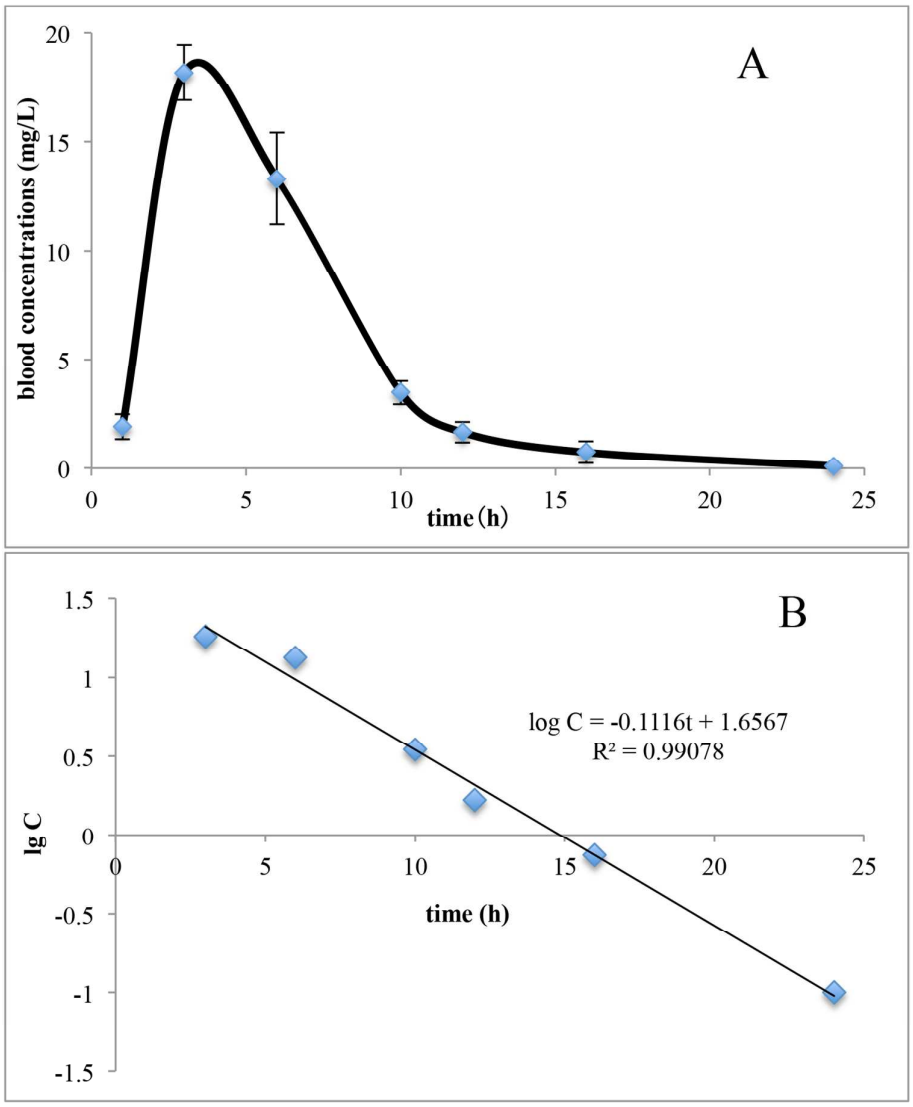
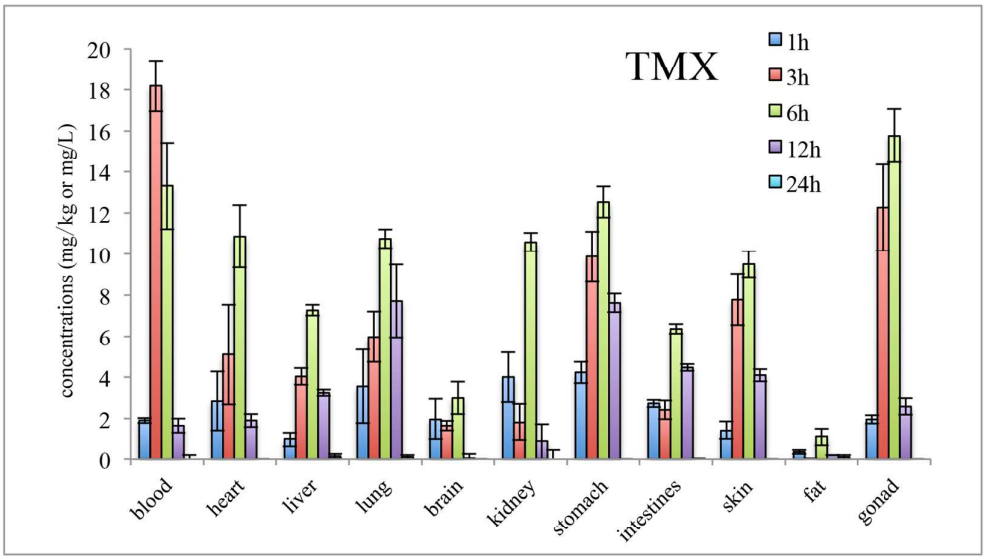
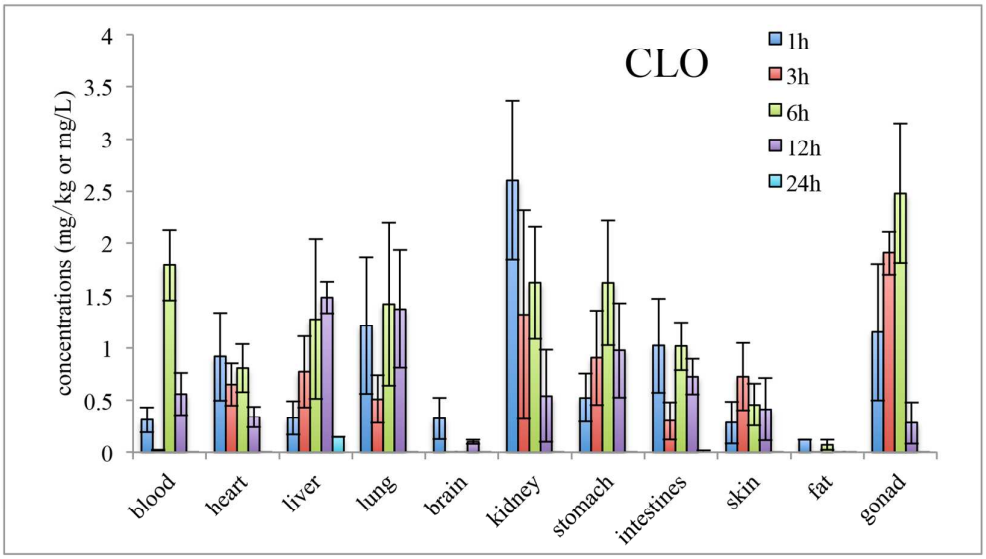

A
blood concentrations (mg/L)
time (h)
B
log C = -0.1116t + 1.6567
R² = 0.99078
lg C
time (h)

Figure. 2


TMX
concentrations (mg/kg or mg/L)
20
18
16
14
12
10
8
6
4
2
0
1h
3h
6h
12h
24h
blood
heart
liver
lung
brain
kidney
stomach
intestines
skin
fat
gonad


CLO
concentrations (mg/kg or mg/L)
4
3.5
3
2.5
2
1.5
1
0.5
0
1h
3h
6h
12h
24h
blood
heart
liver
lung
brain
kidney
stomach
intestines
skin
fat
gonad

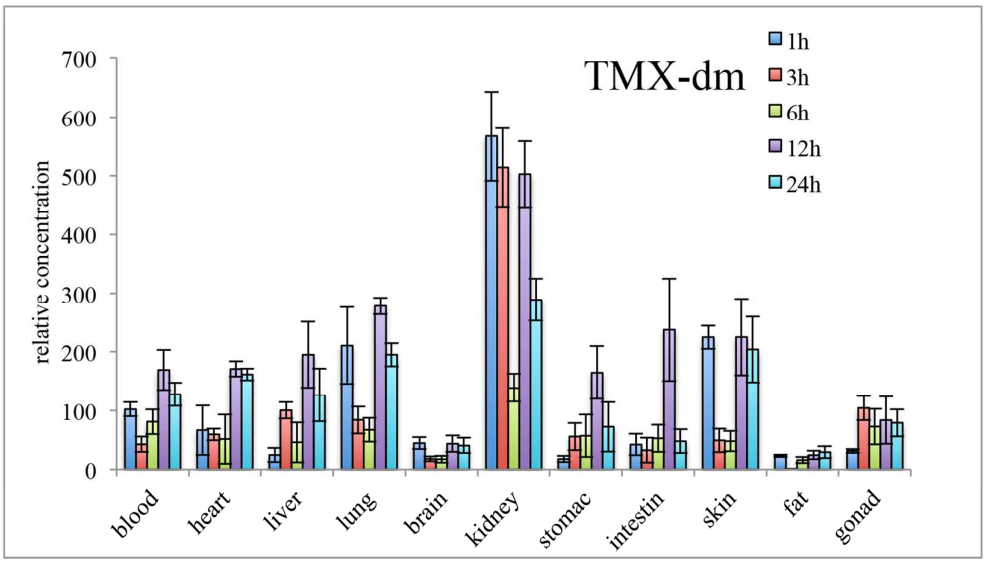

TMX-dm
relative concentration
700
600
500
400
300
200
100
0
1h
3h
6h
12h
24h
blood
heart
liver
lung
brain
kidney
stomac
intestin
skin
fat
gonad

Figure. 3

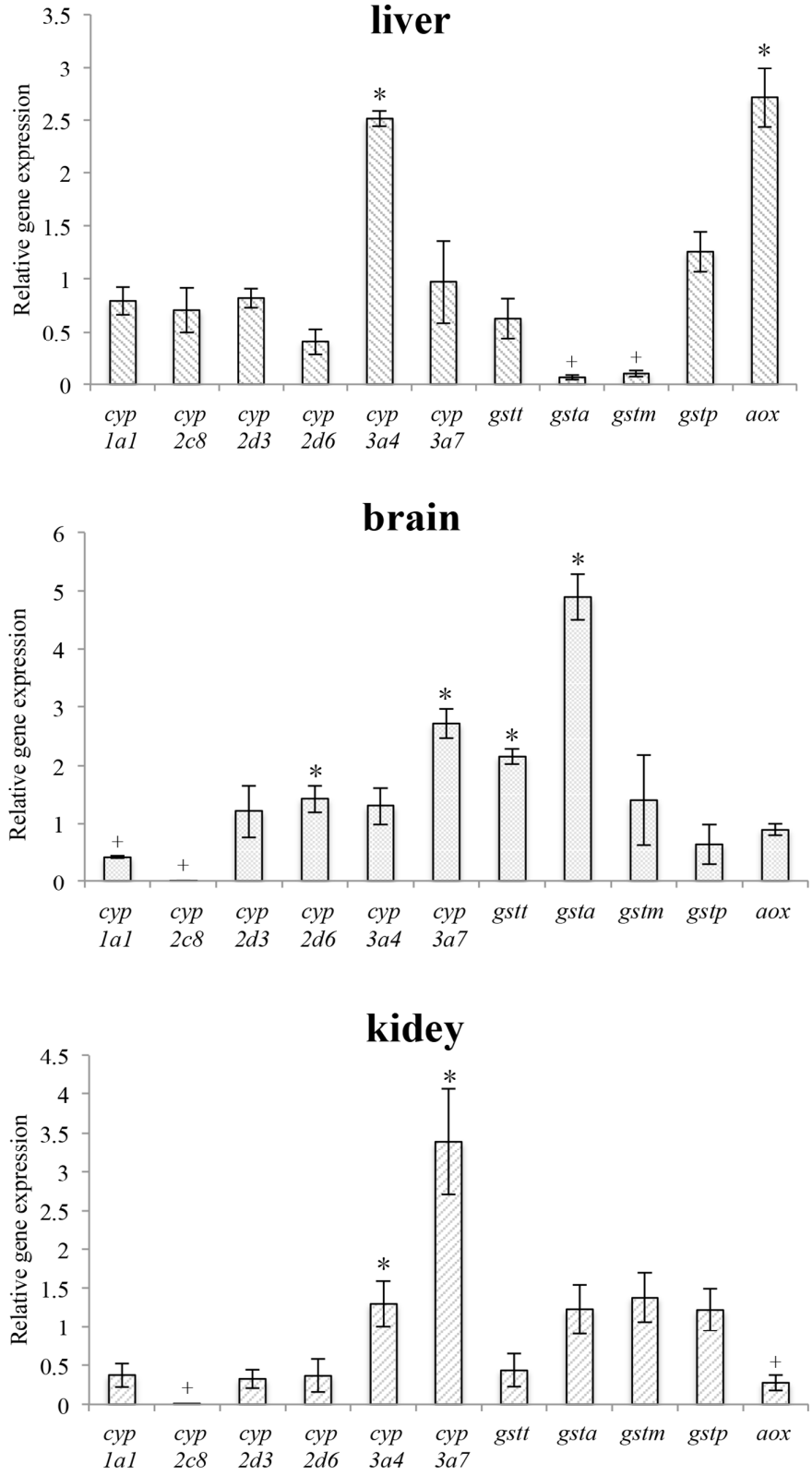

liver
Relative gene expression
3.5
3
2.5
2
1.5
1
0.5
0
cyp 1a1
cyp 2c8
cyp 2d3
cyp 2d6
cyp 3a4
cyp 3a7
gstt
gsta
gstm
gstp
aox
brain
Relative gene expression
6
5
4
3
2
1
0
cyp 1a1
cyp 2c8
cyp 2d3
cyp 2d6
cyp 3a4
cyp 3a7
gstt
gsta
gstm
gstp
aox
kidey
Relative gene expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
cyp 1a1
cyp 2c8
cyp 2d3
cyp 2d6
cyp 3a4
cyp 3a7
gstt
gsta
gstm
gstp
aox

Figure. 4

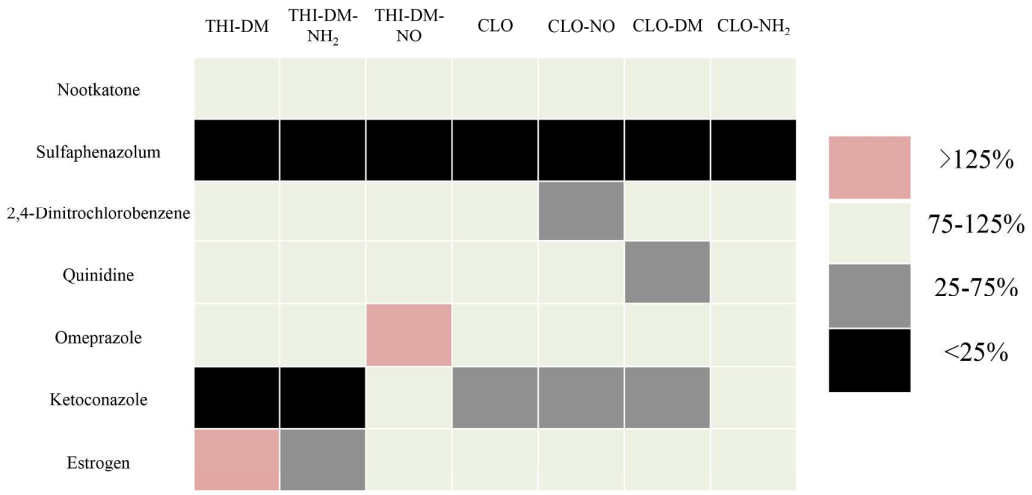
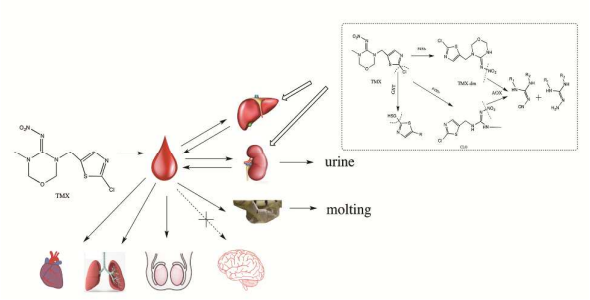

THI-DM
THI-DM-NH2
THI-DM-NO
CLO
CLO-NO
CLO-DM
CLO-NH2
Nootkatone
Sulfaphenazolum
2,4-Dinitrochlorobenzene
Quinidine
Omeprazole
Ketoconazole
Estrogen
>125%
75-125%
25-75%
<25%

Figure. 5



Graphic for table of contents