Thiamethoxam Induced Mouse Liver Tumours

and their Relevance to Humans

Part 1: Mode of Action Studies in the mouse

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ABSTRACT

Thiamethoxam, a neonicotinoid insecticide, which is not mutagenic either *in vitro* or *in vivo*, caused an increased incidence of liver tumours in mice when fed in the diet for 18 months at concentrations in the range 500 to 2500 ppm. A number of dietary studies of up to 50 weeks duration have been conducted in order to identify the mode of action for the development of the liver tumours seen at the end of the cancer bioassay. Both thiamethoxam and its major metabolites have been tested in these studies. Over the duration of a 50-week thiamethoxam dietary feeding study in mice, the earliest change, within one week, is a marked reduction (by up to 40%) in plasma cholesterol. This was followed 10 weeks later by evidence of liver toxicity including single cell necrosis and an increase in apoptosis. After 20 weeks there was a significant increase in hepatic cell replication rates. All of these changes persisted from the time they were first observed until the end of the study at 50 weeks. They occurred in a dose dependent manner and were only observed at doses (500, 1250, 2500 ppm) where liver tumours were increased in the cancer bioassay. There was a clear no-effect level of 200 ppm. The changes seen in this study are consistent with the development of liver cancer in mice and form the basis of the mode of action. When the major metabolites of thiamethoxam, CGA322704, CGA265307 and CGA330050 were tested in dietary feeding studies of up to 20 weeks duration, only metabolite CGA330050 induced the same changes as those seen in the liver in the thiamethoxam feeding study. It was concluded that thiamethoxam is hepatotoxic and hepatocarcinogenic as a result of its metabolism to CGA330050. Metabolite CGA265307 was also shown to be an inhibitor of inducible nitric oxide synthase and to increase the hepatotoxicity of carbon tetrachloride. It is proposed that CGA265307, through its effects on nitric oxide synthase, exacerbates the toxicity of CGA330050 in thiamethoxam treated mice.

INTRODUCTION

Increases in the incidences of mouse liver tumours are a common finding in chronic feeding studies with a wide range of agrochemicals (Carmichael *et al*., 1997). It is also not uncommon to find that the increases occur solely in the mouse liver and are not seen in other organs or in rats in comparable studies. It is equally true that the vast majority of these chemicals are devoid of mutagenic activity and induce their effects by non-genotoxic modes of action. In some cases the modes of action are known and they give a clear indication of the likely human hazard; in others, data are lacking or incomplete resulting in a more conservative approach towards human hazard and risk assessment.

In recent years a common set of guidelines has emerged that outline the types of data needed to establish a mode of action in laboratory animals in order for the animal data to be used as the basis of human hazard and risk assessments. The guidelines are based on the Bradford Hill criteria (Hill, 1965) for establishing causality and have been developed by ILSI (2003) and the US-EPA (EPA, 2003). They provide a rational scientific basis for establishing that changes measured in animals in the short term are causally linked to the development of cancer in the long term. In this paper we have used these guidelines to evaluate the hazards associated with an insecticide, thiamethoxam, which is a mouse liver specific carcinogen.

Thiamethoxam is a neonicotinoid insecticide active against a broad range of commercially important sucking and chewing pests. A comprehensive genotoxicity assessment (including bacterial mutagenicity, gene mutation, cytogenetic, unscheduled DNA synthesis & mouse micronucleus tests) demonstrated that thiamethoxam was not genotoxic. It did, however, cause an increased incidence of liver tumours in male and female Tif:MAGf mice when fed in the diet for 18 months at concentrations up to 2500 ppm (see supplementary data). The total liver adenoma + adenocarcinoma incidence at dose levels of 0, 5, 20, 500, 1250 & 2500ppm was 12, 7, 12, 19, 27 & 45 out of 50 in male mice, and 0, 0, 0, 5, 9 & 32 out of 50 in female mice respectively. 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 2 years (see supplementary data). A series of feeding studies, of up to 50 weeks duration have been conducted in mice in order to establish the early changes, or key events, which lead to liver cancer in mice. Dose responses for these changes have been compared with the tumour responses, temporal relationships have been established and the

changes have been shown to be reproducible in several studies and in two strains of mouse. The major metabolites of thiamethoxam (Figure 1) have been fed to mice and the metabolite responsible for the hepatic changes which precede the development of tumours has been identified. One of the metabolites, CGA322704, has previously been tested for carcinogenicity in CD-1 mice (Federal Register, 2003) and found not to be a liver carcinogen. Comparisons of the effects of this metabolite in Tif:MAGf and CD-1 mice with those of thiamethoxam and its other metabolites were used to give a further insight into the mode of action of thiamethoxam. Metabolite CGA265307 was found to be structurally similar to known inhibitors of inducible nitric oxide synthase (Figure 2). In view of the known role of this enzyme in the development of liver toxicity (Taylor *et al*. 1998; Kim *et al*. 2001; Lala and Chakraborty, 2001; Brennan and Moncada, 2002; Wang *et al*. 2002), the potential of CGA265307 to inhibit inducible nitric oxide synthase has been investigated *in vivo* and *in vitro*. Other possible modes of action have been evaluated in experimental studies. Finally, in order to assess whether infants and children are potentially more susceptible than adults following exposure to thiamethoxam, the sensitivity of young and adult mice to thiamethoxam treatment has been compared.

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-5-ylmethyl)-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%.

Animals

There was no evidence of a sex difference in the outcome of the cancer study in mice and consequently only male animals were used for the mode of action studies. The male

4

Tif:MAGf mice used in these studies were of the same strain and were obtained from the same supplier (RCC Ltd, Biotechnology and Animal Breeding Division, Fullinsdorf, Switzerland) as those used in the cancer study. Male CD-1 mice were supplied by Charles River, Manston Kent, UK. The mice were housed singly in a room with 16-20 air-changes per hour, a temperature of 22±2°C, relative humidity of 55±10%, and a 12-hour light/dark cycle. The animals were acclimated to laboratory conditions for 14 days prior to dosing. Food (see below) and tap water were available throughout the studies *ad libitum*. The animals were not fasted overnight prior to sacrifice the following morning.

Dietary feeding studies

A number of dietary feeding studies were conducted as follows:

A 50-week hepatotoxicity study with thiamethoxam at six concentrations from 0-5000 ppm.

A study of up to 20 weeks duration with thiamethoxam (2500 ppm) and metabolites CGA322704 (2000 ppm) and CGA265307 (500 ppm).

A study of 10 weeks duration with metabolite CGA330050 at dietary concentrations of 500 and 1000 ppm.

The 50 week study was conducted at Syngenta's laboratory in Switzerland (as was the cancer biassay) and the remaining studies at Syngenta's laboratory in the UK. For the 50 week study the test material was admixed with a standard rodent chow, NAFAG 8900 FOR GLP (Nafag, Gossau SG, Switzerland), the same diet that was used in the cancer study. For the studies conducted in the UK the test materials were admixed with CT1 diet supplied by Special Diet Services Limited, Stepfield, Witham, Essex, UK.

The hepatotoxicity of thiamethoxam over a 50-week feeding study

Study design

Young adult male Tif:MAGf mice with a starting body weight of between 30 and 43 g were used for the study. 525 mice were randomly assigned to 35 groups via a computer generated randomization program. Groups of 15 mice each received thiamethoxam at dietary concentrations of 0, 50, 200, 500, 1250, 2500, or 5000 ppm for 10, 20, 30, 40, or 50 weeks.

5

The dose levels included all of those at which tumour incidences were increased in the long term study (500, 1250, 2500 ppm) together with an additional higher dose and two lower dose levels. Clinical observations were made daily and bodyweights and food consumption measured weekly.

Three days before sacrifice, each animal was fitted with an osmotic mini-pump (Alzet, model 1003D, 100μl), 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 1.0 μl/h. The mini-pumps were implanted subcutaneously in the back under slight ether anaesthesia. At sacrifice, blood was collected by cardiac puncture and analysed for alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and cholesterol using standard automated methods. Livers were removed, weighed, and processed for histopathology, cell proliferation measurements and for assessment of apoptosis. A testis was taken as a control for the cell proliferation studies.

Histopathology

The liver and testis samples were processed for paraffin embedding and mounted in one paraffin block (containing three liver and one testis sample). 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, 1995b, 1996). A combined staining for Feulgen and BrdU-immunohistochemistry was performed on liver paraffin sections (including testis) 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

6

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.

The comparative hepatotoxicity of thiamethoxam and metabolites CGA322704 and CGA265307

Study design

Male Tif:MAGf and male CD-1 mice (22 – 30 g bodyweight) were fed on diets containing either 2500 ppm thiamethoxam, 2000 ppm metabolite CGA322704 or 500 ppm metabolite CGA265307 for 1, 10 or 20 weeks. There were 12 animals per group per time point together with an equal number of controls for each test material. The dose levels were chosen on the following basis. 2500 ppm was the highest dose level used in the thiamethoxam cancer bioassay; similarly 2000 ppm was the highest dose tested for metabolite CGA322704 (Federal Register, 2003). The dose of CGA265307 was selected from dose setting studies which showed that 500 ppm of this material in the diet gave comparable blood levels to those seen in mice fed on diets containing 2500 ppm thiamethoxam. The two strains of mice reflect the fact that the carcinogenicity bioassay of thiamethoxam was conducted in Tif:MAGf mice and that of CGA322704 in CD-1 mice. Clinical observations were made twice daily and bodyweights and food consumption measured weekly.

After 1, 10 and 20 weeks 12 mice from each dose group, and from the control group, were killed with an overdose of anaesthetic (halothane). Three days prior to sacrifice the mice were fitted with minipumps containing BrdU as described above. At sacrifice blood was removed by cardiac puncture and the livers removed and weighed. A testis was taken as a control for the cell proliferation studies.

Blood samples were analysed for glucose, urea, creatinine, albumin, total protein, albumin/globulin ratio, total bilirubin, alkaline, phosphatase, alanine aminotransferase,

7

aspartate aminotransferase, creatine kinase, gamma-glutamyl transferase, sodium, potassium, chloride, phosphorus, calcium, cholesterol and triglycerides by standard automated methods.

Histopathology

Livers were processed for histopathological examination (H&E sections, cell proliferation, apoptosis by TUNEL) as described above.

The hepatotoxicity of metabolite CGA330050

The hepatotoxicity of metabolite CGA330050 was assessed in male Tif:MAGf mice (12 animals per group per time point) after 1, 4 and 10 weeks feeding on diets containing 0, 500 and 1000 ppm CGA330050. The protocol and study design were as given above for the 20 week study.

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.

8

Plasma Metabolite analysis

Blood samples collected at each of the time points in the 10, 20 and 50 week studies described above, and liver samples from mice fed on thiamethoxam diets for 10 weeks, were analysed for thiamethoxam and its three major metabolites, CGA322704, CGA330050 and CGA265307.

Plasma was separated from red blood cells by centrifugation at 1000g for 15 minutes at 4°C. Plasma or red blood cells (75 μl) were deproteinated by the addition of an equal volume of ice cold methanol / acetonitrile (4:1 v/v), mixing, and leaving on ice for 60 minutes. The samples were then centrifuged at 14000g for 15 minutes at room temperature and 25 or 50 μl of the supernatant analysed by HPLC as described below.

Liver samples were homogenised in Tris/HCl buffer, pH 7.5, containing 250 mM sucrose to give a 10% w/v homogenate which was centrifuged at 100 g for 15 min. An aliquot of 0.7 ml of the supernatant was diluted with water to a final volume of 1 ml and loaded onto an OASIS HLB (10 mg) SPE cartridge which was equilibrated with 1 ml methanol and 1 ml water. The cartridge was rinsed with 1 ml water followed by 1 ml 10% aqueous methanol. Thiamethoxam and its metabolites were eluted with 1 ml 70% aqueous methanol.

Between 10 and 50 μl of each sample was analysed by HPLC (Schimadzu LC10) using a 250 mm x 4.6 mm Hypersil ODS 5μm column, with 10 mm x 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 minutes, and then rose linearly to 100% methanol/acetonitrile (4:1 v/v) over the next 5 minutes. This concentration was held for 5 minutes, before returning to the starting conditions over a further 5 minutes. The column was allowed to re-equilibrate for 10 minutes 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 minutes respectively. The samples were quantified against standard curves prepared using a range of concentrations of thiamethoxam or each of its metabolites from 0-1000 ng/ml. The limits of detection were 20 ng/ml for thiamethoxam, CGA322704 and CGA265307 and 50 ng/ml for CGA330050.

9

The recovery of the test materials from biological samples was determined by adding thiamethoxam and its metabolites to control whole blood, to plasma and to the 100 g liver supernatant to give concentrations of each component of 5 ug/ml. These samples were extracted and analysed as described above.

Metabolite CGA265307 and inducible nitric oxide synthase (iNOS) inhibition

Inhibition of nitric oxide synthase in vitro

The method used to measure inducible iNOS activity was that described by Rendon *et al*. (1997) using purified iNOS. The ability of CGA265307 to inhibit iNOS activity was determined and compared with that of N-nitro-L-arginine methyl ester (L-NAME) over a range of substrate concentrations from 0-0.5 mM. These experiments were repeated using thiamethoxam and metabolites CGA322704 and CGA330050, at 1 mM concentrations.

Inhibition of nitric oxide synthase in vivo

The hepatotoxicity of carbon tetrachloride is known to be enhanced in mice treated with inhibitors of iNOS (L-NAME) and in iNOS knock-out mice (Morio *et al*. 2001). Consequently, the effect of dietary administration of CGA265307 on carbon tetrachloride hepatotoxicity has been investigated in a study in which 2 groups of male Tif:MAG mice (5 per group) were placed on a diet containing 2000 ppm CGA265307 for 7 days. At 16 hours before termination all of the mice in one group were given a single intra-peritoneal injection of 10 μl/kg carbon tetrachloride in corn oil (10 ml/kg). The other group was given injections of corn oil alone. Two further groups of mice (5 per group) were given control diet for 7 days. At 16 hours before termination, one group was given single intra-peritoneal injections of 10 μl/kg carbon tetrachloride in corn oil (10 ml/kg), the other group was given single intra-peritoneal injections of corn oil alone. The mice were killed with an overdose of halothane and blood collected by cardiac puncture in lithium/heparin tubes. Livers were removed and part of each of the three main lobes placed in formol saline. The livers were trimmed, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H&E) before being examined by light microscopy. Blood samples were centrifuged to separate plasma and

10

alanine aminotransferase and aspartate aminotransferase activities determined by standard automated methods.

The comparative sensitivity of young and adult mice

The sensitivity of adult (15-17 weeks old) and weanling mice (21 days old) has been compared in a study in which thiamethoxam was fed in the diet at concentrations of 0, 500, 1250 and 2500 ppm for 7 days.

Plug positive pregnant female Tif:MAG mice were supplied by RCC Ltd, Biotechnology and Animal Breeding Division, Fullinsdorf, Switzerland. The animals were housed in solid plastic cages under the same environmental conditions as the adults. They received control diet and mains water *ad libitum*. The day of littering (day 1) was noted together with the size of the litters. The pups were sexed on day 7 and remained with the dams until day 18. When the dams were removed, the pups were randomly housed in groups of 6 until the start of the study on day 21 (body weight approx. 8 g). Only male mice were used for the study.

Groups of 6 male adult or 6 male weanling mice were fed on diets containing 0, 500, 1250 and 2500 ppm thiamethoxam for 7 days. The adult animals were housed singly and the weanling mice together by group. Clinical observations and body weights were recorded daily. At the end of the treatment period, all of the mice were killed by exsanguination under terminal anaesthesia induced by halothane vapour. Blood was collected by cardiac puncture and transferred to lithium heparin tubes. Livers were removed and weighed. Plasma was separated from red blood cells by centrifugation at 1000g for 15 minutes at 4°C. Plasma cholesterol, alanine aminotransferase and aspartate aminotransferase were measured using standard automated procedures. Plasma samples were also analysed for thiamethoxam and its major metabolites as described above. Livers were fixed in 10% (w/v) neutral buffered formol saline, dehydrated through an ascending ethanol series and embedded in paraffin wax. Sections (5-7μm) were cut and stained with haematoxylin and eosin.

RESULTS

The hepatotoxicity of thiamethoxam in mice over a 50-week study

11

Clinical signs and mortality were not affected by treatment. The mean daily food consumption was consistently below control level from week 40 at 1250 ppm and from week 9 at 2500 and 5000 ppm. The overall mean food consumption (weeks 1 to 49) amounted to 97, 96, and 95 % of control at these dose levels, respectively.

Organ and Body weights

The mean body weight was consistently below control level at the 2500 and 5000 ppm dose levels (by 8% at 2500 and by 14% at 5000 ppm at week 50). The mean relative liver weight was increased at 2500 ppm (weeks 20 and 40: 111%, and 116% of control, respectively) and at 5000 ppm (weeks 10, 20, 30, 40, 50: 113%, 114%, 117%, 124%, 129% of control, respectively).

Clinical chemistry

The median aspartate aminotransferase activity was increased at 2500 ppm (weeks 20 and 40: 122% and 131% of control, respectively) and at 5000 ppm (all time points; 148 – 210% of control). After combining all time points, increased values were noted at 1250, 2500 and 5000 ppm (116%, 122% and 169% of control, respectively). Alanine aminotransferase activities were increased in a similar manner. After combining all time points, the increases were noted at 1250, 2500 and 5000 ppm (139%, 207% and 256% of control, respectively). The alkaline phosphatase activity was not affected by treatment.

A significant dose dependent reduction in plasma cholesterol levels, at 500 ppm and above, was seen at the earliest time point of 10 weeks and was sustained throughout the study (Table 1). The cumulative data for all time points is shown against dose in Figure 3 and against time for the four highest dose levels in Figure 4.

Histopathology

Increases in hepatocellular hypertrophy, single cell necrosis, apoptosis, inflammatory cell infiltration, pigmentation and fatty change were seen in a dose and time dependent manner at dose levels of 500 ppm and above.

Hypertrophy was characterized by enlarged centrilobular/midzonal hepatocytes with increased amounts of cytoplasmic glycogen, fat, and smooth endoplasmic reticulum and was seen in the 2500 ppm dose group at weeks 30, 40 and 50 and in the 5000 ppm dose group at

all time points (Figure 5). Hepatocellular necroses affected single cells or small groups of cells with mainly centrilobular localization and were often accompanied by inflammatory cells. After combining all time points, increased necroses were seen at 500, 1250, 2500 and 5000 ppm (Figure 6). The pattern of inflammatory cell increases largely followed that for necroses (Figure 5).

Hepatocellular apoptosis, first seen at week 10, affected single cells or small groups of cells, again with mainly centrilobular localization. After combining all time points, significantly increased incidences were observed at 500, 1250, 2500 and 5000 ppm (Figure 6). Pigmentation, which was characterized as lipofuschin (yellow/brown pigment granules), occurred in the cytoplasm of centrilobular hepatocytes, and was increased at the 1250 ppm dose level and above (Figure 5). Occasionally, pigmented Kupffer cells were observed. Significantly increased incidences ($p < 0.05$) of fatty change over control (40%) were observed at 500 (72%), 1250 (82%) and 2500 (79%) ppm but a reduced incidence was observed at 5000 ppm (17%).

Cell proliferation

An increased median BrdU labelling index was observed at 1250 ppm (week 40: 246% of control), at 2500 ppm (weeks 30, 40 and 50: 356%, 422% and 311% of control, respectively) and at 5000 ppm (weeks 10, 30, 40, 50: 211%, 484%, 933%, 485% of control, respectively). These data combined for all time points are shown in Figure 6.

The histopathological examination of the liver described above revealed that the increases in necroses and apoptosis were largely confined to the centrilobular region. Examination of the BrdU labelling index in this region of the livers of mice fed on the 500 ppm diet for 40 weeks revealed a statistically significant increase in the labelling index compared to the same region in control liver (control, 0.15±0.10, 500 ppm 0.36±0.31 $p < 0.05$). This increase was not apparent when comparisons were made across the whole liver. The labelling index was not increased in the centrilobular region at the 200 ppm dose level (0.10±0.07).

Apoptoses (TUNEL)

An increased median TUNEL area density was observed at dose levels of 500 ppm and above. The densities increased with increasing dose and with increasing duration of dosing. After combining all time points, increased median TUNEL area densities were observed at

13

500 ppm (156% of control), at 1250 ppm (188% of control), at 2500 ppm (219% of control), and at 5000 ppm (316% of control).

The comparative hepatotoxicity of thiamethoxam and its metabolites

The hepatotoxicity of metabolites CGA322704 and CGA265307 was compared with that of thiamethoxam in two strains of mouse in a study of up to 20 weeks duration. The findings reported above up to 20 weeks (in the 50-week study) were essentially replicated in the mice fed on a diet containing 2500 ppm thiamethoxam. There was no evidence of a significant strain difference in response in the mice used in this study. Metabolites CGA322704 and CGA265307 induced none of the clinical or histopathological changes seen in the thiamethoxam treated mice. The histopathological data from this study are shown in Table 2.

In contrast, Tif:MAG mice treated with metabolite CGA330050 for up to 10 weeks, revealed essentially the same changes in the liver as those seen with thiamethoxam at this time point. Plasma cholesterol levels were significantly reduced at both dose levels (Figure 7) and histopathological changes in the liver included increases in hepatocellular hypertrophy, single cell necrosis, apoptosis and a significant increase in cell replication rates in treated mice (Table 3).

Plasma metabolites

Plasma metabolites were measured in all of the studies. Thiamethoxam and metabolites CGA322704, CGA330050 and CGA265307 were detected at all time points in the 50-week thiamethoxam study. Data for 10 and 50 weeks are shown in Figure 8. The metabolism of thiamethoxam was linear with dose over the range 500 to 2500 ppm. The concentration of metabolites in the liver was comparable to that in plasma (data not shown). In mice dosed with CGA322704 the only components detected in plasma over the duration of the study (at 1, 10 and 20 weeks) were CGA322704 itself (4.8 – 11.6 ug/ml) and CGA265307 (3.0 – 4.1 ug/ml). In CGA265307 dosed mice (1, 10 and 20 weeks), only the starting material was detected in plasma (3.0 – 4.1 ug/ml), and in mice dosed with CGA330050, the starting material (4.2 ug/ml) and CGA265307 (7.2 ug/ml) were present (measured after 1 week).

14

Metabolite CGA265307 and nitric oxide synthase (NOS) inhibition

The kinetics for the inhibition of iNOS with CGA265307 and L-NAME are shown in figure 9. The data show that both CGA265307 and L-NAME are competitive inhibitors of iNOS with the inhibition constants (Ki) of 0.79 and 0.43 mM respectively. Thiamethoxam and metabolites CGA322704 and CGA330050 did not inhibit iNOS.

When mice were fed on a diet containing 2000 ppm CGA265307 for 7 days and then given a single intraperitoneal injection of 10 μl/kg carbon tetrachloride there was an increase in liver damage compared to mice given carbon tetrachloride alone. Liver damage was assessed by aminotransferase activities (Figure 10) and histopathology (Table 4). There was no evidence of hepatotoxicity in mice fed on the CGA265307 diet alone.

The comparative sensitivity of young and adult mice

There was no evidence of liver toxicity in either weanling or adult mice fed on diets containing thiamethoxam. Liver weights and ALT and AST values from mice in all treated groups were comparable to those in the control groups (data not shown). Plasma cholesterol levels were reduced in adult mice to approximately 70% of control values at the 1250 and 2500 ppm dose levels, and to 78% of control at the 500 ppm dose level. Cholesterol levels were also reduced in weanling mice but to a lesser extent, to 85% of control at the 1250 ppm dose level and 79% at 2500 ppm. The 500 ppm dose level was a clear no effect level in weanlings.

Histopathological examination of the livers of adult mice found a clear treatment related effect in mice fed on the 2500 ppm thiamethoxam diet for 7 days. The changes included increased centrilobular vacuolation and/or decreased eosinophilia. Changes at the lower dose levels were less defined, with a possible weak effect at 1250 ppm and no effect at 500 ppm. In weanling mice, there was also a clear effect at the 2500 ppm dose level with changes similar to those observed in adults but less severe.

The pattern of metabolites in plasma at the end of the study was essentially similar in adult and weanling mice. The actual concentrations of thiamethoxam and its major metabolites in the plasma of weanling mice were up to double those in adult animals (Figure 11).

15

DISCUSSION

Thiamethoxam is not a mutagen, yet it significantly increased the incidences of hepatic tumours in mice in an 18 month feeding study. By contrast, thiamethoxam did not increase tumour incidences at any site in a 2-year feeding study in rats. This phenomenon of a non-genotoxic mouse liver specific carcinogen is not uncommon (Carmichael *et al*. 1997), yet it remains one of the most difficult areas of rodent toxicology to extrapolate to humans. In the absence of other data the default assumption by some regulatory authorities is to assume that these tumours indicate a hazard to human health from the chemical in question. Nevertheless, the very nature of the species specific, organ specific response suggests that such an assumption may be ultra-conservative. In recent years it has been recognised that this issue can only be resolved by first gaining an understanding of the reasons why the tumours develop in the sensitive species. With this insight the lack of response in non-responding species can be resolved and a framework developed to determine the likely hazard to humans. The principle of understanding mode of action in order to evaluate human hazard has been developed by ILSI (2003) and now forms part of the US-EPA Cancer Risk Assessment Guidelines (EPA, 2003). These mode of action guidelines have been used to design a series of studies with thiamethoxam to understand the mode of action of this chemical as a mouse liver carcinogen, and, as described in subsequent papers, to understand the lack of response in rats, and to determine how humans will respond to exposure to thiamethoxam (Green *et al*., 2005; Pastoor *et al*., 2005).

The primary experiment in these studies was a 50 week dietary feeding study in the same strain of mouse that was used in the carcinogenicity study. The carcinogenic dose levels of 500, 1250 and 2500 ppm were used, but the lower dose levels used in the study of 5 and 20 ppm were replaced with dose levels of 50 and 200 ppm in order to give a better dose response curve and a more accurate definition of any no-effect level. A higher dose of 5000 ppm was also used for dose response reasons. The results of this study gave a clear indication of the mode of action of thiamethoxam as a mouse liver carcinogen. Essentially, prolonged exposure to thiamethoxam results in cell death, mainly as single cells dying either by necrosis or apoptosis, which is followed by increased cell replication. The timescale for these changes is extended, cell death occurring only after 10 weeks of feeding and increased cell replication from 20 weeks onwards. Thereafter, the cycle of cell death and cell replication continued for the remainder of the 50 week study. The rates of cell death and replication appeared to be in

16

balance and did not result in a significant increase in liver weights. The small increases in liver weight that did occur were attributed to hypertrophy resulting from increased amounts of cytoplasmic glycogen, fat, and smooth endoplasmic reticulum. Other accompanying changes included lymphocytic infiltration and pigmentation of hepatocytes and Kupffer cells. Thus, the livers of thiamethoxam treated mice undergo a continuous insult, which results in cell death and increased cell replication for at least 30 weeks. Such changes form a well established and accepted mode of action for the development of liver tumours in mice (EPA, 2003). The dose response for these changes followed that for the tumour incidences and significant changes were only seen at carcinogenic dose levels of 500 ppm and above (Figure 12). In addition, the changes had a logical temporal relationship, the biochemical changes including depletion of cholesterol occurred with the first few weeks of the study to be followed by cell death which, in turn, was followed by an increase in reparative cell division (Figure 13).

A question arises as to the cause of the cell death that ultimately leads to the development of liver cancer in mice. Some of the changes in liver biochemistry such as increases in aminotransferase activities are indicative of the subsequent histopathological changes. The most significant and earliest change was a marked reduction in plasma cholesterol, which, as with the histopathological changes, only occurred at doses of 500 ppm and above and had a dose response that was comparable to the tumour dose response. Furthermore, cholesterol levels in treated animals remained lower than those in controls throughout the 50-week study. The correlation between reduced plasma cholesterol, the histopathological changes (key events), and the subsequent development of cancer was absolute in these studies, being seen with thiamethoxam and CGA330050 but not with CGA322704 and CGA265307, nor with thiamethoxam in rats. These data suggest that the changes in cholesterol levels may be causally linked to the subsequent histopathological changes. However, at this point no mechanistic link has been established and cholesterol change must therefore be viewed as an associative event. It is interesting to note the strong correlation that also exists between changes in lipid metabolism in rodents and increases in liver tumours with other chemicals. A large number of drugs have been developed in recent years for the control of lipids (triglycerides and cholesterol) and the prevention of coronary heart disease. These fall into two main classes, the hypolipidemic drugs (triglycerides), and the more recent statins

17

(cholesterol). Both classes have been evaluated in rodent carcinogenicity studies during their development, and the majority of drugs in each class cause liver cancer, particularly in the mouse (MacDonald *et al*. 1988; Gerson *et al*. 1989; Newman and Hulley, 1996; von Keutz and Schulter, 1998). As with thiamethoxam, a causal link has not been established between the effects of these drugs on lipid metabolism and the subsequent development of cancer in rodents.

In order to understand the lack of response in rats, and to provide a possible means of extrapolating the animal data to humans, an understanding of the role of thiamethoxam metabolites in the development of liver cancer in mice is required. To that end, the 3 major metabolites of thiamethoxam were fed to mice in the diet for periods of up to 20 weeks and their hepatotoxicity compared with that of thiamethoxam itself. These studies also gave an opportunity to further test the mode of action indicated by the 50-week study. Metabolite CGA322704 has also been tested for carcinogenicity and shown not to be a liver carcinogen (Federal Register, 2003). Thus, the changes seen in thiamethoxam treated animals should not occur in mice treated with this close structural analogue.   The CGA322704 oncogenicity study also used a different strain of mice (CD-1) to that used in the thiamethoxam studies (Tif:MAGf). Both strains were used in the metabolite studies in order to identify any possible strain differences in response.

The outcome of the metabolite studies was clear and consistent with the known oncogenicity profiles of thiamethoxam and CGA322704. The hepatic changes seen with the hepatocarcinogen thiamethoxam were not seen with CGA322704, which is known not to cause liver tumours in mice (Federal Register, 2003). Metabolite CGA265307 also failed to induce hepatotoxicity in mice in these studies. Consistent with this, the plasma concentrations of CGA265307 were comparable in both CGA322704 and thiamethoxam treated mice providing further evidence that this metabolite alone is not responsible for the liver tumours.   By contrast, metabolite CGA330050 did induce the same changes in the livers of mice as thiamethoxam itself. Again this is consistent with the total data since CGA330050 is not formed from CGA322704. Metabolism studies and comparisons of plasma thiamethoxam concentrations in mice and rats have shown that the blood levels of thiamethoxam are higher in the rat than the mouse at the highest dietary dose concentrations used in the respective cancer bioassays and, *vice versa,* those of CGA330050 are much lower in the rat than the mouse (Green *et al*. 2005). Consequently, it is highly unlikely that

18

thiamethoxam itself plays a role in the development of liver cancer in mice and it can be concluded that metabolite CGA330050 is responsible for the hepatic changes which lead to liver cancer in thiamethoxam treated mice. In the studies which used both strains of mouse, the responses in the livers were identical, as were the metabolite profiles in plasma. Thus, it is reasonable to conclude that the responses seen in mice are not a consequence of the strain used in the cancer studies.

Another possible factor in the development of hepatotoxicity in thiamethoxam treated mice is the role of metabolite CGA265307 and the inhibition of inducible nitric oxide synthase. *In vivo*, nitric oxide, produced from arginine by the nitric oxide synthases, has been shown to have a regulatory role in the development of hepatotoxicity and apoptosis. For example, chemical inhibition of iNOS, or the use of iNOS knock out mice, has been shown to exacerbate chemically induced hepatotoxicity (Morio *et al.* 2001). Nitric oxide is believed to regulate hepatotoxicity and apoptosis by modulating the adverse effects of TNFα released by endothelial cells in response to a toxic challenge (Bradham *et al.* 1998; Taylor *et al.*1998; Luster *et al.* 1999). CGA265307 is identical to the iNOS inhibitor L-NAME in the active part of the molecule, the amino acid function not being a structural component for either potency or selectivity of iNOS inhibitors (Garvey *et al.* 1994, 1997). In the present limited studies CGA265307 was shown to inhibit iNOS *in vitro* and to enhance the toxicity of carbon tetrachloride *in vivo*. It seems likely, therefore, that CGA265307, although not toxic alone, could enhance the hepatotoxicity of metabolite CGA330050.

As part of the risk assessment process, the US-EPA are required to assess the risks to infants and children whenever it appears that their risks might be greater than those of adults (EPA, 2003). Although there are no reasons to suspect that infants and children would be more susceptible than adults to the proposed mode of action of thiamethoxam, the question was addressed experimentally. Such an assessment is problematical in terms of study design, even using experimental animals. It is particularly so for thiamethoxam because histopathological changes are not seen in the liver until 10 weeks after the start of the experiment. Young mice reach maturity at 6-7 weeks, well before the first changes are seen in the liver, and hence any differences between young and adult animals may no longer be apparent in a 10-20 week study. The earliest change, within one week, seen in mice fed on diets containing thiamethoxam was a reduction in plasma cholesterol levels. The correlation between reductions in plasma cholesterol, subsequent changes in liver histopathology and the

19

incidences of liver cancer were absolute, both quantitatively and qualitatively, over a wide range of studies with thiamethoxam and its metabolites in two species. Changes in plasma cholesterol were, therefore, used as a short-term marker for the mode of action of thiamethoxam and a means of comparing the sensitivity of young and adult animals. Plasma cholesterol levels were lowered in adults at all three dose levels, but only at 1250 and 2500 ppm in weanling mice. The magnitude of the response in weanlings at the two higher dose levels was also less than that in adults. Plasma metabolite concentrations were also approximately 2-fold higher in weanling mice reflecting the increased dietary intake in young animals. Overall, the study showed that young mice, despite a significantly higher dietary intake, were at least 2-fold less sensitive than adult mice to the earliest key event in the mode of action of thiamethoxam. It is concluded, based on the results of this study, that infants and children would not be more susceptible than adults following exposure to thiamethoxam.

Other possible modes of action have been investigated in the course of these studies. There is no plausible sequence of events for liver tumour formation by thiamethoxam where interference with nicotinic acetylcholine receptors, the target for neonicotinoids in insects, would represent a key event. As a class, the neonicotinoids have not been found to be oncogenic in rats and mice. Thiamethoxam is not genotoxic in bacteria, eukaryotic cells and mammalian systems. There was no evidence of hepatic peroxisome proliferation (by electron microscopy or from increases in peroxisomal beta-oxidation) in mice fed on diets containing up to 2500 ppm thiamethoxam for 14 days (data not shown) nor was there any evidence, based on hepatic 8-isoprostane F2α, glutathione or α-tocopherol concentrations, of oxidative stress in mice fed on diets containing up to 5000 ppm thiamethoxam for periods up to 50 weeks (data not shown). Thiamethoxam did induce several cytochrome P-450 isoenzymes, but the magnitude of the increases (max 11-fold, CYP2B) were considered insufficient alone to be causally related to the development of liver cancer (data not shown). For example, the level of enzyme induction with thiamethoxam was much lower than that reported for phenobarbital, a known rodent liver carcinogen (Honkakoski *et al*., 1992a, 1992b, Kelley, 1990; Whysner *et al*., 1996).

In summary, a mode of action has been identified for the development of liver tumours in thiamethoxam treated mice which includes marked and sustained cholesterol depletion followed by cell death, both as necrosis and apoptosis, and increased cell replication over a 30 week period. These changes are believed to lead to the tumours seen at 18 months. The

key metabolite inducing these changes has been identified as CGA330050. The development of hepatotoxicity is believed to be enhanced by inhibition of inducible nitric oxide synthase by metabolite CGA265307. The studies described fulfil the criteria identified for an acceptable mode of action, including dose response, temporal relationships, strength, consistency and reproducibility. Table 5 illustrates the strength of the correlation between the early events and the development of tumours. The responses seen with thiamethoxam have been reproduced in studies of 50 and 20 weeks duration, the latter in two strains of mouse. The metabolite studies were internally consistent in that CGA330050 is only formed from thiamethoxam and not from the non-carcinogenic metabolite CGA322704. In all of the studies the dose responses follow that of the tumour response and the temporal relationships follow a logical sequence of biochemical change (starting with plasma cholesterol reduction) leading to cell death followed by increased cell replication followed by the development of tumours.

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21

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FIGURE TITLES AND LEGENDS

Figure 1. Thiamethoxam and its major metabolites in mice.

Figure 2. A comparison of thiamethoxam metabolite CGA265307 and L-nitroarginine methyl ester (L-NAME) and inhibitor of nitric oxide synthases.

Figure 3. Decreases in plasma cholesterol in mice fed on diets containing thiamethoxam for up to 50 weeks.

Data shown are for all time points combined. *$p < 0.05$, **$p < 0.01$

Figure 4. Decreases in plasma cholesterol in mice over the duration of a 50 week feeding study.

Figure 5. Incidences of hepatocellular hypertrophy (A), inflammatory cell infiltration (B) and pigmentation (C) in the livers of mice fed on diets containing thiamethoxam for up to 50 weeks.

Data shown are combined for all time points. *$p < 0.05$

Figure 6. Incidences of single cell necrosis (A), apoptosis (B) and cell replication rates (C) in the livers of mice fed on diets containing thiamethoxam for up to 50 weeks.

At 500 ppm, the increase was significant ($p < 0.05$) only in the centrilobular region when examined separately at 40 weeks. See text. Data shown are for all time points combined. *$p < 0.05$

26

Figure 7. Decreases in plasma cholesterol in mice fed on diets containing CGA330050 for up to 10 weeks.

Figure 8. Thiamethoxam and its major metabolites in the plasma of mice during a 50 week thiamethoxam feeding study.

Figure 9. The inhibition of inducible nitric oxide synthase by metabolite CGA265307 *in vitro*. The inhibition is compared with that of N-nitro-L-arginine methyl ester (L-NAME).

Figure 10. The effect of dietary administration of CGA265307 on carbon tetrachloride (CCl$_4$) hepatotoxicity.

Mice were fed on a diet containing 2000 ppm CGA265307 for 7 days and then given a single oral dose of 0.01 mg/kg CCl$_4$ and killed 16 h later.

Figure 11. A comparison of the concentrations of thiamethoxam and its major metabolites in plasma of weanling and adult mice fed on diets containing thiamethoxam for 7 days.

Figure 12. A comparison of the incidences of single cell necrosis, apoptosis and cell replication rates with those of the tumour incidences in the livers of male mice fed on diets containing thiamethoxam.

The incidence of tumours in control mice has been subtracted from the values shown.

Figure 13. The temporal relationship of the key events in the development of liver cancer in mice fed on a diet containing 2500 ppm thiamethoxam. The associated reductions in plasma cholesterol concentrations are also shown. The values for % change refer to % decrease in

cholesterol values compared to control; for apoptosis and necrosis, to the % increase in the

number of animals per group showing these effects.

Figure 1.

Thiamethoxam:
CGA 293343

CGA 322704

CGA 330050
(plasma metabolite)
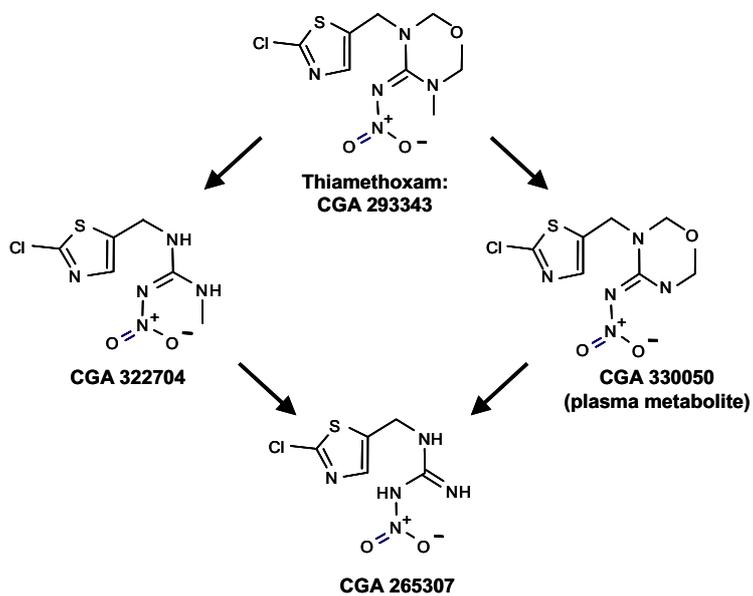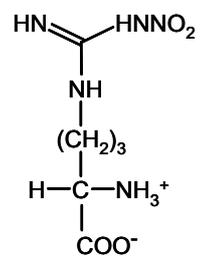
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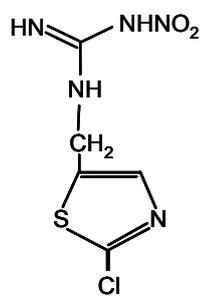
Figure 2.

L-NAME   CGA265307
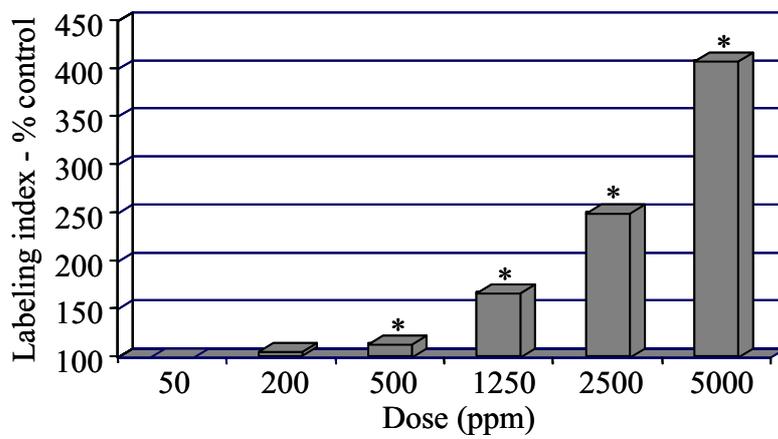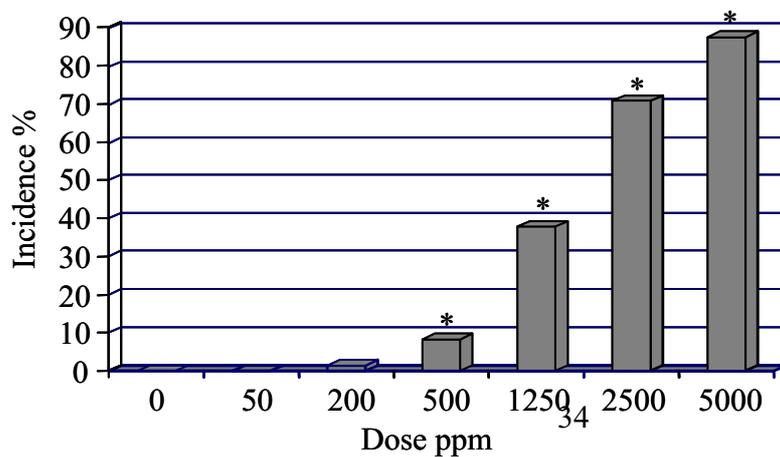
Figure 3.
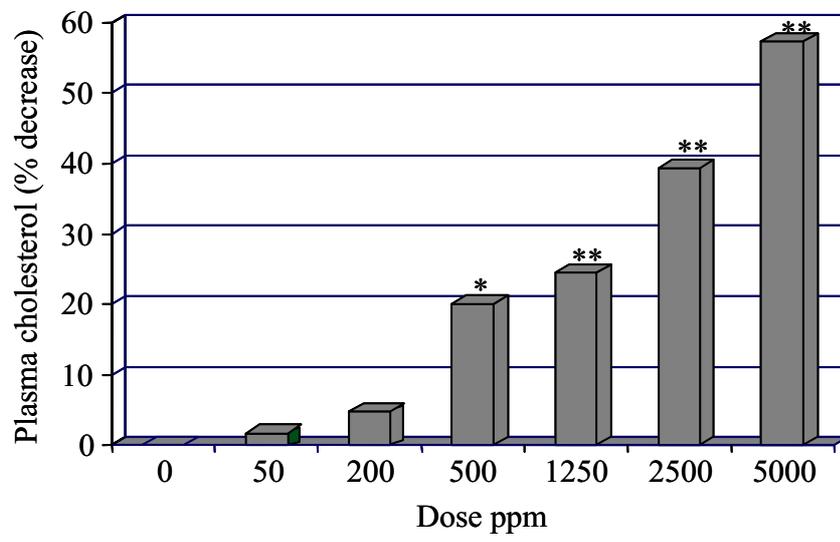
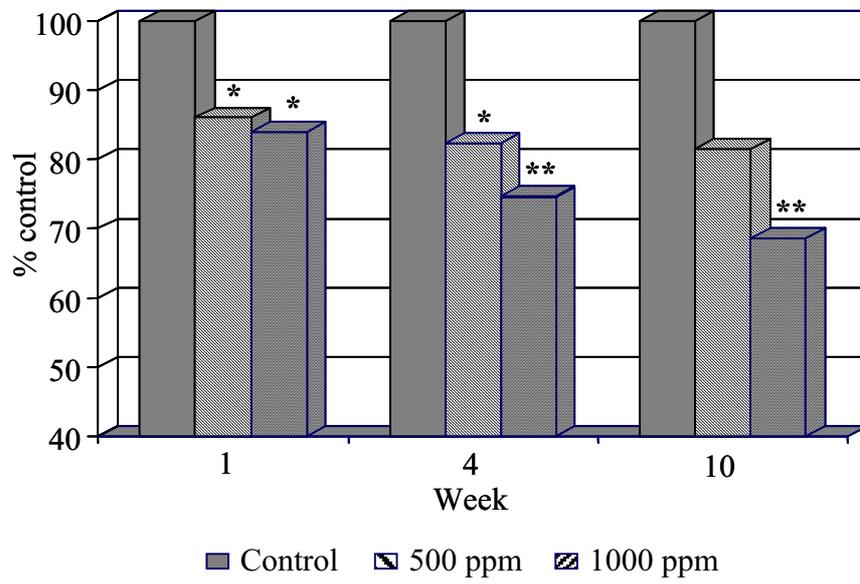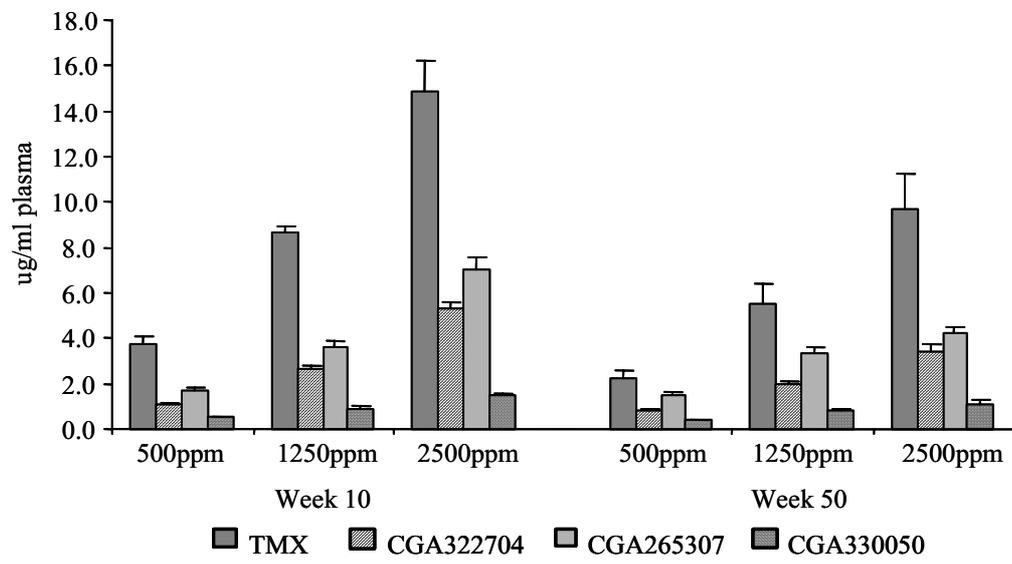Figure 4.
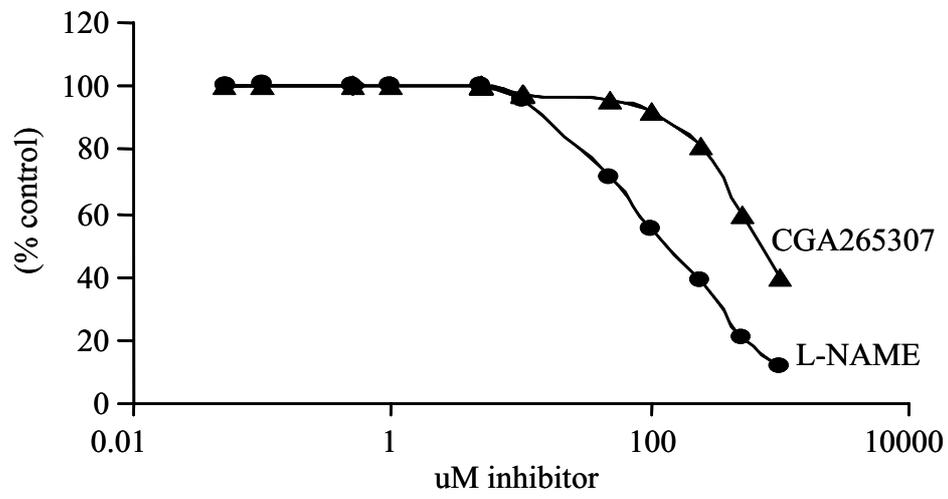
Figure 5.

A.

B.

C.

33
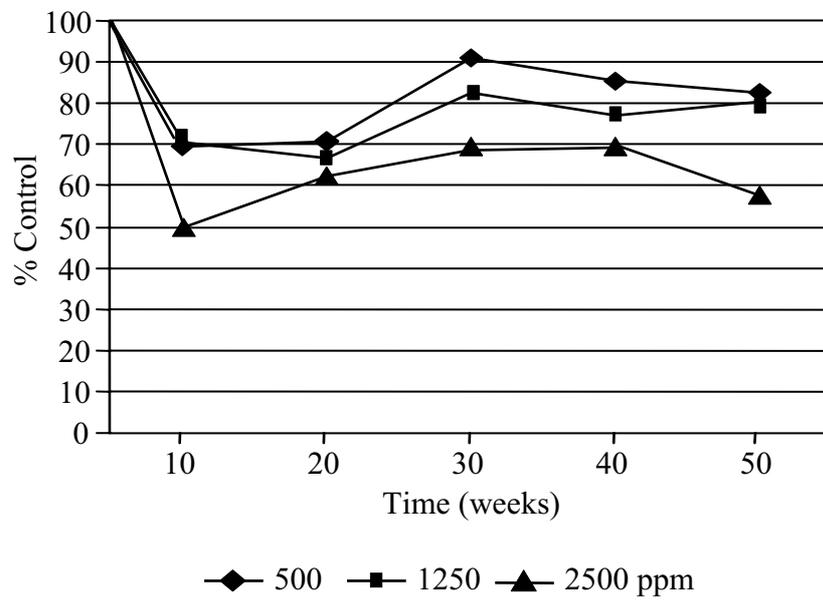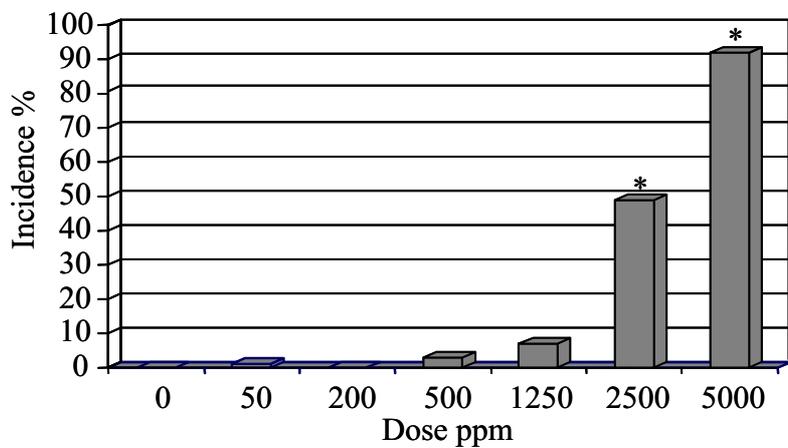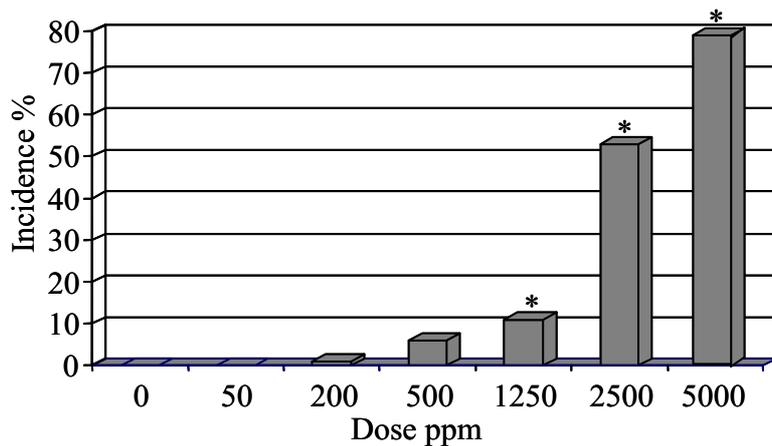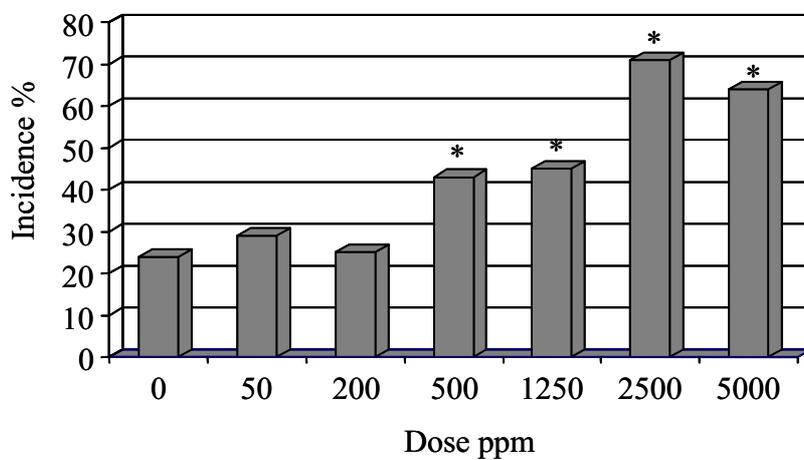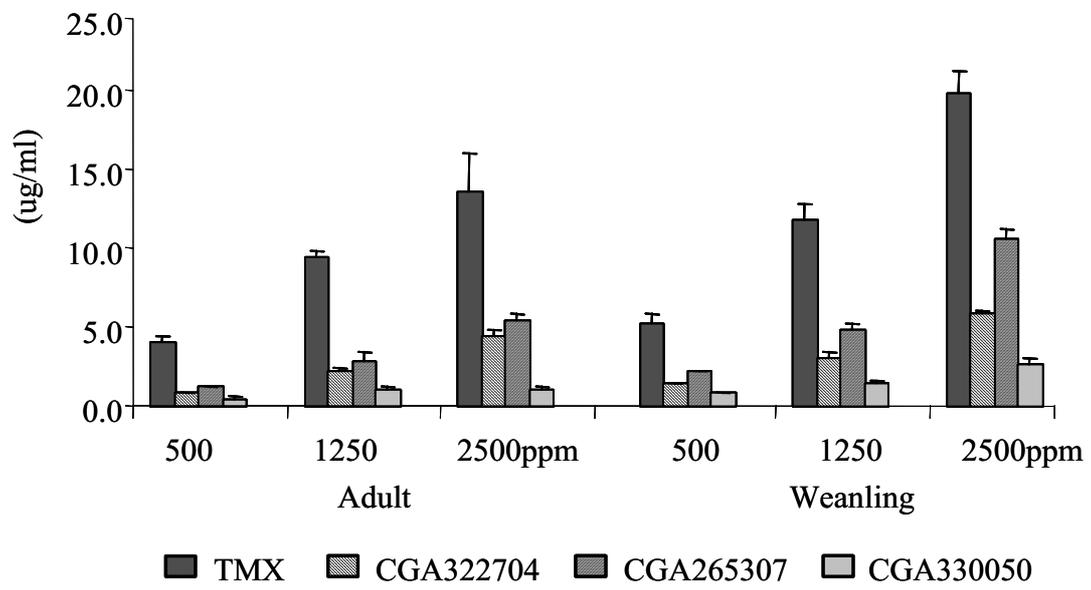
Figure 6.

A.
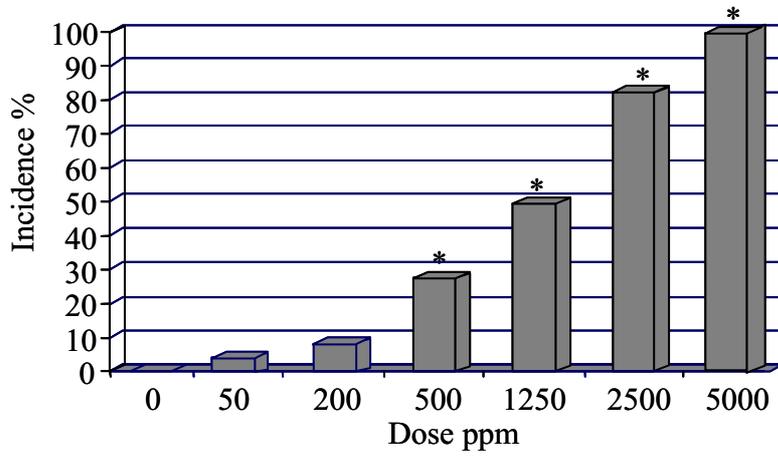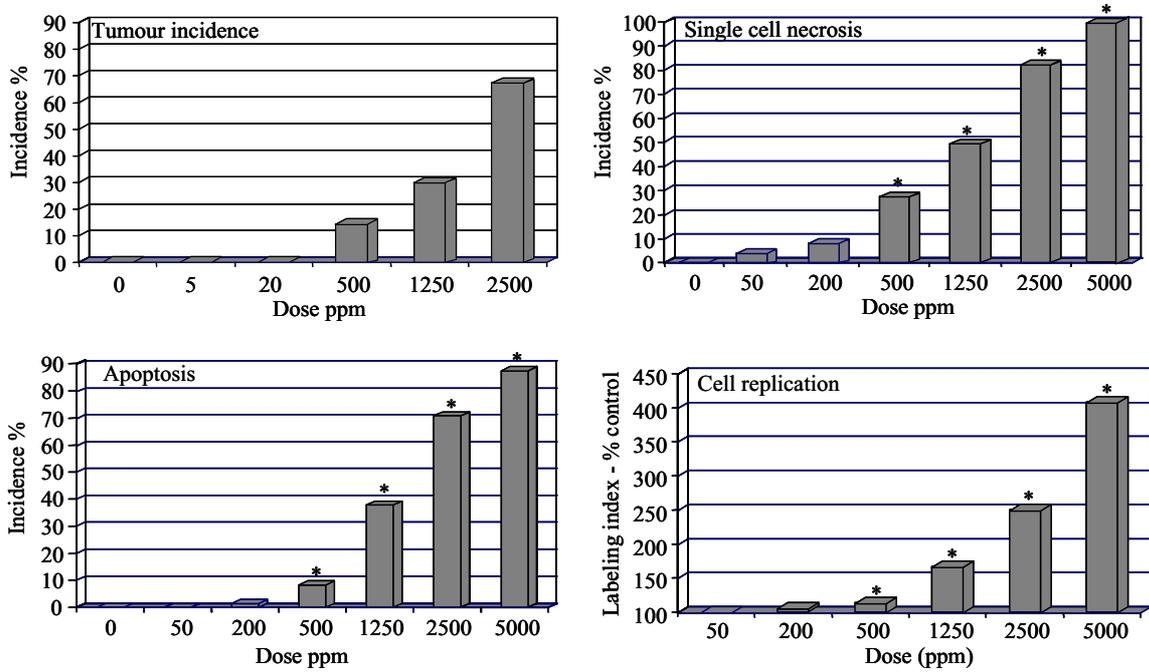

B.

C.

Figure 7

Figure 8.

Figure 9.

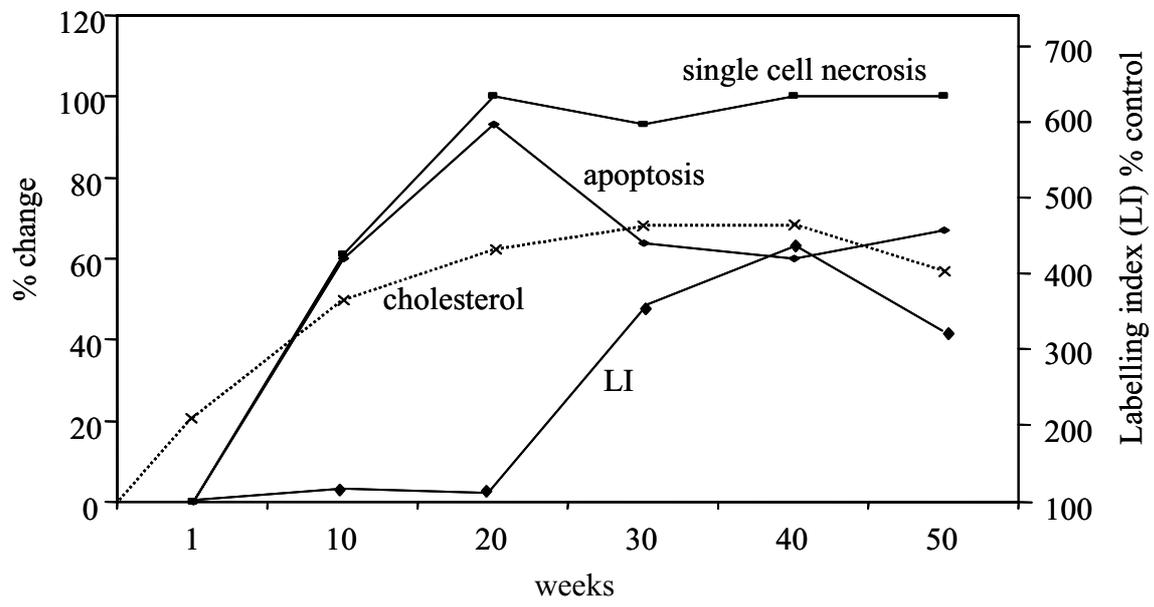Figure 10.
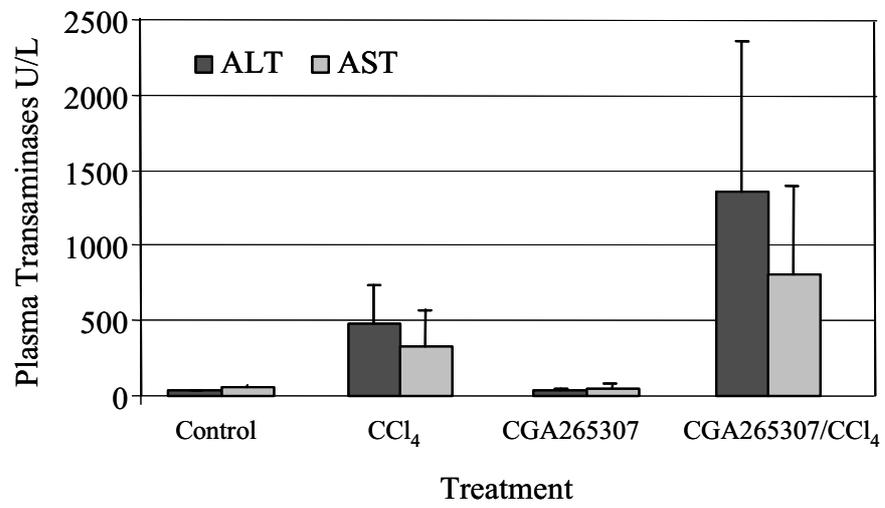
Figure 11.

Figure 11.

Figure 12.

Figure 13.

Table 1. Plasma cholesterol levels in TIF:MAG mice fed on diets containing thiamethoxam for up to 50 weeks

Weeks on diet	Thiamethoxam concentration in the diet ppm						
	0	50	200	500	1250	2500	5000
	Plasma cholesterol (mmol/l)						
10	3.94±0.45	3.64±0.48	3.05±0.85	2.74±0.64*	2.77±0.70*	1.93±0.22**	1.68±0.37**
20	4.31±0.47	3.31±0.81	3.66±0.42	3.12±0.74*	2.94±0.53**	2.75±0.40**	1.41±0.34**
30	3.14±0.66	3.47±1.03	3.63±0.50	3.11±0.32	3.11±0.70	2.18±0.61*	1.47±0.36**
40	3.42±0.44	3.88±0.93	3.69±0.85	2.93±0.86	2.64±0.48*	2.42±0.99	1.61±0.34**
50	3.84±0.44	3.77±0.59	3.40±0.43	3.17±0.63	3.09±0.73	2.21±0.76**	1.86±0.48**
Mean	3.73±0.41	3.61±0.23	3.49±0.27	3.01±0.18*	2.91±0.20**	2.30±0.31**	1.61±0.18**

*$p<0.05$, **$p<0.01$

Table 2. Histopathological findings in the livers of mice fed on diets containing thiamethoxam and metabolites CGA322704 and CGA265307 for up to 20 weeks.

All time points	Control	Thiamethoxam 2500 ppm	CGA322704 2000 ppm	CGA265307 500 ppm
Finding	Tif:MAG mice			
No. Examined	35	36	34	36
Fatty change	-	1	-	-
Apoptosis	1	12**	-	-
Hypertrophy	1	14**	-	-
Single cell necrosis	2	17**	3	2
Inflammatory cell infilt.	4	10	5	10
Mitotic activity	1	4	1	-
Hepatitis	3	1	2	4
Pigmentation	1	6	1	-
Finding	CD-1 mice			
No. Examined	35	36	29	36
Fatty change	-	-	-	-
Apoptosis	-	17**	-	-
Hypertrophy	-	14**	-	-
Single cell necrosis	1	19**	1	-
Inflammatory cell infilt.	4	18**	7	7
Mitotic activity	-	-	-	-
Hepatitis	1	1	-	4
Pigmentation	-	15**	-	-

Data are incidences (number of animals affected per group), ** $p<0.01$

Table 3. Histopathology findings in the livers of Tif:MAG mice fed on diets containing CGA330050 for 10 weeks

Finding	CGA330050 Dietary Concentration		
	Control	500 ppm	1000 ppm
No. Examined	12	12	12
NAD	5	4	1
Hepatocellular hypertrophy	0	0	11**
Single cell necrosis/apoptosis	2	2	6
Inflamm. cell infiltration	2	3	4
Pigmentation	0	0	0
Fatty change	0	1	0
Hepatitis	3	1	2
BrdU labelled hepatocyte nuclei %	0.20±0.11	0.36±0.24	0.57±0.61*

Data are incidences (number of animals affected per group). * $p < 0.05$, ** $p < 0.01$

Table 4. Histopathology findings in the livers of mice fed on a diet containing 2000 ppm CGA265307 for 7 days and then given a single 10ul/kg i.p. dose of carbon tetrachloride

Finding	Control	CCl$_4$ Alone	CGA265307 Alone	CCl$_4$/ CGA265307
No. examined	5	5	5	5
Vacuolation (microvesicular)	0	2	1	5
Hepatocyte hypertrophy	0	1	2	2
Hydropic degeneration	0	0	0	1
Hepatocyte necrosis	0	0	0	1
Subcapsular necrosis	0	2	1	4

Data are incidences (number of animals affected per group).

Table 5. The correlation between early hepatic changes (key events) and the development of tumours in animals fed on diets containing thiamethoxam and its major metabolites.

Test material and Species	Cholesterol depletion	Single cell necrosis	Apoptosis	Cell proliferation	Liver tumours
Thiamethoxam Tif:MAG mouse	✓	✓	✓	✓	✓
Thiamethoxam Tif:RAIf rat	–	–	–	–	–
CGA322704 Tif:MAG mouse	–	–	–	–	–
CD-1 mouse	–	–	–	–	–
CGA265307 Tif:MAG mouse	–	–	–	–	Not tested
CD-1 mouse	–	–	–	–	Not tested
CGA330050 Tif:MAG mouse	✓	✓	✓	✓	Not tested