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Thiamethoxam Induced Mouse Liver Tumors and Their Relevance to Humans
Part 2: Species Differences in Response

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Thiamethoxam is a neonicotinoid insecticide that is not a mutagen, but it did cause a significant increase in liver cancer in mice, but not rats, in chronic dietary feeding studies. Previous studies in mice have characterized a carcinogenicity mode of action that involved depletion of plasma cholesterol, cell death, both as single cell necrosis and as apoptosis, and sustained increases in cell replication rates. In a study reported in this article, female rats have been exposed to thiamethoxam in their diet at concentrations of 0, 1000, and 3000 ppm for 50 weeks, a study design directly comparable to the mouse study in which the mode of action changes were characterized. In rats, thiamethoxam had no adverse effects on either the biochemistry or histopathology of the liver at any time point during the study. Cell replication rates were not increased, in fact they were significantly decreased at several time points. The lack of effect on the rat liver is entirely consistent with the lack of liver tumor formation in the two-year cancer bioassay. Comparisons of the metabolism of thiamethoxam in rats and mice have shown that concentrations of the parent chemical were either similar or higher in rat blood than in mouse blood in both single dose and the dietary studies strongly indicating that thiamethoxam itself is unlikely to play a role in the development of liver tumors. In contrast, the concentrations of the two metabolites, CGA265307 and CGA330050, shown to play a role in the development of liver damage in the mouse, were 140- (CGA265307) and 15- (CGA330050) fold lower in rats than in mice following either a single oral dose, or dietary administration of thiamethoxam for up to 50 weeks. Comparisons of the major metabolic pathways of thiamethoxam *in vitro* using mouse, rat, and human liver fractions have shown that metabolic rates in humans are lower than those in the rat suggesting that thiamethoxam is unlikely to pose a hazard to humans exposed to this chemical at the low concentrations found in the environment or during its use as an insecticide.

Key Words: **thiamethoxam; species differences; hepatotoxicity; metabolism.**

Thiamethoxam is a neonicotinoid insecticide active against a broad range of commercially important sucking and chewing pests. Toxicology tests during development revealed that thiamethoxam was not a mutagen. It did, however, cause an increased incidence of liver tumors in male and female Tif:MAGf mice when fed in the diet for 18 months at concentrations up to 2500 ppm. In marked contrast, there were no increases in cancer incidences either in the liver, or at any other site, in rats fed on diets containing up to 3000 ppm thiamethoxam for two years. A mode of action has been developed, using the framework described by ILSI (2003) and the U.S. EPA (EPA, 2003), which provides a scientific basis for the development of the tumors seen in thiamethoxam treated mice. The mode of action is essentially one of cytotoxicity, cell death, both as single cell necrosis and apoptosis, and increased cell replication rates (Green *et al.*, 2005). The cytotoxicity appears following a sustained decrease in plasma cholesterol concentrations, suggesting that the two phenomena may be causally linked. Having defined a mode of action in mice it is implicit that the biochemical and histopathological components of that mode of action should not occur to any significant degree in rats fed on diets containing thiamethoxam since thiamethoxam is not a carcinogen in the rat. To demonstrate that this is the case would provide further support for the changes seen in the mouse being causally linked to the development of the mouse liver tumors. To that end a dietary feeding study has been conducted in rats in which the same parameters have been measured as those in the equivalent 50-week mouse study described by Green *et al.* (2005). The maximum dietary concentrations used in the two-year rat carcinogenicity study were 1500 ppm in males and 3000 ppm in females. The basis of the lower dose level in males was an increase in nephropathy, mainly attributed to the male rat specific protein α-2μ-globulin, which was not present in

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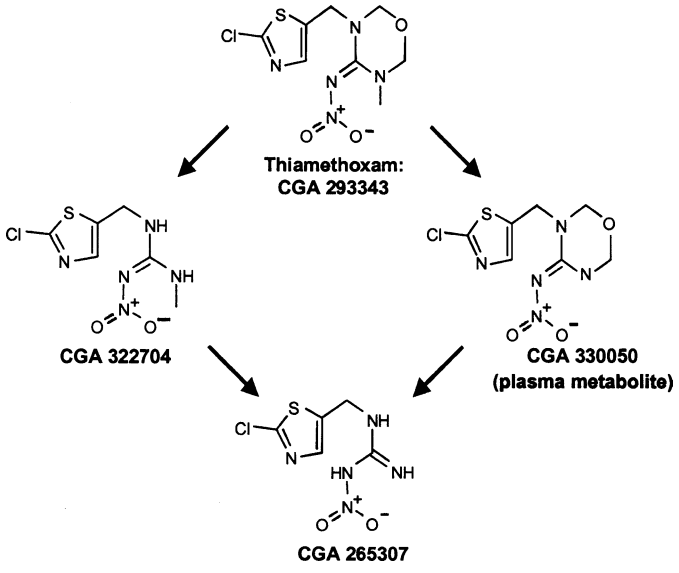

FIG. 1. Thiamethoxam and its major metabolites in mice and rats.

females. In the present study female rats were used in order to expose the liver to the highest concentration of test material used in the bioassay.

The marked species difference in response in the carcinogenicity studies implies either a marked difference in exposure to the test material, a difference in exposure to the metabolites of thiamethoxam or, less likely, a difference in response to the same concentrations of test material or metabolites. The mode of action studies in mice identified two thiamethoxam metabolites (CGA330050 and CGA265307; Fig. 1) which played a role in the development of the hepatotoxicity which in turn resulted in the development of liver cancer. The absence of an increase in liver tumors in the rat cancer bioassay suggests that these metabolites may be present in quantities insufficient to trigger the series of changes that led to cancer in the mouse. In order to ascertain whether this is the case, the metabolism of thiamethoxam has been compared in mice and rats, including an assessment of the concentrations of parent chemical and major metabolites present in blood and plasma following a single po dose of radiolabelled thiamethoxam and following dietary administration of the test material under the conditions of the cancer bioassays. Finally, having compared the metabolism of thiamethoxam in rats and mice *in vivo*, its metabolism has been compared in rat, mouse, and human liver fractions *in vitro* in order to provide a basis for human hazard assessment with this chemical.

MATERIALS AND METHODS

Chemicals. Thiamethoxam(CGA293343;3-(2-chloro-thiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene-N-nitroamine, 98.6%), metabolite CGA265307 (N-(2-chloro-thiazol-5ylmethyl)-N′-nitroguanidine, 99%), and metabolite CGA322704, (N-(2-chloro-thiazol-5-ylmethyl)-N′-methyl-N′-nitroguanidine, 99%) were supplied by Syngenta Crop Protection AG, Basle, Switzerland. Metabolite CGA330050 (3-(2-chloro-thiazol-5-ylmethyl)-[1,3,5]oxadiazinan-4-ylidene-N-nitroamine) was synthesized as described by Maienfisch *et al.* (2001). The structure of the product was confirmed by NMR and mass spectrometry and had a purity of >97%.

Radiolabelled thiamethoxam was also supplied by Syngenta Crop Protection AG, Basle, Switzerland. [Oxadiazin-4-^{14}C] CGA293343 had a specific activity of 2880 kBq/mg and a radiochemical purity of 98.3%. It was diluted with non-radioabelled thiamethoxam (>99% purity) to final specific activities of 311 kBq/mg for mouse studies and 386 kBq/mg for rat studies.

Animals. Female Tif:RAIf rats (140–160 g body weight), male Tif:RAIf rats (180–202 g) and male Tif:MAGf mice (25–34 g body weight) were supplied by RCC Ltd., Biotechnology and Animal Breeding Division, Fullinsdorf, Switzerland. The strains and the supplier were those used for the earlier cancer studies. The animals were housed in rooms with 16–20 air-changes per hour, a temperature of 22 ± 2°C, relative humidity of 50 ± 20%, and a 12 h light/dark cycle. The animals were acclimated to laboratory conditions for up to 14 days prior to dosing. For the metabolism studies, the animals were acclimatized in Plexiglas metabolism cages for 24 h before dosing. Food (CT1 diet supplied by Special Diet Services Limited, Stepfield, Witham, Essex, U.K.) and tap water were available throughout the studies *ad libitum.*

Fifty-week dietary feeding study in the rat. Two-hundred and seventy female rats were randomly assigned to six control and twelve treatment groups with 15 females per group via a computer generated randomization program. Groups of fifteen rats each received thiamethoxam at dietary concentrations of 0, 1000, or 3000 ppm for 1, 10, 20, 30, 40, or 50 weeks. Clinical observations were made daily and bodyweights and food consumption measured weekly.

Three days before sacrifice, each animal was fitted with an osmotic minipump (Alzet, model 2MLl), filled with 5 mg bromodeoxyuridine (BrdU), dissolved in 0.5 M sodium bicarbonate at a concentration of 50 mg/ml. The release rate of the mini-pumps was 10.0 µl/h. The mini-pumps were implanted subcutaneously in the back. The analgesic Ketofen was administered to each animal at least 1 h prior to surgery. The animals were lightly anesthetized with halothane Ph. Eur. vapor during the procedure. At sacrifice, blood was collected by cardiac puncture and analyzed for alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and cholesterol using standard automated methods. Blood samples were also analyzed for thiamethoxam and its major metabolites. Livers were removed, weighed, and processed for histopathology, cell proliferation measurements and for assessment of apoptosis.

Histopathology. The liver samples were processed for paraffin embedding and mounted in one paraffin block. Serial sections were prepared from paraffin blocks, stained with haematoxylin & eosin and examined by light microscopy.

Cell proliferation studies. Replicative DNA synthesis was assessed by immunohistochemical staining of liver sections for nuclear incorporated BrdU, a diagnostic parameter for cell proliferation (Dolbeare, 1995a,b, 1996). A combined staining for Feulgen and BrdU-immunohistochemistry was performed on liver paraffin sections after deparaffinization. Morphometric assessment of BrdU-labelling of hepatocyte nuclei was performed by image analysis (analySIS Pro, Soft Imaging System GmbH, Münster, Germany). Uniform dark brown nuclear staining for incorporated BrdU identified cells in S-phase of the cell cycle. The total number of hepatocyte nuclei and the number of BrdU-labelled hepatocyte nuclei were counted on Feulgen/BrdU-immunohistochemistry stained paraffin sections. The labelling index (LI) for BrdU-positive hepatocytes was calculated as the percentage of labelled nuclei over the total number of nuclei.

Apoptosis. Hepatocellular apoptosis was assessed by TUNEL, i.e., terminal deoxynucleotidyl transferase mediated dUTP nick end labelling histochemistry (Gavrieli *et al.*, 1992). Morphometric assessment of apoptosis was performed by image analysis (analySIS Pro, Soft Imaging System GmbH, Münster, Germany). Measurements included counting and area determination of hepatocellular apoptotic figures (apoptotic hepatocyte nuclei and clusters of apoptotic fragments). The total hepatic tissue area was used as the reference

area. As a measure of apoptotic activity, the area fraction of apoptotic events was evaluated.

Statistical analysis. Arithmetic means with standard deviations were used for descriptive statistics if the data were of normal distribution. Otherwise, medians with 95% confidence intervals were applied.

For the blood chemistry, cell proliferation and apoptosis (TUNEL) data, one-way analysis of variance (ANOVA) was applied (Gad and Weil, 1986) if the data were of normal distribution and equal variance. Otherwise, a Log_{10} transformation was performed. If normality and homoscedasticity were still not given after transformation, a non-parametric Kruskal-Wallis test was used (Kruskal and Wallis, 1952). Treated groups were compared to control groups by Dunnett's test (Dunnett, 1955) if the ANOVA was significant and by Dunn's test (Dunn, 1964) in case of significant Kruskal-Wallis test.

For the macropathology and histopathology data, incidences of macroscopic or microscopic findings were submitted to Fisher Exact Tests (Gad and Weil, 1986) if the sum of observations <100 or to Chi-Square Tests if sum of observations >100. The group-wise comparisons were performed by a sequential step down procedure with respect to difference to control.

All tests were performed using SigmaStat for Windows, Version 2.03, Build 2.03.0 (SPSS Inc.). p-values < 0.05 were considered to be significant.

In Vivo *Metabolism*

Metabolism studies following a single oral dose of radiolabelled thiamethoxam. A single po dose of 100 mg [oxadiazin-4-^{14}C] thiamethoxam/kg body weight was administered to mice and rats in a mixture of polyethylene glycol 200/ethanol 5/3 (v/v) (0.4 ml/kg). Aliquots of the test solutions were radioassayed, and the radiochemical purity of the test substance was confirmed by TLC at the time of dosing.

The animals were placed into open Plexiglas metabolism cages in subgroups of six (mice) or individually (rats), and housed therein for the remainder of the study. Groups of six mice or two rats each were killed 0.5, 1, 2, 4, 6, 8, and 24 h after dosing with a rising concentration of carbon dioxide. Blood was collected by cardiac puncture into heparinized tubes. The radioactivity content was determined and the remaining blood was stored frozen until further analysis.

Blood metabolite analysis. Blood samples were pooled by species and time point. Each sample was extracted with a similar volume of acetonitrile repeatedly (three or four times) until the bulk of the radioactivity had been extracted. For the mouse blood samples >96% of the radioactivity was extracted into acetonitrile and for rat blood >99% was extracted.

Aliquots of whole blood were combusted using a Packard Sample Oxidizer System 387. The $^{14}CO_2$ formed was absorbed in Carbosorb and mixed with the scintillation fluid Permafluor E+ (Packard) prior to liquid scintillation counting using a Packard Tri-Carb scintillation counter. Aliquots of acetonitrile were added directly to scintillation mixture Irga-Safe plus (Packard Instrument Corp.) for the measurement of radioactivity.

Chromatographic systems. HPLC analysis for determination of the metabolite profiles was carried out on a System Gold HPLC system (Beckman). A 5 µm ABZ+Plus C-18 column (250 mm × 4.6 mm) was eluted with a gradient consisting of 100% 0.01 M ammonium formate, pH 5 and changing to 100% methanol over a period of 35 min (3–10 min 15%; 20–30 min 35% methanol). The eluent was monitored with a UV-detector (Module 168) operated at 254 nm and a Radiomatic 500TR radioactivity flow monitor (Packard) with a 500 µl liquid cell and continuous mixing of the eluent with 2 ml/min Flo-Scint A as the scintillant. Typical retention times of the synthetic standards were CGA293343 24.4 min; CGA265307 29.2 min; CGA322704 30.5 min and CGA33005 32.6 min.

Metabolites were also separated by thin layer chromatography using 0.25 mm precoated Si-60 F_{254} plates. The plates were developed in two dimensions using ethylmethylketone/methanol/formic acid/water (80/18/1/1) as the developing solvent system or in one dimension using ethylacetate/2-propanol/formic acid/water (85/13/1/1). Metabolites CGA265307 and CGA330050 co-eluted in these systems. Non-radioactive spots were detected under UV light at 254 nm and radioactive spots by a Bio-Imaging Analyzer, model BAS 5000 (Fuji).

Metabolite identification by LC-MS. Mass spectra of metabolites in the blood of mice were obtained by direct injection of the extracts on a Q-TOF 1 Liquid Chromatograph-Mass Spectrometer (LC-MS) (Micromass). Time-of-flight (TOF) detection scans were obtained in the positive ion mode. Product ion scan mass spectra (ESI/MS/MS) with TOF detection in the positive ion mode were accomplished on selected samples using argon as the collision gas. HPLC for the introduction of samples to the LC-MS was performed with an AQ-ODS column (YMC, Inc.), 12.5 cm × 2 mm ID, and a binary solvent system of 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile (B) at a flow rate of 0.2 ml/min. A linear gradient from 95%A/5% B to 5%A/95% B over 12 min after an initial hold time of 2 min was used.

The mass spectra of CGA293343 and CGA330050 standards were obtained by similar techniques. The mass spectra of CGA322704 and CGA265307 standards were obtained on a TSQ-7000 Atmospheric Pressure Chemical Ionisation (APCI) LC-MS (Finnigan) in the positive ion mode, which produces a similar fragmentation pattern as ESI and ESI/MS/MS

Plasma metabolites following dietary administration of thiamethoxam. In a previous study (Green *et al.*, 2005) male Tif:MAG mice were fed on diets containing 0, 500, 1250, and 2500 ppm thiamethoxam for up to 50 weeks. Blood was collected by cardiac puncture at sacrifices after 1, 10, and 50 weeks of feeding. Blood samples were also collected from rats at each sacrifice during the 50-week study described above. Thiamethoxam and its metabolites CGA322704, CGA265307, and CGA330050 were analyzed in plasma by an HPLC method as described by Green *et al.* (2005).

In Vitro *Metabolism*

Human tissues. Fresh human liver and liver microsomes (n = 6) were obtained from the U.K. Human Tissue Bank, Leicester U.K. and stored at −70°C prior to use. Details of the donors are given in Table 3.

Preparation of hepatic sub-cellular fractions. Rats (n = 12) and mice (n = 20) were sacrificed in a rising concentration of carbon dioxide, the livers removed and washed in ice cold 1.15% potassium chloride pH 7.4. The livers were pooled by species, scissors minced, and homogenates, 30% (w/v) for rat liver and 10 % (w/v) for mouse liver, prepared in 0.01 M potassium phosphate/1.15% (w/v) potassium chloride buffer, pH 7.4, using an Potter Elvehjem homogenizer. The homogenates were centrifuged at 9000 × g for 20 min at 4°C, the supernatant (S9) transferred to fresh tubes and further centrifuged at 105,000 × g for 70 min at 4°C. The supernatant (cytosol) was removed from the microsomal pellet and stored at −70°C. The microsomal pellets were re-suspended in 0.01 M potassium phosphate/1.15% (w/v) potassium chloride buffer, pH 7.4, re-centrifuged at 105,000 × g for 70 min at 4°C, and the pellet re-suspended in the same buffer (1:1 w/v original tissue wet weight to buffer) before being stored at −70°C. Human liver microsomes were prepared in an identical manner except that the livers were homogenised individually using an Ultra Turrax homogeniser.

Protein concentrations, for all tissue fractions, were determined using the method of Lowry *et al*. (1951).

Microsomal incubations. Thiamethoxam (0.05 mM to 10.0 mM), CGA322704 (0.05 mM to 2.0 mM), or CGA330050 (0.05 mM to 5.0 mM) was dissolved in 10 µl of DMSO and incubated with 0.75 mg of either human, rat, or mouse liver microsomal protein, 5 mM magnesium chloride, and 1 mM NADPH in 0.1 M Tris-HCl buffer pH 7.4, for 15 min at 37°C in a total incubation volume of 1 ml. Under these conditions, all product formation was linear with respect to protein concentration and incubation time.

The reaction was started by the addition of the NADPH following a 5 min pre-incubation. Reactions were terminated by the addition of 1 ml ice-cold methanol and cooling to 0°C. The tubes were allowed to stand on ice for 30 min before the protein was removed by centrifugation at 1000 × g for 10 min. The supernatant was decanted into HPLC vials.

TABLE 1
Labelling Index of Hepatocytes in the Livers of Rats Fed on Diets Containing Thiamethoxam

Dietary conc. Time (weeks)	0 ppm	1000 ppm Hepatocyte labelling index %	3000 ppm
1	9.6 ± 5.8	5.9 ± 3.2*	6.9 ± 3.5
10	6.5 ± 3.9	4.1 ± 2.1*	3.9 ± 2.6*
20	3.2 ± 1.1	1.8 ± 1.5**	3.8 ± 1.6
30	6.9 ± 3.2	3.3 ± 1.3**	3.4 ± 2.1**
40	3.5 ± 2.4	2.9 ± 1.0	1.6 ± 1.5**
50	1.5 ± 0.8	1.7 ± 0.9	0.7 ± 0.2**

Note. Results are expressed as the percentage (mean ± SD) of BrdU-labelled hepatocytes over the total number of hepatocytes.
*$p < 0.05$
**$p < 0.01$.

Between 10 and 50 µl of each sample was analyzed by HPLC (Schimadzu LC10) using a 250 mm × 4.6 mm Hypersil ODS 5 µm column, with 10 mm × 4.6 mm Hypersil ODS 5 µm guard column. The initial mobile phase consisted of 90% water and 10% methanol/acetonitrile (4:1 v/v). The gradient rose linearly to 45% methanol/acetonitrile (4:1 v/v) over 25 min, and then rose linearly to 100% methanol/acetonitrile (4:1 v/v) over the next 5 min. This concentration was held for 5 min, before returning to the starting conditions over a further 5 min. The column was allowed to re-equilibrate for 10 min prior to the injection of the next sample. The flow rate of the mobile phase was 0.75 ml/min, and the column eluent was monitored with a UV detector set at 254 nm. Approximate retention times for thiamethoxam, CGA265307, CGA322704, and CGA330050, were 21.0, 23.5, 25.5, and 27.5 min, respectively.

Cytochromes P-450 1A1, 2D6, 3A4, and 2E1 activities were measured in human livers as described by Lake (1987) and McKillop *et al.* (1998), respectively.

Michaelis-Menten constants Km and Vmax were calculated by non-linear regression of the initial velocity on the substrate concentration using GraFit data fitting software (Leatherbarrow, 1998). Initial estimates were derived from Hofstee plots.

RESULTS

Fifty-Week Dietary Feeding Study in the Rat

There were no adverse effects on food consumption, body-weights, organ weights, or clinical signs, nor were there any toxicologically significant changes in either blood or urine clinical chemistry parameters, that were attributable to treatment with thiamethoxam at either dose level. Similarly, there were no histopathological changes in the liver at any time point that were associated with treatment with thiamethoxam. The number of apoptotic bodies was not increased over control at either dose level. Although there was no evidence of hepatocyte proliferation at either dose level, a reduction of mononuclear hepatocyte S-phase was seen after administration of 1000 ppm up to 30 weeks and throughout the study, except at weeks 1 and 20, after administration of 3000 ppm thiamethoxam (Table 1). The binuclear hepatocytes that constitute 7–10% of hepatocytes are shown in this study to be relatively less likely to enter cell division; their maximum individual labelling index was 1.20% in controls and 0.80% in treated animals.

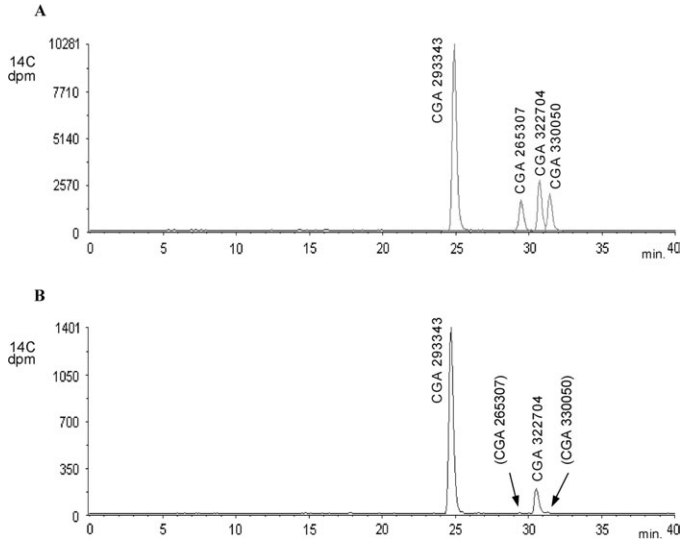

FIG. 2. Representative HPLC profiles of blood from mice and rats given a single oral dose of ^{14}C-thiamethoxam. (A) Mice, 1-h blood sample, (B) rats, 4-h blood sample.

In Vivo *Metabolism*

Metabolites in blood following a single oral dose of ^{14}C-thiamethoxam. In order to determine the thiamethoxam metabolites to which tissues are exposed in mice and rats, quantitative profiles of metabolites in whole blood were obtained at various time points after a single po dose of [oxadiazin-4-^{14}C] thiamethoxam (100 mg/kg). Typical HPLC profiles for mice and rats are shown in Figure 2. In mice, there were four major components that co-chromatographed with the standards for CGA293343, CGA322704, CGA265307, and CGA330050. In rats, the profiles contained two major components that co-chromatographed with CGA293343 and CGA322704. Minor radioactive peaks were visible in the HPLC profile from rats that matched the retention times of CGA265307 and CGA330050 (Fig. 2). Metabolites in mouse blood were quantified by HPLC and those in rat blood by TLC.

To further confirm the identities of the metabolites detected in blood, the extract from mice at the 2 h time point was analyzed by LC-MS. The ESI spectra and the ESI/MS/MS product ion spectra of two major components in blood matched those of the synthetic standards CGA293343 ($[M+H]^+$ at m/z 292, −100%, and CGA330050 ($[M+H]^+$ at m/z 278, −100%,) run under similar conditions, thus confirming their structures. Mass spectra for the other two major metabolite peaks were consistent with the structures of CGA322704 ($[M+H]^+$ at m/z 250, −100%), and CGA265307 ($[M+H]^+$ at m/z 236, −100%,), and matched closely with the mass spectra of these reference standards obtained earlier under slightly different conditions (APCI).

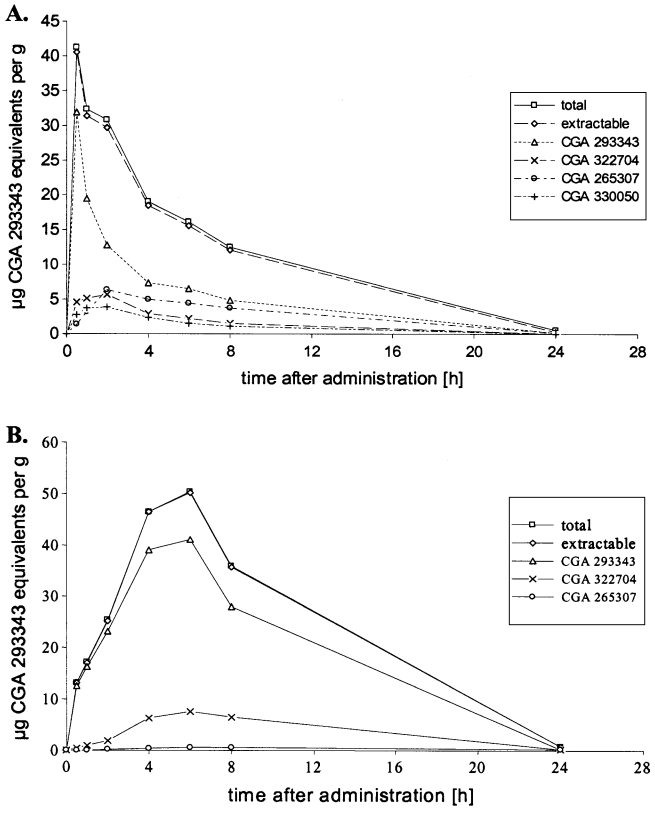

FIG. 3. Blood levels of total radioactivity and of individual metabolites in mice (A) and rats (B) following a single po dose of ^{14}C-thiamethoxam. Mouse metabolites were quantified by HPLC and rat metabolites were quantified by TLC. CGA265307 and CGA330050 co-eluted in the same TLC region, hence CGA265307 in rats reflect the sum of CGA265307 + CGA330050.

The overall time course of metabolites in blood from mice and rats at each of the time points up to 24 h after administration is displayed in Figure 3. The content of parent CGA293343 reached a maximum value at the earliest time point of 0.5 h in mice, indicating rapid absorption. Content of the three metabolites rose more slowly, with peak concentrations at 2 h. In rats, absorption was less rapid, with maximum concentrations of CGA293343 and of total radioactive residues achieved at 6 h; the concentration of metabolites CGA322704 and CGA265307 (which included co-eluting CGA330050) followed a similar time course. In both species, excretion was rapid with significantly lower blood concentrations of total radioactivity observed at 24 h.

Plasma metabolites following dietary administration of thiamethoxam. The concentrations of thiamethoxam and its major metabolites in mouse and rat plasma (HPLC analysis) after 1, 10, and 50 weeks feeding at the highest concentrations used in the cancer bioassays, 2500 and 3000 ppm respectively, are shown in Figure 4. Although the concentrations of thiamethoxam were relatively similar, the concentrations of the three metabolites were noticeably greater in mouse plasma than in rat. The concentrations of CGA265307 were approximately 22-fold greater in mouse plasma than in rat plasma after one week of feeding. After 10 weeks feeding, the concentration of CGA265307 in mouse plasma had increased approximately 3.6-fold whereas that in rat plasma had reduced, the difference between the two species now being approximately 140-fold. The difference between the two species for metabolite CGA330050 was up to 15-fold over the duration of the study. The overall reduction for both species in the concentrations of thiamethoxam and its metabolites at 50 weeks compared to 10 weeks reflects the lower food consumption at the later time point, approximately 40% lower on a mg/kg bodyweight basis.

In Vitro *Metabolism*

The rates of metabolism of thiamethoxam to CGA322704 and CGA330050 were measured in hepatic microsomal fractions prepared from mice, rats, and humans. In addition, the rates of metabolism of these two metabolites to CGA265307 were also determined using the same fractions. Metabolism could not be detected in the absence of NADPH suggesting that these reactions were catalyzed by isoforms of cytochromes P-450. Figure 5 shows a comparison of each metabolic rate in the three species, based on HPLC analysis of each incubation mixture. From these data rate constants Km and Vmax were calculated and are shown in Table 2. Metabolic rates for all four reactions were significantly greater in mouse microsomal fractions than in those from either rats or humans. Comparisons were made for the conversion of thiamethoxam to CGA265307 by the two pathways (i.e., via CGA322704 or CGA330050) by multiplying the differences in rate between species for each step in the pathway (Table 2). On this basis the rates in mouse liver were 54-fold (via CGA322704) and 87-fold (via CGA330050) higher than those in rat liver and 371-fold and 238-fold higher respectively than those in human liver.

The Km values obtained for the metabolism of thiamethoxam to CGA322704 and CGA 330050 in human liver were exceptionally high and the highest values clearly exceeded any concentration that could be achieved *in vivo* and, in some cases, these estimates of Km exceeded the solubility of thiamethoxam in water. Because of this variability and the fact that Vmax would never be reached in some of these individuals it was felt that initial rates of metabolism, Vi, may provide a better comparison between species. In all species each metabolic transformation had a linear rate ($R^2 > 0.99$) up to substrate concentrations of 0.1mM and Vi values at this concentration are also given in Table 2. 0.1 mM is also representative of the concentrations of thiamethoxam found in the blood of rats and mice at the dietary concentrations used in the cancer bioassays. In practice, the species differences in Vi were comparable to those expressed as intrinsic clearance (Vmax/Km) with human values still very much lower than those in the mouse.

Of the four metabolic rates measured, that from CGA330050 to CGA265307 was significantly higher than those of the

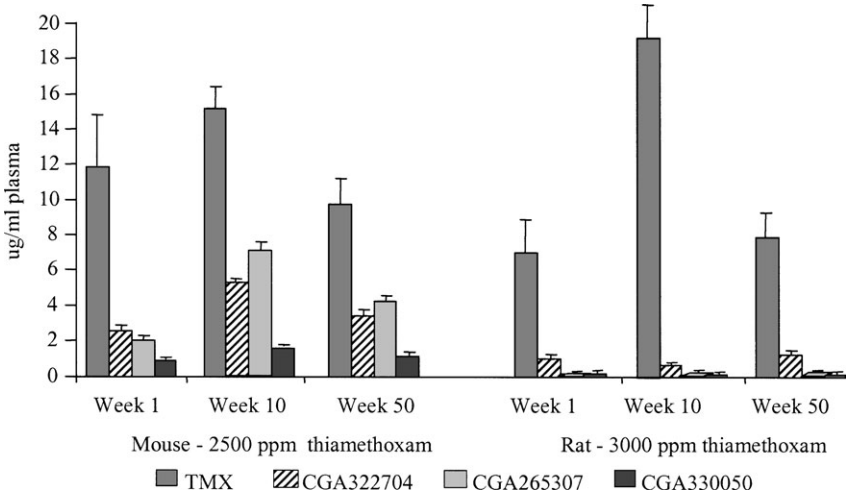

FIG. 4. A comparison of the plasma concentrations of thiamethoxam and its major metabolites in female rats and male mice following dietary feeding at the highest dose levels used in the respective carcinogenicity studies.

others, the rate of metabolism of CGA330050 being greater than its rate of formation from thiamethoxam in all three species. This difference in relative rates is consistent with the low levels of this intermediary metabolite found *in vivo*.

Cytochromes P-450 1A1, 2D6, 3A4, and 2E1 activities in the human liver samples used in this study are given in Table 3. The activities are comparable to those reported for humans and confirm that these samples were metabolically viable.

DISCUSSION

In a previous study, a number of changes were identified in the livers of mice fed on diets containing thiamethoxam for up to 50 weeks (Green *et al.*, 2005), which were consistent with the subsequent development of the tumors seen at 80 weeks in the carcinogenicity study. These changes included depletion of plasma cholesterol, increases in hepatic transaminases, and histopathological evidence of single cell necrosis, apoptosis, and an increase in cell replication rates. Accompanying changes included hypertrophy, inflammatory cell infiltration, pigmentation, and fatty change. None of these changes were seen in rats given thiamethoxam in the diet for 50 weeks at dose levels of up to 3000 ppm, the highest dietary concentration used in the rat carcinogenicity study. In fact the only significant change in the rat liver was a reduction in cell proliferation rates. Consequently, the findings of this study, and those of the previous mouse study, are entirely consistent with the outcome

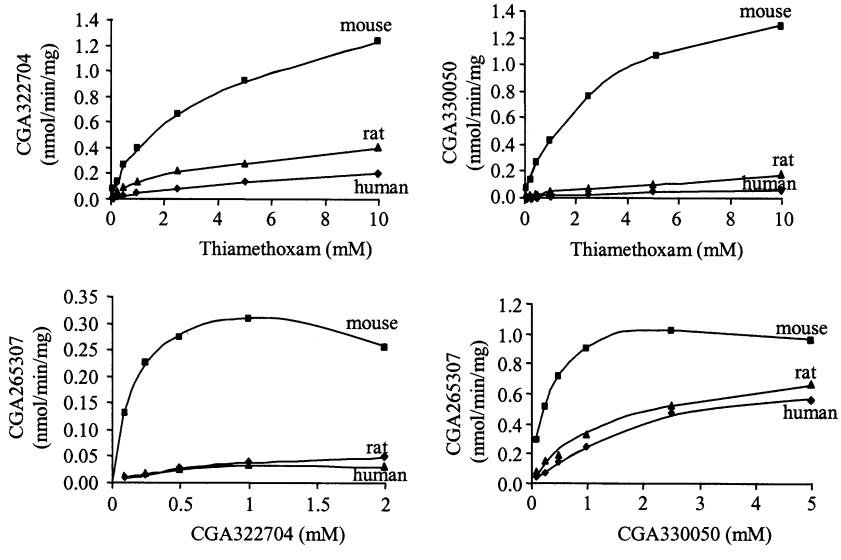

FIG. 5. The *in vitro* metabolism of thiamethoxam and its major metabolites in mouse, rat, and human liver microsomal fractions.

TABLE 2
The *in Vitro* Metabolism of Thiamethoxam and Its Major Metabolites in Mouse, Rat, and Human Liver Microsomal Fractions

Metabolic conversion/species	Vi (nmol/min/mg)	Km (mM)	Vmax (nmol/min/mg)	Vmax/Km
Thiamethoxam to CGA322704				
Mouse	0.074	3.26	1.59	0.49
Rat	0.025	3.05	0.49	0.16
Human ($n = 6$)	0.006 ± 0.002	11.95 ± 5.96	0.48 ± 0.40	0.04
CGA322704 to CGA265307				
Mouse	0.130	0.12	0.31	2.54
Rat	0.010	0.25	0.04	0.14
Human ($n = 6$)	0.007 ± 0.004	0.94 ± 0.64	0.08 ± 0.06	0.08
Thiamethoxam to CGA330050				
Mouse	0.065	2.96	1.67	0.56
Rat	0.008	2.64	0.14	0.05
Human ($n = 6$)	0.004 ± 0.003	4.44 ± 4.44	0.10 ± 0.12	0.02
CGA330050 to CGA265307				
Mouse	0.289	0.26	1.07	4.17
Rat	0.076	1.82	0.93	0.51
Human ($n = 6$)	0.036 ± 0.021	1.98 ± 1.93	0.88 ± 1.10	0.45

Note. Vi was measured at 0.1 mM; values are means ± SD.

of the respective carcinogenicity studies and clearly demonstrate the causal relationship between cell death and reparative cell division, and the development of liver cancer.

The qualitative species difference in hepatotoxicity and carcinogenicity between mice and rats suggests either that mice respond very differently to thiamethoxam than rats, or that the internal dose of thiamethoxam and its metabolites is very different between mice and rats. The latter is by far the more common explanation for species differences in carcinogenicity between mice and rats. Consequently, the metabolism of thiamethoxam was compared in rats and mice *in vivo* following a single oral dose and following dietary administration at the highest dose levels used in the cancer studies. A unique data set has been acquired in that thiamethoxam and its major metabolites (Figs. 1 and 2) have been measured in mouse and rat plasma at 10 week intervals during dietary feeding studies for periods of up to 50 weeks. Blood or plasma metabolite profiles also have the distinct advantage that they reflect exposure of the target organ, the liver, far better than urinary metabolites.

The profiling of blood samples following a single oral dose of [oxadiazin-4-^{14}C] thiamethoxam in mice confirmed the presence of thiamethoxam (CGA293343), CGA322704, CGA265307, and CGA330050 as the major metabolites. In rats, the same components were present, but CGA265307 and CGA330050 were present in much smaller quantities than in mouse blood and represented only a very small percentage of the total material in blood following a single oral dose. No other metabolites were detected in the blood of either species in these studies.

Continuous dietary administration of 2500 ppm thiamethoxam to mice resulted in an induction of metabolism with a 3.6-fold increase in CGA265307 concentrations over a 10 week feeding period. In rats, the metabolism of thiamethoxam decreased on continuous feeding of a 3000 ppm diet with a resultant increase in the concentrations of thiamethoxam itself in plasma and a reduction in those of its metabolites. As a result, the species difference in metabolism was much greater after prolonged dietary administration than that observed following a single oral dose. Interestingly, the concentrations of thiamethoxam were either similar or even higher in rat blood than in mouse blood in both the single dose and the dietary studies. This finding strongly suggests that thiamethoxam itself is unlikely to play a role in the development of liver tumors and supports the conclusion from the mouse mode of action studies that metabolites CGA265307 and CGA330050 are responsible. These metabolites were present in 140- and 15-fold greater concentrations respectively in the mouse than the rat. Thus, the lower concentrations of these metabolites *in vivo* provide an explanation for the lack of response in the rat liver in the long-term studies.

Although major species differences were established between mice and rats *in vivo*, comparisons between rodents and humans were, of necessity, made using liver fractions *in vitro*. The four major steps in the metabolism of thiamethoxam to CGA265307 (Fig. 1) all occurred at significantly higher rates in mouse liver microsomal fractions than in the same fraction prepared from rat liver. Overall, the product of the intrinsic clearance rates (Vmax/Km) for conversion of thiamethoxam to CGA265307 via CGA322704 was 54-fold lower in rats than mice, and that via CGA330050 was 87-fold lower, differences that are consistent with the differences in CGA265307 plasma concentrations *in vivo*. The fact that the *in vitro* measurements reflect the species differences between mice and rats *in vivo* provides reassurance that human *in vitro* data are an accurate reflection of the fate of thiamethoxam in humans *in vivo*.

The human metabolic rates were significantly lower than those in mouse liver, and similar to, or lower than, those in rat liver. The human rates exhibited a spread in values typical of human liver samples (up to an order of magnitude—see Table 2). Comparing the mean values, the product of the intrinsic clearance rates (Vmax/Km) for the conversion of thiamethoxam to CGA265307 via CGA322704 was 371-fold lower than in mouse liver and that via CGA330050 was 238-fold lower. The remarkably high human Km values seen in the human livers for the metabolism of thiamethoxam to CGA322704 and CGA 330050 suggests that metabolism by these pathways would be minimal under any foreseeable conditions of exposure. For example, the concentration of thiamethoxam in the plasma of rodents exposed to 2500 or 3000 ppm thiamethoxam in the diet was in the range 0.04–0.06 mM whilst the mean Km values for the two pathways in humans are in the range 4.44 to 11.95 mM. The basis for the very high Km values measured in humans compared to rodents

TABLE 3
Available Details for the Human Liver Donors

Donor	Age	Sex	Cause of death	CYP1A1 (pmol/min/mg protein)	CYP2D6 (pmol/min/mg protein)	CYP3A4 (nmol/min/mg protein)	CYP2E1 (nmol/min/mg protein)
A	41	Female	Myocardial infraction	8.8	82.9	1.79	1.53
B	68	Male	Liver resection	—[a]	—[a]	—[a]	—[a]
C	38	Male	Intracerebral haemorrhage	31.8	71.5	7.60	2.13
D	40	Female	SAH	91.7	134.2	3.64	1.57
E	46	Male	Intracranial bleed	31.6	98.0	1.28	1.81
F	50	Male	Head injury	13.5	86.3	1.66	2.01

Note. All donors were Caucasians. Only donor E had a recent drug history (noradrenaline/cefuroxime).
[a]Insufficient sample to measure cytochrome P-450 activities.

is unknown but it may indicate that different isoforms of cytochromes P-450 metabolise thiamethoxam (by the same pathways) in humans than in rodents.

In conclusion, comparisons of the hepatotoxicity of thiamethoxam in mice and rats at the dose levels used in the cancer bioassays demonstrated a clear causal relationship between changes which only occurred in the mouse liver and the development of liver tumors. A metabolic basis for this difference in response has been identified and comparisons *in vitro* with human liver fractions have shown metabolic rates in humans are equal to or even lower than those in the rat. However, metabolic rates alone do not fully reflect the differences between mice and rats and humans. The use of metabolic rates in isolation would exclude less quantifiable factors such as the role of nitric oxide or indeed the unique susceptibility of the mouse liver. Given that human metabolic rates are similar to rats, a more accurate assessment of human hazard would appear to come from the observation that there was no evidence whatsoever for cholesterol reduction, or for any of the key events which lead to cancer, in rats fed on diets containing 3000 ppm thiamethoxam for 50 weeks. It seems highly unlikely that thiamethoxam will pose a carcinogenic hazard to humans exposed to this chemical either from the environment or during its use as an insecticide.

REFERENCES

Dolbeare, F. (1995a). Bromodeoxyuridine: A diagnostic tool in biology and medicine, Part I: Historical perspectives, histochemical methods and cell kinetics. *Histochem. J.* **27**(5), 339–369.

Dolbeare, F. (1995b). Bromodeoxyuridine: A diagnostic tool in biology and medicine, Part II: Oncology, chemotherapy and carcinogenesis. *Histochem. J.* **27**(12), 923–964.

Dolbeare, F. (1996). Bromodeoxyuridine: A diagnostic tool in biology and medicine, Part III. Proliferation in normal, injured and diseased tissue, growth factors, differentiation, DNA replication sites and in situ hybridization. *Histochem. J.* **28**(8), 531–575.

Dunn, O. J. (1964). Multiple comparisons using rank sums. *Technometrics* **6,** 241–252.

Dunnett, C. W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Stat. Assoc.* **50,** 1096–1121.

EPA (2003). Draft Final Guidelines for Carcinogen Risk Assessment, U.S. Environmental Protection Agency, Washington DC.

Gad, S. C., and Weil, C. S. (1986). *Statistics and Experimental Design for Toxicologists.* The Telford Press, Caldwell, NJ.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.* **119,** 493–501.

Green, T, Toghill, A., Lee, R., Waechter, F., Weber, E., and Noakes, J. (2005). Thiamethoxam induced mouse liver tumors and their relevance to humans. Part 1: Mode of action studies in the mouse. *Toxicol. Sci.* **86,** 36–47.

ILSI (2003): Meek, M. E., Bucher, J. R., Cohen, S. M., Dellarco, V., Hill, R. N., Lehman-McKeeman, L. D., Longfellow, D., Pastoor, T., Seed, J., and Patton, D. E. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol.* **33**(6), 591–653.

Kruskal, W. H., and Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. *J. Amer. Stat. Assoc.* **47,** 583–621.

Lake, B. G. (1987). Preparation and characterisation of microsomal fractions for studies of xenobiotic metabolism. In *Biochemical Toxicology, A Practical Approach* (K. Snell and B. Mullock, Eds.), pp. 183–215. Oxford IRL Press, Oxford, U.K.

Leatherbarrow, R. J. (1998). *GraFit* Version 4.0. Erithacus Software Ltd, Staines, U.K.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Maienfisch, P., Huerlimann, H., Rindlisbacher, A., Gsell, L., Dettwiler, H., Haettenschwiler, J., Sieger, E., and Walti, M.(2001). The discovery of thiamethoxam: A second generation neonicotinoid. *Pest. Management Sci.* **57,** 165–176.

McKillop, D., Wild, M. J., Butters, C. J., and Simcock, C. (1998). Effects of propofol on human hepatic microsomal cytochrome P450 activities. *Xenobiotica* **2,** 845–853.