From: Debbie Stubbs
To: <coshita@oehha.ca.gov>
Date: 5/5/2009 11:38 AM
Subject: Thiamethoxam: Response to Prioritization of Chemicals for Consultation by the CIC
Attachments: Thiamethoxam_SyngentaResp_Prioritization_CIC_consult 5-5-09.pdf; CARC_Thiamethoxam_6-13-05.pdf; T003075-03 Wt of Evidence Liver 12-16-03.pdf

Dear Ms. Oshita,

Attached please find a document containing comments from Syngenta Crop Protection, Inc regarding the potential selection for evaluation by the CIC of thiamethoxam. The document outlines the reasons why Syngenta believes that thiamethoxam does not meet the necessary selection criteria. In addition, two additional supporting documents are attached that are referenced in the response document. One document is the most recent USEPA Report of the Cancer Assessment Review Committee (CARC) dated June 13, 2005. The second document is a Weight of Evidence document that is referenced in the CARC document.

We appreciate the opportunity to provide these comments and we are willing to discuss any questions you may have. Please contact me via e-mail or at the numbers below.

Regards,

Debbie Stubbs l Senior Regulatory Manager l State Affairs Dept. l
Syngenta Crop Protection l P.O. Box 18300 l Greensboro, NC 27419

Ph: 336-632-2449 Fax: 336-632-2884

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Thiamethoxam

Syngenta Response to the Proposed Inclusion of Thiamethoxam on the List of Chemicals Submitted to the Carcinogen Identification Committee (March 2009)

Response

DATA REQUIREMENT(S):	Not Applicable
AUTHOR(S):	Fernando A. Suarez, M.S., DABT
COMPLETION DATE:	May 5, 2009
PERFORMING LABORATORY:	Syngenta Crop Protection, Inc. 410 Swing Road Greensboro, NC 27409 USA
LABORATORY PROJECT ID:	Report Number: T001848-09 Task Number: T001848-09
SPONSOR:	Syngenta Crop Protection, Inc 410 Swing Road Post Office Box 18300 Greensboro, NC 27419-8300 USA

STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The assessment contained in this volume was not conducted in accordance with EPA Good Laboratory Practices, 40 CFR Part 160.

Study Director: There is no GLP study director for this volume.

Fernando A. Suarez, M.S., DABT
Author

05/05/09
Date

Performing Laboratory: Syngenta Crop Protection, Inc.
410 Swing Road
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1.0 INTRODUCTION

The California Office of Environmental Health Hazard Assessment (OEHHA) requested public comment regarding the list of chemicals submitted in March 2009 to the Carcinogen Identification Committee (CIC) for review. OEHHA requested CIC to prioritize the proposed list to determine which chemicals should proceed to the next stage of the evaluation process.

The proposed list of chemicals was developed according to the established Process for Prioritizing Chemicals for Consideration under Proposition 65 by the State's Qualified Experts. According to this procedure, OEHHA applied human and animal data screens to chemicals in the tracking database, and performed subsequent toxicological evaluations on the selected chemicals that were flagged. Based on the results of genotoxicity tests and long-term feeding studies in rats and mice, thiamethoxam (CAS No. 153719-23-4), was included on the proposed list to the CIC for consideration.

Although thiamethoxam was initially classified in 2001 by the US EPA as "likely to be carcinogenic in humans", current scientific evidence demonstrates that thiamethoxam should not be considered for listing as a carcinogen under Proposition 65. Syngenta has submitted numerous studies to EPA that demonstrate that the mode of action for mouse liver tumors would not be operative in humans. Based on this information, the United States Environmental Protection Agency changed its original cancer classification to "not likely to be carcinogenic in humans (US EPA, 2005; 2007).

The present document summarizes the design and results of the studies identified by OEHHA as relevant during the preliminary toxicological evaluation of thiamethoxam, and provide the rationale and evidence that support the exclusion of thiamethoxam from the CIC list.

2.0 RELEVANT STUDIES IDENTIFIED BY OEHHA DURING THE PRELIMINARY TOXICOLOGICAL EVALUATION

2.1 Genotoxicity Studies

The mutagenic potential of thiamethoxam has been thoroughly tested. The test battery was designed to evaluate *in vitro* point mutations in prokaryotic and eukaryotic cells, *in vitro* and *in vivo* clastogenic potential in somatic cells, and DNA damaging potential measured as unscheduled DNA synthesis. The *in vitro* point mutation and clastogenicity studies were performed both with and without a metabolic activation system to investigate the effect of metabolites of thiamethoxam on the test systems. Additionally, the mutagenic potential of thiamethoxam was investigated in an Ames test using microsomal S9 activation system from mice pre-treated (induced) with thiamethoxam. None of the studies revealed any genotoxic effects of thiamethoxam at the DNA level, the gene level or the chromosome level of organization, either with or without metabolic activation (Table 1).

TABLE 1 Summary of Thiamethoxam Genotoxicity Studies

Study type	Test system	Concentrations or doses tested	Result	Reference
***In vitro* studies**				
Bacterial gene mutation assay	*S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537; *E. coli* WP2 uvr A	*S. typhimurium* & *E. coli*: 312.5, 625, 1250, 2500 and 5000 µg/plate.	not mutagenic with and without metabolic activation in S. typhimurium and E. coli	Hertner, 1995a
Bacterial gene mutation assay / metabolic activation with mouse liver microsomes from animals pre-treated with thiamethoxam for 14 days at 3 dose levels	*S. typhimurium* strains TA 98, TA 100, TA 102, TA 1535, TA 1537	312.5, 625, 1250, 2500 and 5000 µg/plate.	not mutagenic with and without metabolic activation in S. typhimurium	Deparade, 1999
Mammalian gene mutation assay	Chinese hamster V79 cells	61.67 to 2220.0µg/ml DMSO w/out activation; 123.33 to 3330.0µg/ml DMSO with activation	not mutagenic with and without metabolic activation	Ogorek, 1996
Mammalian cytogenetic test Chromosome aberrations	Chinese hamster ovary cells (ATTC CCL 61)	283.75 to 2270.0µg/ml DMSO w/out activation; 1135.0 to 4540.0µg/ml DMSO with activation	not clastogenic with and without metabolic activation	Zeugin, 1996
Unscheduled DNA repair	Rat hepatocytes	13.01 to 1665µg/ml culture medium	no induction of unscheduled DNA synthesis	Ogorek, 1996
Unscheduled DNA repair	Mouse hepatocytes	7.3, 14.6, 29.2, 58.4, 117 and 235µg/ml culture medium	no induction of unscheduled DNA synthesis	Ogorek, 2000
***In vivo* studies in somatic cells**				
Micronucleus test	5 male and 5 female (Tif: MAGf, SPF) mice per group and time point	312.5, 625 and 1000mg/kg (all males and in females sacrificed 16 hours post-application) 312.5, 625 and 1250 mg/kg bw (females of the 24 and 48 hours groups)	not clastogenic or aneugenic	Hertner, 1995b

2.2 Carcinogenicity Studies

The toxicity profile of thiamethoxam has been thoroughly tested over the full range of short, intermediate and long-term repeat dose studies, including carcinogenicity studies in rats and mice. Thiamethoxam was not carcinogenic to the rat, however, there was an increased incidence of neoplastic and non-neoplastic alterations in the liver of mice at high dose levels. The study summaries are included below.

2.2.1 18-Month Studies in Male and Female Tif:MAGf(SPF) Mice

Study Design: Groups of 60 male and 60 female albino mice (Tif:MAGf, SPF strain, body weight range 20.9 - 42.0g, source Ciba-Geigy Ltd., Switzerland) received thiamethoxam orally for 78 weeks, by admixture in the diet, at concentrations of 0, 5, 20, 500, 1250 and 2500ppm. Ten animals/sex/group were designated for clinical laboratory investigations and 50 animals/sex/group for the oncogenicity study. Additional groups of 10 animals/sex in the control and high dose groups were similarly treated with thiamethoxam for 35 weeks and sacrificed for interim evaluation.

Findings: Survival incidence and the incidences of single/multiple palpable masses were not affected by treatment in either sex. Clinical signs were restricted to a slightly increased incidence of abdominal distension in females at 1250ppm and in males at 1250 and 2500ppm. This finding correlated histologically, in most cases, with benign or malignant liver tumors.

At week 35, the only effects on organ weights attributable to treatment were increased liver and adrenal weights in females treated at 2500ppm. At week 79, mean carcass weight was reduced in males at 2500ppm. Liver weights were increased dose-dependently in both sexes at 500, 1250 and 2500ppm. Other significant differences at 2500ppm are considered to be incidental to treatment because of an effect on body weight (male brain weight), no histological correlate (male kidney weight) or aberrant mean value in the control group due to the presence of tumors (female spleen weights).

There were no treatment-related gross lesions at necropsy in week 35. Macroscopic examination at week 79 revealed an increased incidence of masses and nodules in the liver at doses ≥500ppm, particularly in male groups.

Liver histopathology performed at week 35 revealed non-neoplastic lesions only. A treatment-related increase occurred in the incidences of inflammatory cell infiltration, necrosis of single hepatocytes, Kupffer cell pigmentation, and hepatocyte hypertrophy in both sexes at 2500ppm. The severity ranged from minimal to moderate. At week 79, treatment-related, non-neoplastic lesions in the liver occurred at ≥500ppm, comprising increased incidences of focal cellular alteration (mainly eosinophilic), inflammatory cell infiltration, necrosis of single hepatocytes, hepatocellular hypertrophy, increased mitotic activity, deposition of pigments (lipofuscin and hemosiderin), and hyperplasia of Kupffer cells. The lesions ranged in severity from minimal to marked and their incidences generally showed a positive dose-relationship.

Treatment-related, non-neoplastic effects in tissues other than liver were increased incidences and severity of splenic extramedullary hematopoiesis and gastric mucosal epithelial hyperplasia in both sexes at 2500ppm. The latter lesion was frequently accompanied by mild inflammatory cell infiltration, and in males, could be correlated with grossly observable thickening of the stomach.

At 79 weeks, the incidence of hepatocellular adenoma was significantly greater than concurrent and historical control levels in both sexes at ≥500ppm. The incidence of hepatocellular adenocarcinoma was also significantly greater than concurrent and historical control levels in females at 1250 ppm and in both sexes at 2500 ppm.

There were no treatment-related effects at dose levels of 5 ppm and 20 ppm in male or female mice.

2.2.2 Two-Year Study in Male and Female Sprague-Dawley Rats

Study Design: Groups of 70 male and 70 female Sprague-Dawley rats (strain Tif: RAIf, SPF, body weight range 114.2 - 224.7g, source Ciba-Geigy Ltd., Switzerland) were administered thiamethoxam orally for 104 weeks, by admixture in the diet, at concentrations of 0, 10, 30, 500 or 1500ppm (males) and 0, 10, 30, 1000 or 3000ppm (females).

Findings: Survival incidence was not affected by treatment, there were no clinical signs of an adverse effect of treatment and the incidences of single and multiple palpable masses were unaffected by treatment at all dose levels. Body weight development in all male groups was unaffected by treatment, but females at 3000ppm had slightly depressed weight gain from week 3 until termination, at which time cumulative weight gain was depressed by 12.6%

There were no treatment-related effects on organ weights at any dose level, at either 52 or 104 weeks. *Post mortem* examination of animals sacrificed after 52 and 104 weeks, and animals that died spontaneously between 52 and 104 weeks, revealed no treatment-related macroscopic lesions.

Micropathology: 52-Week sacrifice: No treatment-related neoplastic lesions occurred in animals sacrificed at 52 weeks or that died during the first 52 weeks. Only 3 malignant and 7 benign neoplasias were identified. All were of the type that spontaneously occurs in this strain of rat, and the distribution between the control and treated groups did not indicate an effect of treatment. Non-neoplastic, treatment-related lesions at 52 weeks were increased incidences of renal tubular regenerative changes, chronic tubular lesion and tubular basophilic proliferation of the kidneys in males at 500 and 1500ppm. Minimally increased incidences of renal tubular and pelvic lymphocytic infiltration also occurred at 1500ppm, but without tubular hyaline change. These alterations are considered to represent the sequelae of alpha-2μ-globulin mediated nephropathy. The kidneys of females at all dose levels were unaffected.

104-Week Sacrifice: Treatment-related, non-neoplastic changes in the kidneys and liver occurred in animals sacrificed at 104 weeks or that died between weeks 53 and 104. In the kidneys, a slightly increased incidence of slight/moderate chronic nephropathy occurred in males at 1500ppm accompanied by a minimal increase in incidence of lymphocytic infiltration of the renal cortex at 1500ppm. Two animals also showed tubular hyaline change. In the liver, a treatment-related increase occurred in the incidence of slight/moderate focal cellular alteration, generally of the clear cell subtype, in females at 3000ppm. All other non-neoplastic findings in the liver and kidneys were of a similar nature, severity and incidence in treated and control groups. In other tissues, some non-neoplastic lesions occurred at slightly higher incidences in treated groups than in controls, but in all instances these are considered unrelated to treatment with thiamethoxam since their occurrence is not dose-related, or the incidence is within the historical control range or known to occur spontaneously in aged rats of this strain.

All neoplastic findings, both malignant and benign, occurring at 104 weeks are considered incidental to treatment with thiamethoxam since the incidences in treated and control groups

are similar, or the inter-group distribution shows no relationship to dose level, or the incidences are within historical control ranges and the lesions are known to occur spontaneously in aged rats.

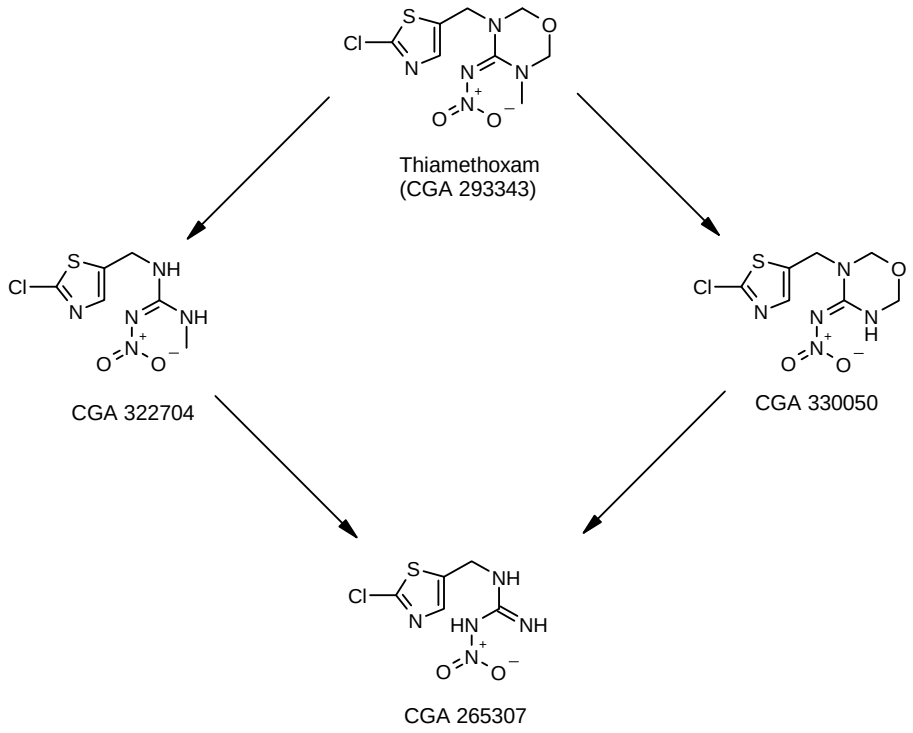
2.3 Mechanistic Studies

As a result of the development of liver tumors, in both male and female mice receiving thiamethoxam in the diet at concentrations of 500 ppm or more for 78 weeks, a comprehensive program of investigative work was designed to understand the mode of action of thiamethoxam on the carcinogenic response observed in mice. Results from these experiments identified a number of key events associated with the development of liver tumors. This information was compiled in a comprehensive Weight of Evidence (WOE) report that was submitted to the US EPA for evaluation (Green et al., 2003a). Also, major components of this mode of action work were subsequently published in a toxicology peer-reviewed journal (Green et al., 2005a, Green et al., 2005b, Pastoor et al., 2005). The WOE document provides a thorough review of the new data available on thiamethoxam and its metabolites, and describes in detail the likely mode-of-action of thiamethoxam in the development of liver tumors in mice. A summary of the mode of action is provided below.

A number of key events were identified in the animal experiments that were associated with the carcinogenic response. These include a reduction in plasma cholesterol, hepatocellular cytotoxicity in the form of single cell necrosis and apoptosis, and an increase in cell replication rates in the liver. The changes occur in a dose-dependent manner and are seen only at the dose levels where cancer incidences were increased (≥500 ppm), but not a lower dose levels (200 ppm and below). They form a rational temporal sequence with biochemical changes leading to cell death followed by increased cell replication. These events are also highly dependent on the duration of dosing; cholesterol changes are seen within one week, cytotoxicity after 10 weeks of dosing and cell replication increases after 20 weeks. The changes are reproducible between studies and between strains of mice and the early changes are rapidly reversible when the test diets are removed.

Further work demonstrated that certain major metabolites of thiamethoxam (CGA330050 and CGA265307) played a key role in the mode of action in mice, and the relative rates of formation of these metabolites explained the species difference in response between mice and rats (Figure 1).

FIGURE 1 Major Metabolic Pathways of Thiamethoxam



None of the described key events were seen in rats fed on diets containing thiamethoxam, nor were they seen in mice fed a diet containing CGA322704, a non-carcinogen. The key metabolite responsible for the hepatic changes in mice has been identified as CGA330050, a plasma metabolite that is formed from thiamethoxam but not CGA322704. These hepatic changes are exacerbated by another important metabolite, CGA265307 (fig 1). Comparative *in vivo* metabolism studies in mice and rats demonstrated that metabolism of thiamethoxam in mice produced substantially higher amounts of CGA330050 and CGA265307, whereas metabolism in rats proceeded primarily via CGA322704.

Finally, *in vitro* comparison of rates of metabolism of thiamethoxam and its metabolites CGA322704 and CGA330050 were performed in liver microsomes from mouse, rat, and human. The rate of formation of the key metabolites CGA330050 and CGA265307 was at least 54-fold lower in rats than in mice. In humans, the rate of this conversion was even lower than in rats, the non-responding species.

3.0 DISCUSSION

Currently established criteria to evaluate the human relevance of animal mode-of-action data require scientific information to address the following elements: i) specificity, consistency and strength of the data, ii) elimination of alternate explanations, and, iii) determination of the likelihood that the proposed mode-of-action could be operative in humans (ILSI., 2003).

Overall, the data obtained from the thiamethoxam research program and presented in the weight of evidence report strongly support the proposed mode-of-action for mouse liver tumors. First, the key events described in the proposed mode-of-action occurred in a logical temporal sequence and displayed dose-response relationships consistent with the findings of the carcinogenicity studies. In addition, these key events were shown not to occur in rats, a non-responding species. The key events (i.e., biochemical changes, necrosis, apoptosis, and increased cell replication rates) are biologically plausible, as they are known to be associated with the development of liver tumors in mice. Finally, the key events were shown to be reproducible in two strains of mice and to be in complete concordance with the metabolic data.

Second, alternative modes-of-action were specifically studied and excluded. For example, the experimental data specifically excluded: a) potential genotoxic activity of thiamethoxam, b) induction of peroxisome proliferation by thiamethoxam, c) production of oxidative stress by thiamethoxam, d) modulation of glutathione biosynthesis, and e) induction of CYP450 enzymes to levels known to be associated with hepatocarcinogenesis.

Third, the WOE report addressed the plausibility that the animal mode of action could be operative in humans. Insufficient amounts of the active metabolites CGA330050 and CGA265307 would be formed in humans to produce hepatotoxic effects necessary for subsequent liver tumor development, and therefore, this characterized mode-of-action for liver tumors in mice would not be operative in humans.

4.0 CONCLUSIONS

When thiamethoxam was originally registered in the US, the US EPA classified it as “likely to be carcinogenic in humans”, based on liver tumors at high dose levels in an 18-month mouse study (US EPA, 2001). Following scientific criteria defined by the International Life Science Institute (ILSI, 2003), Syngenta developed an extensive investigative program designed to characterize the thiamethoxam mode of action for liver tumors in mice. Results from this work are summarized in a weight-of-evidence evaluation document that was submitted to the US EPA (Green et al., 2003). On Jun 13, 2005 the Cancer Assessment Review Committee published the final report of the evaluation of the carcinogenic potential of thiamethoxam. On May 22, 2007, the Health Effects Division published the “Thiamethoxam Human Health Risk Assessment” for thiamethoxam food uses in a wide range of crops (US EPA 2007).

In essence, this research program demonstrated that thiamethoxam-induced tumors in mice are species-specific, and arise through a non-genotoxic mode of action characterized by a series of key events that include: perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. The key metabolites, CGA330050 and CGA265307, responsible for the key events in the mouse are not formed in sufficient quantities in the rat to begin the chain of key hepatic effects, thereby explaining the lack of a carcinogenic response in the rat. In humans, in vitro studies show that these two key metabolites are formed in significantly lower amounts in

humans than in rats, the non-responding species. Because humans would not generate sufficient amounts of the key metabolites responsible for this mode of action in mice, the combination of factors leading to the development of liver tumors in mice would not be present in humans. Therefore, it is concluded that humans would not be at risk of developing liver tumors as a result of exposure to thiamethoxam.

Based on the results from toxicity studies, mechanistic studies, and the evaluation of the overall weight of evidence, the carcinogenic mode of action of thiamethoxam is considered to be well understood. USEPA concluded that the proposed mode-of-action of thiamethoxam is not operative in humans, and that thiamethoxam does not pose a carcinogenic risk to humans. Accordingly, thiamethoxam should not be considered for listing as a carcinogenic toxicant under Proposition 65.

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Rec'd
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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

TXR No.: 0053465

MEMORANDUM

DATE: June 13, 2005

SUBJECT: **THIAMETHOXAM:** Report of the Cancer Assessment Review Committee
PC Code: 060109

FROM: Jessica Kidwell, Executive Secretary Jessica Kidwell
Cancer Assessment Review Committee
Health Effects Division (7509C)

TO: Alan Levy, Toxicologist (RAB2)
Mike Doherty, Risk Assessor (RAB2)
Health Effects Division (7509C)

Dan Kenny, PM
Insecticide/Rodenticide Branch, Registration Division (7505C)

The Cancer Assessment Review Committee met on April 27, 2005 to evaluate the carcinogenic potential of Thiamethoxam. Attached please find the Final Cancer Assessment Document.

cc: J. Pletcher
Y. Woo

CANCER ASSESSMENT DOCUMENT

EVALUATION OF THE CARCINOGENIC POTENTIAL OF ***THIAMETHOXAM***

PC Code 060109

FINAL
June 13, 2005

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

DATA PRESENTATION: Alan C. Levy
Alan Levy, Toxicologist

DOCUMENT PREPARATION: Jessica Kidwell
Jessica Kidwell, Executive Secretary

COMMITTEE MEMBERS IN ATTENDANCE: (Signature indicates concurrence with the assessment unless otherwise stated).

Karl Baetcke

Lori Brunsman, Statistician — JR for LB

William Burnam, Chair

Marion Copley

Vicki Dellarco

Kit Farwell

Nancy McCarroll — for Nancy McCarroll

Tim McMahon

Jess Rowland

Linda Taylor — for LT

NON-COMMITTEE MEMBERS IN ATTENDANCE (Signature indicates concurrence with the pathology report)

Doug Wolf, Pathologist, EPA/ORD/NHEERL (conference call) — See attached sheet

OTHER ATTENDEES: Kelly Schumacher (HED/RAB2), Karlyn Bailey (HED/RAB2), Christina Swartz (HED/RAB2)

DATA PRESENTATION: _____
Alan Levy, Toxicologist

DOCUMENT PREPARATION: _____
Jessica Kidwell, Executive Secretary

COMMITTEE MEMBERS IN ATTENDANCE: (Signature indicates concurrence with the assessment unless otherwise stated).

Karl Baetcke _____

Lori Brunsman, Statistician _____

William Burnam, Chair _____

Marion Copley _____

Vicki Dellarco _____

Kit Farwell _____

Nancy McCarroll _____

Tim McMahon _____

Jess Rowland _____

Linda Taylor _____

NON-COMMITTEE MEMBERS IN ATTENDANCE (Signature indicates concurrence with the pathology report)

Doug Wolf, Pathologist, EPA/ORD/NHEERL (conference call) _____

OTHER ATTENDEES: Kelly Schumacher (HED/RAB2), Karlyn Bailey (HED/RAB2), Christina Swartz (HED/RAB2)

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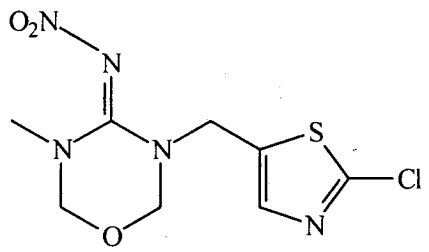
I. INTRODUCTION

On April 27, 2005 the Cancer Assessment Review Committee of the Health Effects Division of the Office of Pesticide Programs met to re-evaluate the carcinogenic potential of Thiamethoxam.

II. BACKGROUND INFORMATION

Thiamethoxam, **PC Code 060109** (4H-1,3,5-oxadiazin-4-imine, 3-[(2-chloro-5-thiazoly)methyl]tetrahydro-5-methyl-N-nitro-; CAS No. 153719-23-4) is a broad spectrum insecticide belonging to the class of compounds call neonicotinoids. The molecular weight is 291.7 and the molecular formula is $C_8H_{10}ClN_5O_3S$. Thiamethoxam is currently registered for food uses. The chemical structure is as follows:



In a chronic toxicity/carcinogenicity study in rats, 50 rats/sex/dose received thiamethoxam at dietary levels of 0, 10, 30, 500 or 1500 ppm for males (0, 0.41, 1.29, 21, or 63 mg/kg/day, respectively) and 0, 10, 30, 1000, or 3000 ppm for females (0, 0.48, 1.56, 50.3, or 155 mg/kg/day, respectively) for 24 months (MRID 44718708). In the carcinogenicity study in mice, thiamethoxam was administered in the diet to 50 mice/sex/group at 0. 20, 500, 1250, or 2500 ppm (0, 0.65, 2.63, 63.8, 162 or 354 mg/kg/day for males and 0, 0.89, 3.68, 87.6, 215, or 479 mg/kg/day for females, respectively) for 18 months (MRID 44703326).

In 1999, Syngenta Crop Protection (then Novartis, Inc.) submitted a weight-of-evidence document that was reviewed by EPA. In June 2000, thiamethoxam was classified as "Likely to be Carcinogenic to Humans" by the EPA and a linear low-dose extrapolation approach for the quantification of human cancer risk based on the most potent liver tumor response observed in mice (US EPA, 2000). At the time, this approach was supported by the lack of confirmation of the carcinogenic mode of action for mouse liver tumors induced by thiamethoxam. EPA suggested that additional data were needed to supplement the proposed mode of action.

These data have been submitted to EPA and were reviewed by the CARC for the April 2005 meeting. They include the following:

1. Characterization of thiamethoxam-related effects on the mouse liver at intervals over a 50-week period at 6 dose levels, including those where increased tumor incidences had been seen (MRIDs 46161513, 46161518, 46161521).

2. A 50-week study in rats at dietary concentrations of 0, 1000 and 3000 ppm thiamethoxam (MRID 46161514).

3. A comparative investigation of the hepatotoxicity of thiamethoxam and a number of its metabolites in two strains of mice in studies of up to 20 weeks duration (MRID 46161513).

4. A recovery study in mice (MRID 46161508)

5. A comparison of the hepatotoxicity of thiamethoxam in young (weanling) and adult mice (MRID 46161509).

6. Kinetics and metabolism studies following low and high single oral doses in mice (MRIDs 46161502, 46161504, 46161505, 46161512).

7. A comparative study in mice and rats of the kinetics of thiamethoxam and its metabolites following a single high oral dose and following dietary administration for up to 50 weeks (MRIDs 46161503, 46161506, 46161507).

8. Comparisons of the *in vitro* metabolism in rat, mouse and human liver (MRID 46161506).

A summary of the registrant's position was submitted and is entitled "Weight of Evidence for a Mode of Action for Thiamethoxam - Related mouse liver tumors (MRID 46161501). In this document the registrant presented the weight of evidence from these studies that supports a plausible mode of action explanation for the increased incidence of hepatic tumors in mice in a long-term thiamethoxam dietary feeding study.

III. MODE OF ACTION

The CARC followed the EPA's final guidelines for Carcinogen Risk Assessment (March 29, 2005) and the ILSI-RSI publication "A Framework for Human Relevance Anaysis of Information on Carcinogenic Modes of Action" (ILSI, 2003) for the evaluation of carcinogenic reponse in animal studies and in determination of the relevance to humans. The Hill criteria (Hill, 1965) for causation were used as a general guide.

1. Did the registrant provide sufficient evidence to establish a plausible mode of action for liver tumors in the mouse? Yes, the CARC concluded that the available data are sufficient to support a plausible non-linear mode of action for liver carcinogenicity of thiamethoxam in mice. The CARC agrees with the registrant that, "A series of key events has been identified that form the basis for the mode of action for the development of liver tumors in mice. These include perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. In addition, these changes occur in a dose-dependent manner and are only seen at dose levels where tumor incidences are increased."

A. Key Events

Increased liver tumors (combined adenomas and carcinomas) were observed in long-term carcinogenicity studies in male and female mice at dietary concentrations of 500, 1250, and 2500 ppm of thiamethoxam. A series of time- and dose-related key events that form the basis for the mode of action for the formation of mouse liver tumors were identified at dose levels where tumor incidences were increased. Progressive steps in the mode of action include the following:

1. *Bioactivation of thiamethoxam* to a reactive key metabolite CGA330050, which induced the same changes in mouse liver as thiamethoxam itself (MRID 46161501)

2. *General perturbation of hepatocyte metabolism.* Early stage general perturbation of hepatocyte metabolism was caused by metabolite CGA330050 and included lowering of plasma cholesterol (within one week of dosing), induction of cytochrome P-450 isoenzymes, decreased protein synthesis, glycogen, and lipid accumulation (MRID 46161513, MRID 46161508). The earliest and most significant event in the process was the marked reduction in plasma cholesterol levels that occurred at ≥500 ppm (a dose level where tumor incidences were also increased) over 1-10 weeks of exposure (MRID 46161508).

3. *Hepatotoxicity and apoptosis (necrosis).* At this stage (8-50 weeks), signs of hepatotoxicity are seen (hepatocellular hypertrophy, lymphocytic infiltration and pigmentation of hepatocytes and Kupffer cells, clinical chemistry changes, single

hepatocyte necrosis, and apoptosis). The incidence and severity of hepatocellular hypertrophy was increased at 30-50 weeks in mice treated with 2500 ppm thiamethoxam and at all time points in mice treated with 5000 ppm (MRID 46161521). There was a statistically significant 3-10 fold increase in the number of apoptotic bodies in the centrilobular region of the liver in male mice treated with ≥500 ppm for 59 days or longer (MRID 46161520). Necrosis was increased in a dose-related manner in mice treated with 500 ppm at week 40, at weeks 20, 40, and 50 in mice treated with 1250 ppm and at all time points in mice treated with 2500 or 5000 ppm (MRID 46161521). Overall, the data show that thiamethoxam treatment induces clear time- and dose-dependent histopathological changes of hypertrophy and induction of apoptosis as well as hepatocellular necrosis.

4. *Sustained Increased Cell Proliferation:* Sustained increases in cell proliferation (240%), measured specifically in centrilobular hepatocytes (the region of the liver in which single cell necrosis and apoptosis were concentrated), were observed in male mice at 500 ppm at 40 weeks (MRID 46161518). (In this study, no earlier measurements were made.)

Events leading to Hepatic Neoplasia

Studies where mice were fed diets of 0-5000 ppm thiamethoxam for 1-50 weeks: MRIDs 44703407, 44703406, 46161508, 46161509, 46161521, 46161513.

Study Weeks	Observation
1-10	**Liver Dysfunction:** decreased protein synthesis, glycogen and lipid accumulation, reduced cholesterol, cytochrome P-450 induction and hepatocellular hypertrophy
10-50	**Hepatotoxicity:** increased ALAT/ASAT, pigmentation, inflammatory cell infiltration, hepatocellular hypertrophy, single cell necrosis and increased apoptosis
20-50	**Sustained increase in cell replication rates**
50-80	**Neoplasia**

B. Reversibility of Effects

Preneoplastic changes induced by thiamethoxam are considered to be reversible. Continued administration of thiamethoxam is necessary to progress to stages beyond hepatotoxicity (including cell death, increases in cell replication and permanent cell transformation to neoplasia). Mice were fed 2500 ppm of thiamethoxam for 4 weeks and then given control diet for 4 weeks (MRID 46161508). After 4 weeks of treatment, plasma cholesterol was reduced to

63% of control value, liver weights were increased by 9%, and there was a reduction of eosinophilia of centrilobular hepatocytes. After 2 weeks on the control diet, plasma cholesterol levels had returned to control values and liver histology was normal. Liver weights were normal within 4 weeks.

C. Mouse Strains

Hepatic tumors and events described above were observed in the Tif:MAG mouse. Long-term studies with thiamethoxam metabolite CGA322704 (tested as clothianidin; Federal Register, 2003) are believed to have used the CD-1 mouse. Therefore, males of both strains were given diets of 0 or 2500 ppm thiamethoxam for 1, 10 or 20 weeks. Parameters examined were the same as those in the 50-week studies: liver biochemistry, histopathology, apoptosis (TUNEL) and cell replication rates (MRID 46161513).

At all time points, plasma cholesterol levels decreased. At 10 and 20 weeks, aminotransferase activities were increased and histopathological changes noted at these time points in the 50-week study were observed. An increase in cell replication rates was seen at 20 weeks. The magnitude of the changes observed in this study was comparable to those seen in the 50-week study. All changes were seen in both strains and to a similar degree.

D. Metabolites

The ability of the major metabolites of thiamethoxam (CGAA322704, CGA330050, and CGA265307) to induce characteristic hepatic lesions observed with thiamethoxam was investigated in feeding studies in mice up to 20 weeks duration (MRID 46161510, 46161513).

CGA322704 is reported not to induce cancer in mice (or rats). In the 20-week study at 2000 ppm in the diet (same as highest dose in a chronic feeding study), the metabolite did not reduce plasma cholesterol, cause hepatotoxicity nor increase cell replication rates. Thiamethoxam (2500 ppm) in the same study was hepatotoxic and increased cell replication rates.

CGA330050 (500 and 1000 ppm; dose levels selected to mimic systemic exposure to this metabolite following high-dose levels of thiamethoxam) induced the same liver changes as those seen after thiamethoxam administration (reduced plasma cholesterol levels as well as histological changes: increased hypertrophy, single cell necrosis, apoptosis and cell replication at 10 weeks).

CGA265307 (500 ppm; a dose selected to mimic systemic exposure to this metabolite following a feeding level of 2500 ppm thiamethoxam) did not induce any hepatic lesions seen in thiamethoxam treated mice.

E. CGA265307 and Inhibition of Nitric Oxide (NO) Synthase

This metabolite is structurally similar to a number of known NO synthase inhibitors. NO produced by these enzymes is known to have a regulatory role in the liver in modulating the adverse effects of TNF_{α} released from endothelial cells during chemically induced hepatotoxicity. Therefore, inhibition of these enzymes could exacerbate the cytotoxicity, necrosis and apoptosis that are believed to lead to cell replication and tumors in the livers of mice fed thiamethoxam (MRID 46161511). The CARC considered this to be an ancillary event, not part of the key events.

F. Lack of Effects on Rat Liver

No thiamethoxam-related increases in liver tumors were reported in the 2-year rat study (males - high dose of 1500 ppm due to kidney toxicity; females - high dose of 3000 ppm as the MTD). The 50-week mouse study was repeated in female Tif:Ralf rats at doses of 0, 1000 or 3000 ppm for 1, 10, 20, 30, 40 or 50 weeks (15 rats/group). Livers were examined microscopically for morphological changes, replicative DNA synthesis was assessed by nuclear incorporation of BrdU as a diagnostic parameter for cell proliferation and the apoptotic activity was assessed by TUNEL-histochemistry. Clinical chemistry parameters were measured at each time point. Metabolic enzyme activities were measured after 1 and 10 weeks (MRID 46161519).

None of the changes seen in the mouse 50-week study were seen in the rat study. **The key events leading to hepatocarcinogenesis are only seen in the mouse.**

G. The Role of Metabolism in Species Specificity

Time related metabolic profiles in plasma of mice and rats is listed below (MRID 46161501).

Test Article	Mice (2500 ppm)			Rats (3000 ppm)		
	Week 1	Week 10	Week 50	Week 1	Week 10	Week 50
Thiamethoxam	12	15	10	7	19	8
CGA322704	3	5	3	[illegible]	1	1
CGA265307	2	7	4	<1	<1	<1
CGA330050	1	2	1	<1	<1	<1

UNITS ≐ µg/mL plasma; This table was made from Figure 6, p. 22 of MRID 46161501.

H. Cross-Species Comparison of Thiamethoxam Metabolic Conversion Rates

Metabolic pathway	Relative rate
	mouse : rat
Thiamethoxam to CGA265307 via CGA322704	54 : 1
Thiamethoxam to CGA265307 via CGA330050	87 : 1
	mouse : human
Thiamethoxam to CGA265307 via CGA322704	371 : 1
Thiamethoxam to CGA265307 via CGA330050	238 : 1

The above table indicates that the conversion of thiamethoxam to the metabolite CGA265307 via either pathway (CGA322704 or CGA330050) is greater in the mouse than in either the rat or human.

I. Summary of Mode of Action

The CARC agrees with the Registrant's summary of the mode of action as follows: "A number of key events have been identified which are believed to lead to liver cancer in mice fed diets containing thiamethoxam. These include a reduction in plasma cholesterol, cytotoxicity in the form of single cell necrosis and apoptosis, and an increase in cell replication rates. The changes occur in a dose-dependent manner and are seen only at the dose levels where cancer incidences were increased. They form a rational temporal sequence with biochemical changes leading to cell death followed by increased cell replication. These events are also highly dependent on the duration of dosing; cholesterol changes are seen within one week, cytotoxicity after 10 weeks of dosing and cell replication increases after 20 weeks. The changes are reproducible between studies and between strains of mice and the early changes are rapidly reversible when the test diets are removed."

"None of these changes were seen in rats fed on diets containing thiamethoxam, nor were they seen in mice fed a diet containing CGA322704, a non-carcinogen. The key metabolite responsible for the hepatic changes in mice has been identified as CGA330050, a metabolite that is formed from thiamethoxam but not CGA322704. These hepatic changes are exacerbated by another important metabolite, CGA265307."

"The correlation between key events and liver tumors is absolute within these studies. It is consistent with the species differences in carcinogenicity and internally consistent with the known metabolism of thiamethoxam."

2. Is the Animal Mode of Action Plausible in Humans?

A. Key Events Across Species

Sufficient evidence for a mode of action in the mouse has been established. To be relevant to humans, the key events that were identified in the test species should be concordant in humans. The concordance table below represents this concept and analysis, where each key event is matched across species and directly to humans.

Key Event	Mouse	Rat	Human
Generation of critical metabolites: CGA330050 and CGA265307	Yes	Yes	Yes (1)
CGA330050-related liver "dysfunctional" changes: ↓cholesterol biosynthesis; ↓ protein synthesis; ↑ P-450	Yes chol=500 ppm[a] protein=2500 ppm	No; (insufficient CGA 330050)	Unlikely (1) (insufficient CGA 330050)
CGA265307 inhibition of inducible nitric oxide synthase (iNOS). **The CARC considered this to be an ancillary event.**	Yes 2500	No; (insufficient CGA 265307)	Unlikely; (insufficient CGA 265307)
Hepatotoxicity (clinical chemistry; histopathology; apoptosis)	Yes cl chem=2500 ppm histo=2500 ppm apop≥500 ppm necrosis≥500 ppm	No	*In vivo* data not available
Sustained increase in cell replication	Yes ≥500 ppm	No	*In vivo* data not available
Tumors	Yes 500 ppm~↑13% 1250 ppm~↑30% 5000 ppm ~↑65%	No	Unlikely

(1) = based on *in vitro* data
a = dose at which effect occurred

The CARC concluded that while the mode of action is plausible in humans, it is dependent on a sufficient amount of the active metabolite being persistently formed to drive the toxicity and regenerative proliferation. The table shows that the progression of key events in rats and humans does not go beyond the generation of CGA330050 and CGA265307. The CARC concluded that the *in vitro* data showed that the metabolic capability of humans is more similar to rats than mice; however, the *in vitro* human data are too limited to draw quantitative kinetic conclusions.

B. Are Young Animals more Sensitive than Adults?

Comments:

1. histopathological changes in the liver not seen until 10 weeks
2. mice reach maturity at 6-7 weeks
3. earliest key event: one week, reduction of plasma cholesterol

Study:

Groups of 6 male weanling mice (21 days old) and groups of 6 male adult mice (15-17 weeks old) were fed diets of 0, 500, 1250 and 2500 ppm thiamethoxam for 7 days (MRID 46161509). Plasma cholesterol was measured at the end of the study. Dietary intake was higher in weanlings than adults (about 2x based on concentration of the major metabolites at the end of the study). Plasma cholesterol levels were lowered in adults at all 3 levels, but only at 1250 and 2500 ppm in weanlings. The magnitude of the response in weanlings at the two higher doses was also less than in adults.

Conclusion:

Infants and children would not be more susceptible than adults.

3. Conclusions

The CARC agreed with the registrant that a plausible mode of action has been established for the development of liver tumors in a mouse bioassay with thiamethoxam. It is concluded that the liver tumors in the mouse arise through a non-genotoxic mode of action characterized by a series of key events that include: perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. Neither the key events nor an increase in liver tumors are seen in rats fed on diets containing up to 3000 ppm thiamethoxam. The key metabolites, CGA330050 and CGA265307, responsible for the key events in the mouse are not formed in sufficient quantities in the rat and explain the lack of a carcinogenic response in this species.

A sufficient amount of active metabolite must be produced along with persistent exposure to the active metabolite to lead to the hepatotoxic/regenerative proliferative/neoplastic response in the mouse. Limited human *in vitro* metabolism studies suggest that humans are more similar to the rat compared to the mouse in producing the active metabolite. The rat does not develop tumors following treatment with thiamethoxam. Thus, the mouse appears to be uniquely sensitive to this mode of action. Because of the threshold nature of the mode of action and the unique sensitivity of the mouse, it is concluded that humans are unlikely to be at risk for developing tumors

following exposures to thiamethoxam.

IV. WEIGHT-OF-EVIDENCE CONSIDERATION

A. Hazard Identification

Mouse

Dietary administration of thiamethoxam was associated with increased incidence of liver tumors in both sexes of mice.

In male mice, the incidences of liver adenomas, carcinomas, and combined adenomas and/or carcinomas for the control, 5, 20, 500, 1250, 2500 ppm dose groups, respectively, were as follows:

Adenomas:	11/50 (22%),	5/50 (10%),	10/49 (20%),	17/50 (34%),	27/48 (56%),	40/50 (80%)
Carcinomas:	1/50 (2%),	4/48 (8%),	2/46 (4%),	5/50 (10%),	7/46 (15%),	20/49 (41%)
Combined:	12/50 (24%),	7/50 (14%),	12/49 (24%),	19/50 (38%),	27/48 (56%),	45/50 (90%)

Male mice had significant increasing trends, and significant differences in the pair-wise comparisons of the 2500 ppm dose group with the controls, for hepatocellular adenomas, carcinomas, and adenomas and/or carcinomas combined, all at $p<0.01$. There were also significant differences in the pair-wise comparisons of the 1250 ppm dose group with the controls for hepatocellular adenomas and adenomas and/or carcinomas combined, both at $p < 0.01$, and for hepatocellular carcinomas at $p < 0.05$.

In female mice, the incidences of liver adenomas, carcinomas, and combined adenomas and/or carcinomas for the control, 5, 20, 500, 1250, 2500 ppm dose groups, respectively, were as follows:

Adenomas:	0/48 (0%),	0/50 (0%),	0/49 (0%),	5/44 (11%),	8/47 (17%),	31/49 (63%)
Carcinomas:	0/48 (0%),	0/50 (0%),	0/49 (0%),	0/44 (0%),	2/47 (4%),	11/49 (22%)
Combined:	0/48 (0%),	0/50 (0%),	0/49 (0%),	5/44 (11%),	9/47 (19%),	32/49 (65%)

Female mice had significant increasing trends, and significant differences in the pair-wise comparisons of the 2500 ppm dose group with the controls, for hepatocellular adenomas, carcinomas, and adenomas and/or carcinomas combined, all at $p<0.01$. There were also significant differences in the pair-wise comparisons of the 500 ($p< 0.05$) and 1250 ($p< 0.01$) ppm dose groups with the controls for hepatocellular adenomas and adenomas and/or carcinomas combined. The incidence of carcinomas in females at 500 and 1,250 ppm, although not statistically significant, exceeded the concurrent and historical control values. The increases in these tumors in males at 500 ppm were considered to be

biologically significant and were supported by a dose-related increase in the incidence and severity of non-neoplastic lesions.

The incidences of adenomas and carcinomas at ≥500 ppm exceeded the historical control ranges (males: 10%-46% and 0%-24%, respectively; females: 0%-8% and 0%-2%, respectively). The CARC reaffirmed the previous CARC's conclusion that the liver tumors in male and female mice were treatment-related.

Dosing at the highest dose was considered adequate and not excessive based on decreased body weight gains in both sexes and histopathological changes in the liver and kidney.

Rat

Thiamethoxam was not carcinogenic in male or female rats dose levels of up to 1500 ppm in males and 3000 ppm in females. The CARC revisited the adequacy of dose issue according to HED's Dose Selection Paper. They determined that dosing in male rats at 1500 ppm was adequate based on the results of the 90 day study. In the 90-day study at 1250 ppm, there was decreased body weight/body weight gain of 15%/22%, increased creatinine, and kidney pathology (chronic tubular lesions) in males rats. In female rats, dosing at 3000 ppm could have been tested higher; however, since the mode of action is known and the regulatory doses are below 3000 ppm, the CARC determined that a new rat study will not impact the risk assessment.

B. Mutagenicity

Thiamethoxam was negative in both *in vitro* and *in vivo* mutagenicity assays.

C. Mode of Action

The CARC agreed with the registrant that a plausible mode of action has been established for the development of liver tumors in a mouse bioassay with thiamethoxam. It is concluded that the liver tumors in the mouse arise through a non-genotoxic mode of action characterized by a series of key events that include: perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. These changes occurred in a time- and dose-dependant manner and are only seen at dose levels where tumor incidences are increased. Neither the key events nor an increase in liver tumors are seen in rats fed on diets containing up to 3000 ppm thiamethoxam. The key metabolites, CGA330050 and CGA265307, responsible for the key events in the mouse are not formed in sufficient quantities in the rat and explain the lack of a carcinogenic response in this species.

While humans are *qualitatively* capable of generating the active hepatotoxic metabolite, limited *in vitro* human metabolism studies suggest humans appear to be more like the rat in that they are less efficient at producing the active metabolite. The mode of action is dependant on a sufficient amount of active metabolite persistently produced to drive the carcinogenic response. Humans are unlikely to be at risk of developing liver tumors as a result of exposure to thiamethoxam due to these two characteristics (the threshold response coupled with the difference in species sensitivity).

V. CLASSIFICATION OF CARCINOGENIC POTENTIAL

In accordance with the EPA's *Final Guidelines for Carcinogen Risk Assessment* (March, 2005), the CARC classified Thiamethoxam as **"Not Likely to be Carcinogenic to Humans"** based on convincing evidence that a non-genotoxic mode of action for liver tumors was established in the mouse and that the carcinogenic effects are a result of a mode of action dependent on sufficient amounts of a hepatotoxic metabolite produced persistently. Although humans are qualitatively capable of producing the active metabolite, thiamethoxam is unlikely to pose a cancer risk to humans unless sufficient amounts of metabolites are persistently formed to drive a carcinogenic response. Based on limited *in vitro* human metabolism data and comparative metabolism studies in the mouse and rat, the human appears to be more like the rat, which does not develop tumors after thiamethoxam treatment. Lastly, the non-cancer assessment is sufficiently protective of the key events (perturbation of liver metabolism, hepatotoxicity/regenerative proliferation) in the animal mode of action and, thus, cancer is not an issue.

VI. QUANTIFICATION OF CARCINOGENIC POTENTIAL

Not Required.

VII. BIBLIOGRAPHY

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THIAMETHOXAM: ASSESSMENT

TITLE

Weight of Evidence for a Mode of Action for Thiamethoxam-Related Mouse Liver Tumors

DATA REQUIREMENT

Not Applicable

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VOLUME 1 OF 1 OF STUDY

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Title: NAFTA Insecticide Team Leader .

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Study Director: There is no GLP study director for this volume.

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Summary

Thiamethoxam is a mouse-specific non-genotoxic liver carcinogen. Tumor incidences were not increased in the rat under comparable conditions. Each of the criteria defined by the US-EPA and ILSI for establishing a mode of action has been addressed in order to explain the increase in liver tumors in thiamethoxam-treated mice. The conclusions are as follows:

A series of key events that form the basis of the mode of action has been identified in the mouse liver. These include perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. In addition, these changes occur in a dose-dependent manner and are only seen at the dose levels where tumor incidences are increased.

Furthermore, these changes occur in a logical temporal sequence, with early (0 –10 weeks) biochemical changes leading to toxicity and cell death (10 – 50 weeks) and increased cell replication (20 – 50 weeks of feeding).

None of these changes occurred in the rat long-term study with thiamethoxam, suggesting that significant differences exist between mice and rats in the metabolism of thiamethoxam. CGA322704, a metabolite of thiamethoxam, did not increase the incidence of mouse liver tumors in a dietary feeding study.

CGA330050, a metabolite of thiamethoxam that is not formed from CGA322704, induced the same changes in the mouse liver as thiamethoxam itself. Therefore, CGA330050 is considered to be the active metabolite of thiamethoxam.

CGA265307, a major metabolite in the mouse, has been shown to be an inhibitor of nitric oxide synthases and is believed to exacerbate the mild hepatotoxicity induced by CGA330050.

The changes seen in mice are consistent, reproducible between studies, and internally consistent with the known metabolism of thiamethoxam.

Markedly lower amounts of CGA330050 and CGA265307 are formed in rats than in mice. These major quantitative differences in metabolism explain the lack of response in the rat. Comparisons *in vitro* show that humans produce even less of these metabolites than the rat.

A number of experimental studies have been conducted that explored alternative modes of action, but no plausible alternative modes of action have been identified.

Comparisons of the sensitivity of young and adult mice to thiamethoxam show that young mice are at least 2-fold less sensitive than adults. Thus, there is no evidence to suggest that children are at greater risk than adults from thiamethoxam.

Therefore, a plausible mode of action has been elucidated for the development of liver tumors in a mouse bioassay with thiamethoxam. It is concluded that the tumors are specific to the mouse and arise through a non-genotoxic mode of action characterized by a series of key events that include: perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. The key metabolites, CGA330050 and CGA265307, responsible for the key events in the mouse are not formed in sufficient quantities in the rat to begin the chain of key hepatic effects, thereby explaining the lack of a carcinogenic response in the rat.

In humans, *in vitro* studies show that these two key metabolites are formed in significantly lower amounts in humans than in rats, the non-responding species. Because humans would not generate sufficient amounts of the key metabolites, the combination of factors leading to the development of liver tumors in mice would <u>not</u> be present in humans. Therefore, it is concluded that humans would not be at risk of developing liver tumors as a result of exposure to thiamethoxam.

1.0 Introduction

An increased incidence of liver tumors (combined adenomas and adenocarcinomas) was observed in a long-term study in male and female mice at dietary concentrations of 500, 1250 and 2500 ppm thiamethoxam (Bachmann, 1998a) (Table 1). Thiamethoxam was not carcinogenic in the rat at dose levels of up to 1500 ppm in males and 3000 ppm in females (Bachmann, 1998b).

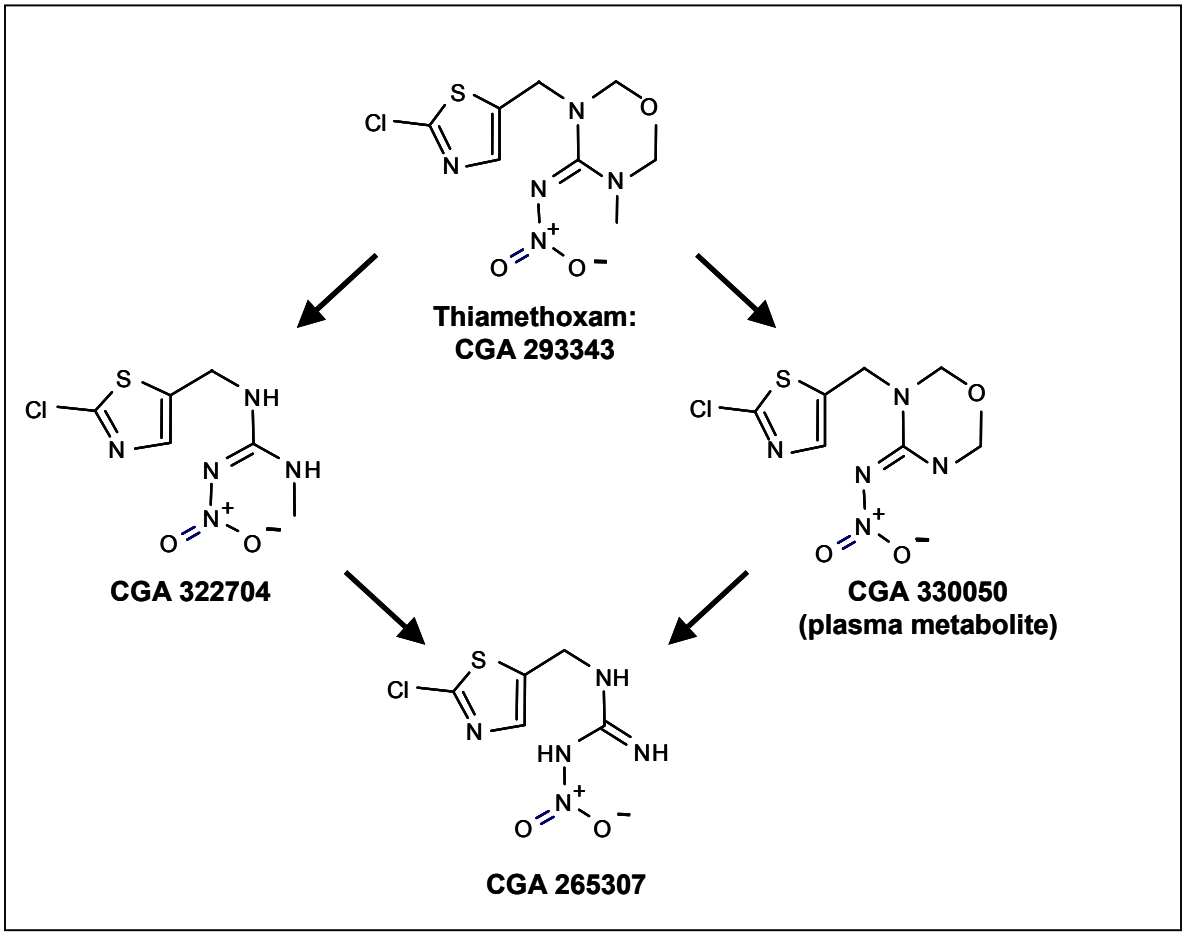
Table 1. Incidence of Liver Tumors (combined adenoma and adenocarcinoma) in Tif:MAGf Mice (Bachmann, 1998a)

Male Mice	Dose-ppm	0	5	20	500	1250	2500
	Dose-mg/kg-day	0	0.65	2.63	63.8	162	354
	Incidence	**12/50**	**7/50**	**12/50**	**19/50**	**27/50**	**45/50**
Female Mice	Dose-mg/kg-day	0	0.89	3.68	87.6	215	479
	Incidence	**0/50**	**0/50**	**0/50**	**5/50**	**9/50**	**32/50**

Thiamethoxam is not mutagenic in any *in vitro* or *in vivo* assay, including a bacterial gene mutation assay (Hertner, 1995a); a mammalian gene mutation assay (Ogorek, 1996a); a mammalian cytogenetic assay (Zeugin, 1996); unscheduled DNA repair (Ogorek, 1996b); nor a micronucleus test (Hertner, 1995b). In addition, thiamethoxam was not mutagenic in bacteria using induced mouse liver S9 for metabolic activation (Deparade, 1999). Therefore, thiamethoxam is not a genotoxic carcinogen. The question then arises as to the mode of action of thiamethoxam as a mouse liver carcinogen and the relevance to human health of the non-genotoxic, mouse-specific hepatic tumor responses.

A large number of metabolites of thiamethoxam have been identified in mice and rats (Thanei, 1998; Mewes, 1998). The major metabolites present *in vivo*, either in urine or plasma, following either a single oral dose or repeat dietary administration, are thiamethoxam itself and metabolites CGA322704, CGA265307 and CGA330050 (Figure 1) (Mewes, 2000; Briswalter, 2002a, b, c, d; Green, 2003a).

Figure 1. Major Metabolic Pathways of Thiamethoxam



In 1999, Syngenta Crop Protection (then Novartis, Inc.) submitted a weight-of-evidence document (Weber et al., 1999) that was reviewed by EPA (EPA, 2000). In that review, EPA suggested that additional data would be needed to supplement the proposed mode of action. These data have been collected and include:

- Characterization of thiamethoxam-related effects on the mouse liver at intervals over a 50-week period at six dose levels, including those where increased tumor incidences had been seen (Noakes, 2003a; Weber, 2003; Soames, 2003).
- A 50-week study in rats at dietary concentrations of 0, 1000 and 3000 ppm thiamethoxam. (Noakes, 2003b).

- A comparative investigation of the hepatotoxicity of thiamethoxam and a number of its metabolites in two strains of mouse in studies of up to 20 weeks duration. (Noakes, 2003a).
- A recovery study in mice (Green, 2003b).
- A comparison of the hepatotoxicity of thiamethoxam in young (weanling) and adult mice (Green, 2003c).
- Kinetics and metabolism studies following low and high single oral doses in mice (Mewes, 2000; Briswalter, 2002a, c, d).
- A comparative study in mice and rats of the kinetics of thiamethoxam and its metabolites following a single high oral dose and following dietary administration for up to 50 weeks (Briswalter, 2002b, c; Green, 2003a; Green, 2002).
- Comparisons of the *in vitro* metabolism of thiamethoxam in rat, mouse, and human liver (Green, 2002).

The purpose of this document is to present the weight of evidence from these studies that supports a plausible mode of action explanation for the increased incidence of hepatic tumors in mice in a long-term thiamethoxam dietary feeding study. Based on this explanation, further conclusions are drawn regarding the relevance of these thiamethoxam-related mouse liver tumors to human health.

2.0 Postulated Mode of Action and Key Events

A number of time- and dose-related key events have been identified that characterize a mode of action that leads to liver cancer in thiamethoxam-treated mice. Several progressive steps in the mode of action include an early stage mild hepatic "dysfunction" caused by the metabolite CGA330050 (characterized by lowering of plasma cholesterol, induction of cytochrome P-450 isoenzymes, decreased protein synthesis and glycogen and lipid accumulation), which is exacerbated by another specific thiamethoxam metabolite, CGA265307 (via inhibition of nitric oxide synthase), leading to hypertrophy, lymphocytic infiltration, and pigmentation of hepatocytes and Kupffer cells. Single hepatocyte necrosis,

apoptosis and increased cell proliferation follow in the later stages, which finally leads to the formation of hepatic tumors (Goldsworthy et al., 1993; Butterworth and Eldridge, 1995).

A series of biochemical and histopathological events that occur in a sequence, leading to hepatic neoplasia, have been characterized in studies where mice were fed diets containing 0-5000 ppm thiamethoxam for periods from 1 – 50 weeks (Trendelenburg, 1998; Weber, 1998; Green, 2003b, c; Weber, 2003; Noakes, 2003a). The results of these studies show the following progression of events:

Study Week	Observation
1-10	**Liver dysfunction:** Decreased protein synthesis, glycogen and lipid accumulation, reduced cholesterol, cytochrome P-450 induction and hepatocellular hypertrophy.
10-50	**Hepatotoxicity:** Increased ALAT/ASAT, pigmentation, inflammatory cell infiltration, hepatocellular hypertrophy, single cell necrosis and increased apoptosis
20-50	**Sustained increase in cell replication rates.**
50-80	**Neoplasia**

Evidence for each of these areas is provided below.

2.1 Progression of Hepatic Key Events

2.1.1 1-10 weeks: Liver dysfunction

The earliest stages of thiamethoxam's effect on the mouse liver (within one week of dosing) are characterized by histological changes, including accumulation of glycogen and lipid in centrilobular regions of the liver. These histological changes are accompanied by several signature biochemical changes, including:

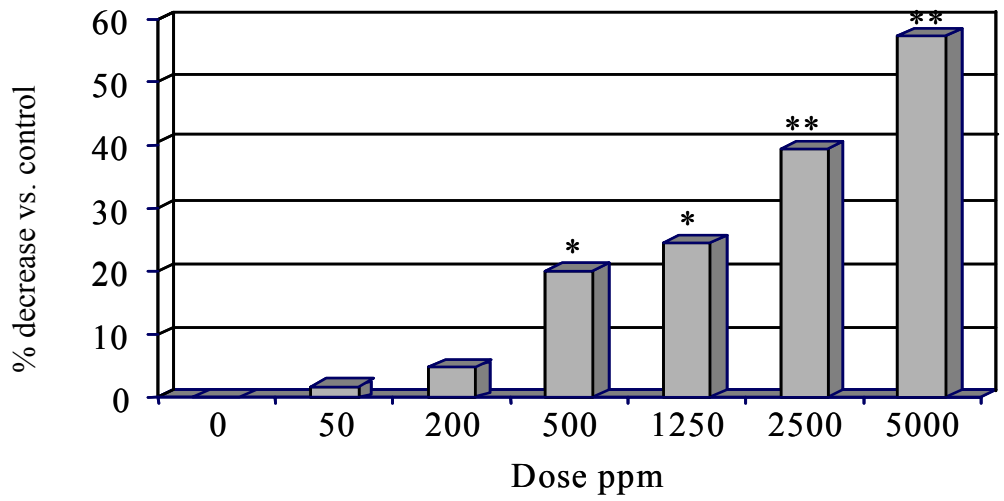
Protein synthesis: A statistically significant reduction in plasma total protein was observed in mice fed on diets containing 2500 ppm thiamethoxam for 20 weeks (Noakes, 2003a).

Cytochrome P-450 induction: A significant increase in hepatic cytochrome P-450, principally of the 2B family, was seen in mice fed on diets containing 0, 100, 500 or

2500 ppm thiamethoxam for 14 days (Trendelenburg, 1998). Thiamethoxam was found to be a moderate (up to 9-fold) inducer of mouse Cyp2b isoenzymes, and both pentoxyresorufin O-depentylase and benzyloxyresorufin O-debenzylase activity. However, these moderate increases alone were considered to be of insufficient magnitude to be causally linked to the development of liver cancer.

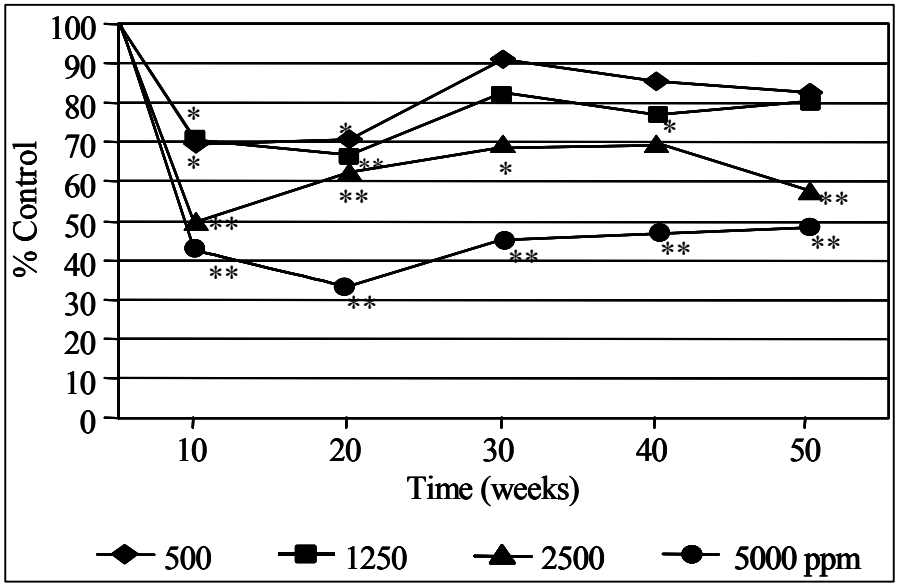
Plasma cholesterol changes: The earliest and most significant change in mice fed on diets containing thiamethoxam is a marked reduction in plasma cholesterol levels. Cholesterol levels were reduced in a dose dependent manner at dose levels of 500 ppm thiamethoxam and above (Figure 2) (Green, 2003b). Furthermore, they remained lower than controls throughout the 50-week study (Figure 3).

Figure 2. Dose-Related Decreases in Plasma Cholesterol (mean values over 50 week study)



* Statistically significant ($p<0.05$)
** Statistically significant ($p<0.01$)

Figure 3. Cholesterol Decreases During 50-Week Study



* Statistically significant ($p<0.05$)
** Statistically significant ($p<0.01$)

There is strong evidence that changes in lipid metabolism in rodents leads to an increase in liver tumors. 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 such as the fibrates, and the more recent statins. 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 (Newman and Hulley, 1996). Thiamethoxam is not a peroxisome proliferator and it has minimal effects on plasma triglycerides *in vivo*. In specifically lowering plasma cholesterol its mode of action resembles that of the statin family of cholesterol lowering drugs. All of the major statins are rodent liver carcinogens, the mouse being the more sensitive species (MacDonald *et al*. 1988; Gerson *et al*. 1989; Newman and Hulley, 1996; von Keutz and Schulter, 1998). Thus, it is probable that changes in cholesterol biosynthesis and/or

metabolism are an early and critical key event in the mode of action of thiamethoxam as a mouse liver carcinogen.

2.1.2 10-50 weeks: Hepatotoxicity

At this stage of continuous dosing in mice, frank signs of hepatotoxicity can be seen (Noakes, 2003a; Weber, 2003). The following statistically significant changes were observed:

Plasma cholesterol changes: As seen in the 50-week study, reductions in plasma cholesterol are sustained throughout this period (Figure 3; Green, 2003b).

Plasma clinical chemistry changes: Changes in several markers of liver damage are seen. The median aspartate aminotransferase activity (ASAT) was increased at 2500 ppm (weeks 20 and 40) and at 5000 ppm (from week 10 to 50). Upon combining the data from all time points, increased ASAT values were noted at 1250, 2500 and 5000 ppm (116%, 122% and 169% of control, respectively) (Weber, 2003).

The median alanine aminotransferase activity (ALAT) was significantly increased at 1250 ppm (weeks 40 and 50), at 2500 ppm (from week 10 to 50) and at 5000 ppm (from week 10 to 50). Upon combining all time points, increased ALAT values were noted at 1250, 2500 and 5000 ppm (139%, 207% and 256% of control, respectively) (Weber, 2003).

Hepatocellular hypertrophy was increased in incidence and/or mean severity at 2500 ppm (from week 30 to 50) and at 5000 ppm (all time points). Upon combining all time points, increased incidences were observed at 2500 and 5000 ppm (Figure 4a). This change was characterized by enlarged centrilobular/mid-zonal hepatocytes with increased amounts of cytoplasmic glycogen, fat, and smooth endoplasmic reticulum (Weber, 2003).

Pigmentation was increased in incidence and/or mean severity at 500 ppm (week 50), at 2500 ppm (from week 20 to 50), and at 5000 ppm (all time points). Upon

combining all time points, increased incidences were observed at 1250, 2500 and 5000 ppm. Yellow/brown pigment granules in the cytoplasm of centrilobular hepatocytes characterized this lesion. Occasionally, pigmented Kupffer cells were observed (Weber, 2003).

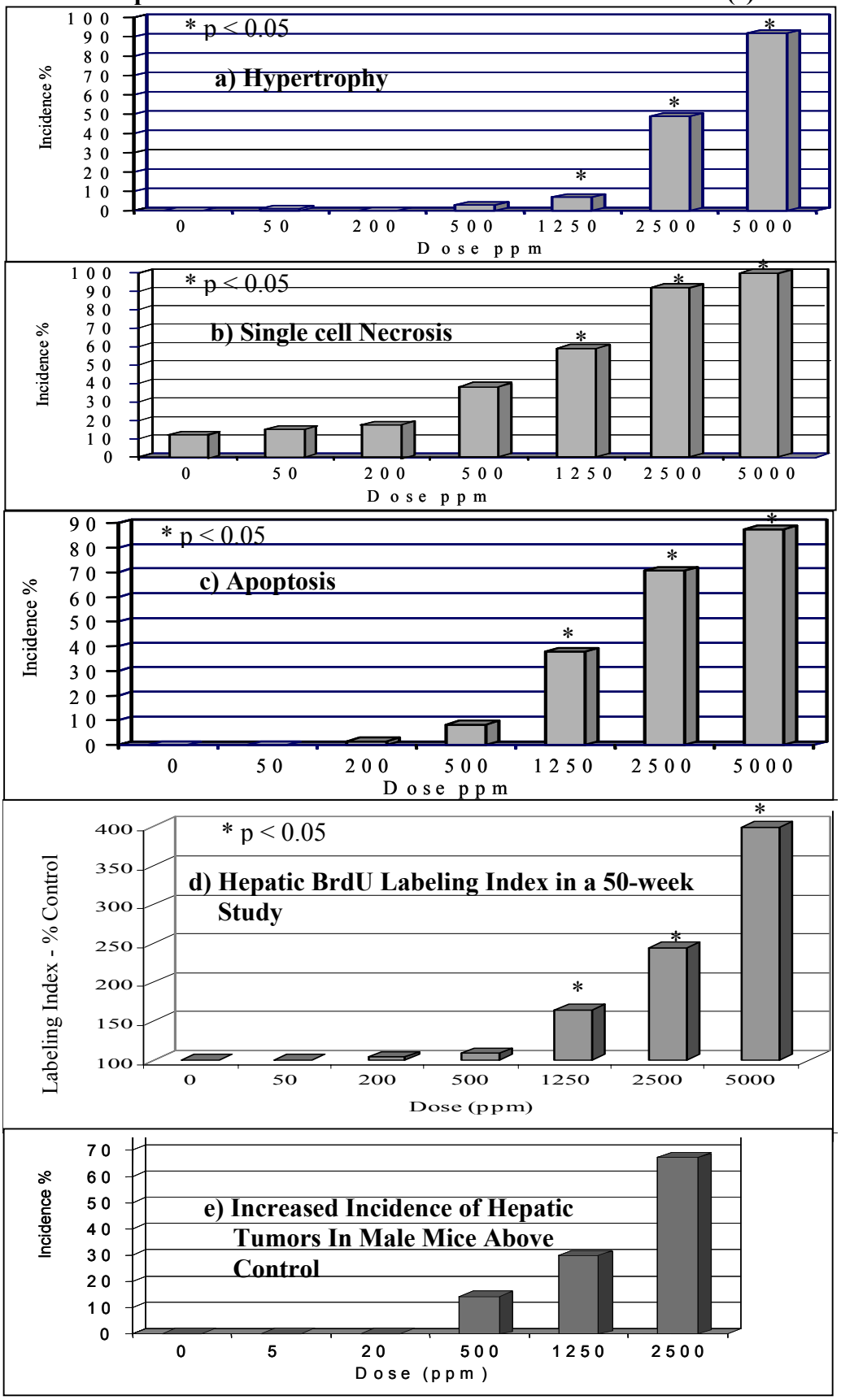
Hepatic Single Cell Necrosis was increased in incidence and/or mean severity at 500 ppm (week 40), at 1250 ppm (from week 20 to 50), at 2500 ppm (all time points), and at 5000 ppm (all time points). Upon combining all time points, increased incidences were observed at 500, 1250, 2500 and 5000 ppm (Figure 4b). Hepatocellular necroses affected single cells or small groups of cells with mainly centrilobular localization and were often accompanied by inflammatory cells (Weber, 2003).

Inflammatory cell infiltration was increased in incidence and/or mean severity at 500 ppm (week 50), at 1250 ppm (weeks 40 and 50), at 2500 ppm (weeks 20, 40 and 50), and at 5000 ppm (from week 30 to 50). Upon combining all time points, increased incidences were observed at 500, 1250, 2500 and 5000 ppm: (Weber, 2003).

Hepatocellular apoptosis. Histologically, this was increased in incidence and/or mean severity at 1250 ppm (weeks 20 and 30), at 2500 ppm (from week 10 to 50), and at 5000 ppm (from week 10 to 50). Upon combining all time points, increased incidences were observed at 500, 1250, 2500 and 5000 ppm (Figure 4c). Hepatocellular apoptoses affected single cells or small groups of cells with mainly centrilobular localization (Weber, 2003).

Quantitative assessment of the extent of apoptosis using TUNEL area density showed an increase at 500 ppm (weeks 30 and 40), at 1250 ppm (weeks 30 and 40), at 2500 ppm (weeks 30, 40 and 50), and at 5000 ppm (from week 10 to 50). Upon combining all time points, increased median TUNEL area densities were observed at 500 ppm (156% of control), at 1250 ppm (188% of control), at 2500 ppm (219% of control), and at 5000 ppm (316% of control) (Weber, 2003).

Figure 4. **Dose response of hepatocellular changes over 10-50 weeks (a-d); comparison with the liver tumor incidence over 80 weeks (e)**



2.1.3 20-50 weeks: Sustained Increase In Cell Replication Rate

An increased median BrdU labeling index was observed at 1250 ppm (week 40), at 2500 ppm (weeks 30 to 50) and at 5000 ppm (weeks 10, 30, 40, and 50). Upon combining all time points, increased median BrdU labeling indices were observed at 1250 ppm, 2500 ppm, and 5000 ppm (Figure 4d). These increases were determined across the whole liver lobule (Weber, 2003). When the increases in cell replication rates were measured specifically in centrilobular hepatocytes, the region of the liver in which single cell necrosis and apoptosis was concentrated, a significant increase in cell replication rates was also observed in the 500 ppm dose group (week 40, 240%) (Soames, 2003).

2.1.4 Summary of key events

A series of key events has been identified that form the basis of the mode of action for the development of liver tumors in mice. These include perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. In addition, these changes occur in a dose-dependent manner and are only seen at the dose levels where tumor incidences are increased. No significant hepatic changes were observed at 200 ppm in the mouse.

2.2 Reversibility of effects

Preneoplastic hepatocellular changes induced by thiamethoxam are considered to be reversible events. Progression to the next stages beyond hepatotoxicity, including cell death, increases in cell replication and permanent cell transformation to neoplasia, requires sustained administration of thiamethoxam.

To test the reversibility of the key events, mice were fed on a diet containing 2500 ppm thiamethoxam for four weeks and then given control diets for four subsequent weeks (Green, 2003b). At the end of the 4-week treatment period, plasma cholesterol levels were reduced to 69% of concurrent control values, liver weights were increased by 9%, and eosinophilia of centrilobular hepatocytes was reduced. Two weeks after return to control diet, plasma cholesterol levels had returned to control values and liver histology was normal. Liver

weights returned to normal within 4 weeks. Thus, the early liver dysfunction occurring in mice fed on diets containing thiamethoxam is readily reversible.

This 4-week study did not produce cytotoxicity (of a severity that leads to cell death), which is consistent with other studies where it was not observed until 10 weeks or more of administration (Noakes, 2003a; Weber, 2003).

2.3 Results in another mouse strain

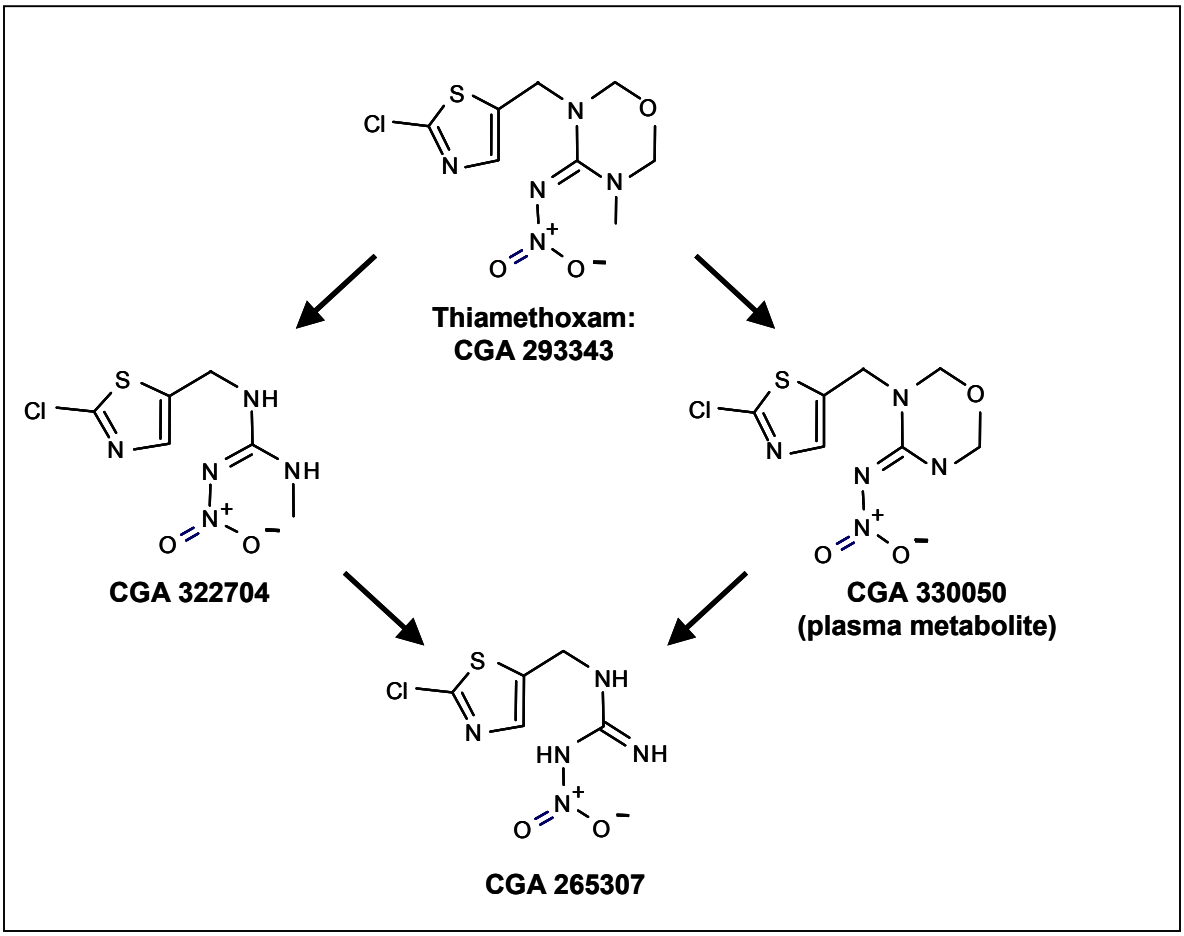
Both the hepatic tumors and the key events described above were seen in the Tif:MAG mouse. The long-term studies with thiamethoxam metabolite CGA322704 (tested as clothianidin; Federal Register, 2003) are believed to have used the CD-1 mouse. In order to investigate possible strain effects on the outcome of these studies, both male Tif:MAG mice and male CD-1 mice were fed on diets containing 0 and 2500 ppm thiamethoxam for 1, 10 or 20 weeks. The end-points monitored in this study were identical to those in the 50-week studies, namely, liver biochemistry, histopathology, apoptosis (TUNEL) and cell replication rates (Noakes 2003a).

Decreases in plasma cholesterol levels were seen at all time points. At 10 and 20 weeks, aminotransferase activities were increased and the histopathological changes noted at these time points in the 50-week mouse study were observed. An increase in cell replication rates was seen at 20 weeks. The magnitude of the changes seen in this study was comparable to those seen in the 50-week mouse study. All of these changes were seen in both Tif:MAG and CD-1 mice to a similar degree. Thus the key events are not mouse-strain specific.

2.4 The role of metabolites in the mode of action

The ability of the major metabolites of thiamethoxam, CGA322704, CGA265307 and CGA330050 (Figure 5) to induce the characteristic hepatic lesions seen with thiamethoxam was investigated in a series of feeding studies in mice of up to 20 weeks duration (Noakes, 2003a, Green, 2003d).

Figure 5. Major Metabolic Pathways of Thiamethoxam



2.4.1 CGA322704

CGA322704 is reported not to induce cancer in mice (or rats) in chronic feeding studies (Federal Register, 2003). The results from this cancer study provided a further opportunity to test the correlation between the changes identified as key events in the thiamethoxam studies and the development of liver cancer.

CGA322704 (2000 ppm in the diet; a dose level selected to match the highest dose level in a chronic feeding study) did not reduce plasma cholesterol, cause hepatotoxicity, nor increase cell replication rates in a 20 week mouse feeding study (Noakes, 2003a). Thiamethoxam (2500 ppm in the diet), used as a positive control in the same study, was hepatotoxic and increased cell replication rates. CGA322704's lack of liver carcinogenicity and lack of any

of the responses in the liver seen in mice fed on diets containing thiamethoxam confirmed the correlation between the key events and liver cancer.

2.4.2 CGA330050

CGA330050 (500 and 1000 ppm in the diet; dose levels selected to mimic systemic exposure to this metabolite following high dose feeding levels of thiamethoxam) induced the same changes in the livers of mice as those seen after dietary administration of thiamethoxam. Plasma cholesterol levels were reduced significantly and the histopathological examination of the livers revealed an increase in hypertrophy, single cell necrosis, apoptosis and increased cell replication at 10 weeks, all of which are clear indicators of the early stages that are involved in the formation of liver tumors in mice. Therefore, CGA330050 is seen to play a clear role in the development of hepatic lesions (Green, 2003d).

2.4.3 CGA265307

CGA265307 (500 ppm in the diet, a dose level selected to mimic systemic exposure to this metabolite following a feeding level of 2500 ppm thiamethoxam) did not induce any of the hepatic lesions seen in thiamethoxam treated mice when tested in a 20 week study alongside thiamethoxam and CGA322704 (Noakes, 2003a).

2.4.4 CGA265307 and inhibition of NO synthase

Although not hepatotoxic, CGA265307 potentially impacts on the development and severity of the lesions caused by metabolite CGA330050. CGA265307 is structurally similar to a number of known inhibitors of nitric oxide synthases (Green, 2003e).

Nitric oxide produced by these enzymes is known to have a regulatory role in the liver in modulating the adverse effects of TNFα released from endothelial cells during chemically induced hepatotoxicity. Thus, inhibition of these enzymes could exacerbate the cytotoxicity, necrosis and apoptosis that are believed to lead to cell replication and tumors in the livers of mice fed on diets containing thiamethoxam.

CGA265307 has been shown to inhibit purified nitric oxide synthases *in vitro*, acting as a competitive inhibitor with the normal substrate arginine (Green, 2003e). *In vivo*, carbon tetrachloride was shown to be more hepatotoxic in mice fed on a diet containing CGA265307 at 2000 ppm for 7 days than in mice fed on control diet (Green, 2003e). Furthermore, the blood levels of CGA265307 in this experiment were comparable to those seen in mice fed on diets containing 2500 ppm thiamethoxam, the highest dose level used in the mouse cancer study. It is concluded, therefore, that inhibition of nitric oxide synthases by CGA265307 is likely to exacerbate the hepatotoxicity of thiamethoxam (initiated by CGA330050) that leads to cell death, increased cell replication and the development of liver tumors in mice.

2.5 Mouse specificity

2.5.1 Lack of Effects on Rat Liver

There was no treatment related increase in liver tumors in the rat bioassay. In the male rat the highest dose of 1500ppm was limited by kidney toxicity. A high dose level of 3000ppm was the MTD in female rats. The 50-week study in mice was, therefore, repeated in female Tif:RaIf rats (Noakes, 2003b). The purpose of the study was to establish a correlation between the key events identified in the mouse and the development of liver cancer, on the basis that these events should not occur in the rat because thiamethoxam is not a carcinogen in this species.

Groups of 15 female rats were treated with thiamethoxam admixed into the diet at 0, 1000 or 3000 ppm for 1, 10, 20, 30, 40, or 50 weeks. Liver sections were examined microscopically for morphological changes, replicative DNA synthesis was assessed by nuclear incorporation of BrdU as a diagnostic parameter for cell proliferation, and the apoptotic activity was assessed by TUNEL-histochemistry. A wide range of clinical chemistry parameters was measured at each time point. Metabolic enzyme activities were measured after 1 and 10 weeks (Waechter, 2003).

None of the changes seen in the mouse 50-week study were seen in rats fed on diets containing 1000 or 3000 ppm thiamethoxam for 50 weeks. Throughout the study, at all time points, livers were morphologically normal and indistinguishable from controls. There were no increases in cell replication rates. Clinical chemistry parameters, including plasma cholesterol and the aminotransferases (ASAT and ALAT), were unchanged (Noakes, 2003b). Likewise, a wide range of cytochrome P-450 isoenzymes, epoxide hydrolase, UDP-glucuronyltransferase, gamma-glutamylcysteine synthetase and glutathione S-transferase activities were essentially unaffected by treatment (Waechter, 2003).

Thus, the key events leading to hepatocarcinogenesis are only seen in the mouse.

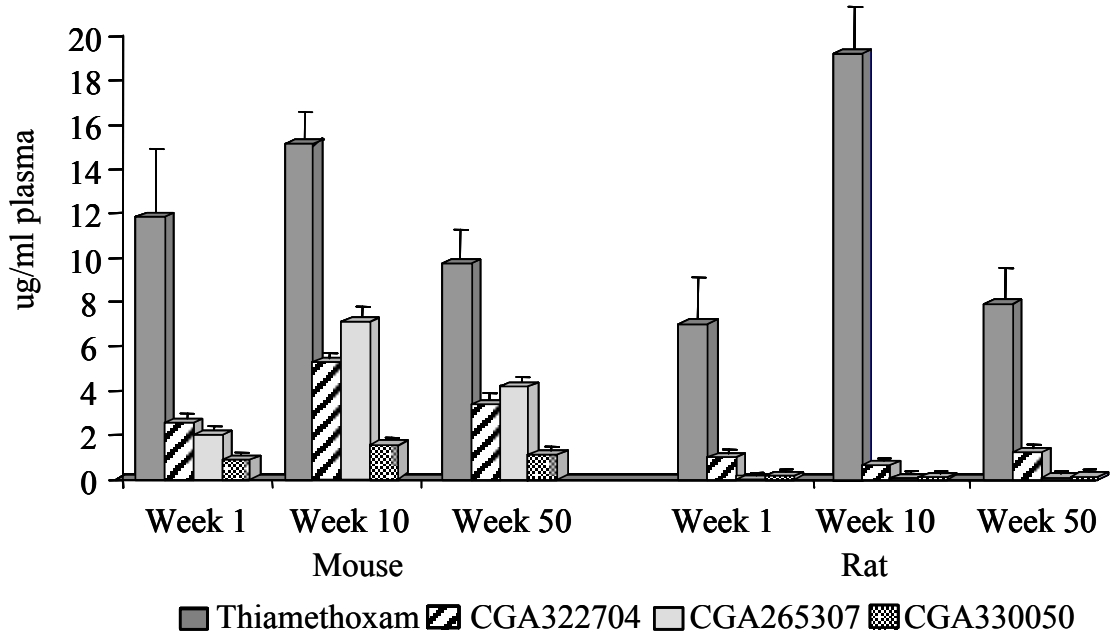
2.5.2 The Role of Metabolism in species specificity

Two metabolites, CGA330050 and CGA265307, have been shown to play a role in the development of the hepatotoxicity that leads to liver tumors in mice. CGA330050 is responsible for the reductions in plasma cholesterol and the subsequent cell death and increased cell replication. CGA265307 is believed to exacerbate these responses by inhibiting nitric oxide synthases.

Comparative metabolism studies in mice and rats have shown significant species differences in the formation of the key metabolites, CGA330050 and CGA265307 (Green, 2002; 2003a). Following a single 100 mg/kg oral dose of C-14 thiamethoxam, the rat excretes 73% of an administered dose as unchanged thiamethoxam, whereas the mouse excretes only 39% into the urine as thiamethoxam. The increased metabolism of thiamethoxam in the mouse is reflected in urine as significantly greater amounts of metabolite CGA265307, 19% of the dose in mouse urine, but only 1.9% in rat urine. The urinary excretion of CGA322704 is similar in both species, suggesting that the route to CGA265307 via CGA330050 is much more important in the mouse (Thanei, 1998; Briswalter, 2002a, 2002b, 2002c). CGA330050 is not excreted to any significant extent in urine but is observed, together with thiamethoxam and the other major metabolites in plasma.

Plasma metabolites have been measured at intervals during many of the dietary feeding studies in which thiamethoxam and its metabolites were administered to mice and rats (Green, 2003a). These data give a more accurate indication of the internal doses under the conditions of the cancer studies and hence a more accurate measure of the contribution of metabolism to the species differences in carcinogenicity between mice and rats. Thiamethoxam and its major metabolites, CGA322704, CGA265307 and CGA330050 were measured in plasma collected from female Tif:RaIf rats and male Tif:MAG mice fed on diets containing 3000 (rats) or 500, 1250 and 2500 ppm (mice) thiamethoxam for up to 50 weeks (Figure 6).

Figure 6. Time-Related Metabolic Profiles in Plasma of Mice fed 2500ppm and Rats fed 3000ppm



Plasma metabolite profiles have also been compared in two strains of male mice, Tif:MAG and CD-1, following dietary administration of 2500 ppm thiamethoxam, 2000 ppm CGA322704 or 500 ppm CGA265307 for up to 20 weeks. In addition, CGA330050 has been fed to male Tif:MAG mice at a dietary concentration of 1000 ppm and plasma metabolite profiles measured after 1 week of feeding.

The principal findings from these studies were as follows:

- Thiamethoxam and its metabolites are rapidly cleared in both rats and mice following a single oral dose.
- The metabolism of thiamethoxam in mice was linear with dose between dietary concentrations of 500 and 2500 ppm.
- The blood levels of thiamethoxam were comparable in mice and rats at the highest dose levels used in the cancer studies.
- The metabolism of thiamethoxam to CGA265307 was induced in mice, but not rats, during the first 10 weeks of feeding.
- Major species differences in the levels of two circulating metabolites were apparent. The concentrations of CGA265307 and CGA330050 were up to 140 and 15-fold greater, respectively, in mice than in rats at roughly equivalent dietary feeding levels of thiamethoxam.
- In plasma samples taken from Tif:MAG and CD-1 mice (Noakes, 2003a), there were no significant strain differences in the metabolism of thiamethoxam.
- After feeding of CGA330050 to Tif:MAG mice at 1000 ppm for one week, CGA 265307 was the major metabolite generated (Green, 2003a).
- Overall, the mouse's rate of metabolism through CGA330050 is much higher than the rat, clearly supporting the finding that the thiamethoxam → CGA330050 → CGA265307 pathway accounts for the hepatotoxic effects that lead to liver tumors in the mouse.

Thus, the quantitative differences between mice and rats in the metabolism of thiamethoxam, and specifically in the formation of key metabolites CGA330050 and CGA265307, is entirely consistent with the species difference in the hepatocarcinogenicity of this chemical in mice and rats.

2.5.3 *In vitro* species comparison

In vivo metabolism studies in mice and rats have provided insight into the species difference in the carcinogenicity of thiamethoxam. Plasma concentrations of CGA330050 are much higher in the mouse than the rat, and the mouse produces approximately 10-fold more CGA265307 than the rat after a single dose and up to 140-fold more after multiple doses.

In vitro comparisons have also been made of thiamethoxam's metabolism in mouse, rat and human liver fractions (Green, 2002). Rate constants (Km and Vmax) were determined for each of the metabolic steps shown in Figure 5. Figure 7 graphically represents the substantial difference between mice and rats or humans in the conversion rate of precursors (X-axis) to metabolites (Y-axis). The flux of thiamethoxam to CGA330050 and CGA265307 is 54-87 times greater in the mouse than the rat and 238-371 times greater than human microsomal preparations, as shown in Table 2. The high metabolic rates in the mouse correlate well with the CGA330050/CGA265307-related hepatic effects that lead to neoplasia.

Figure 7. Metabolism of Thiamethoxam in Rat, Mouse and Human Liver Microsomal Fractions

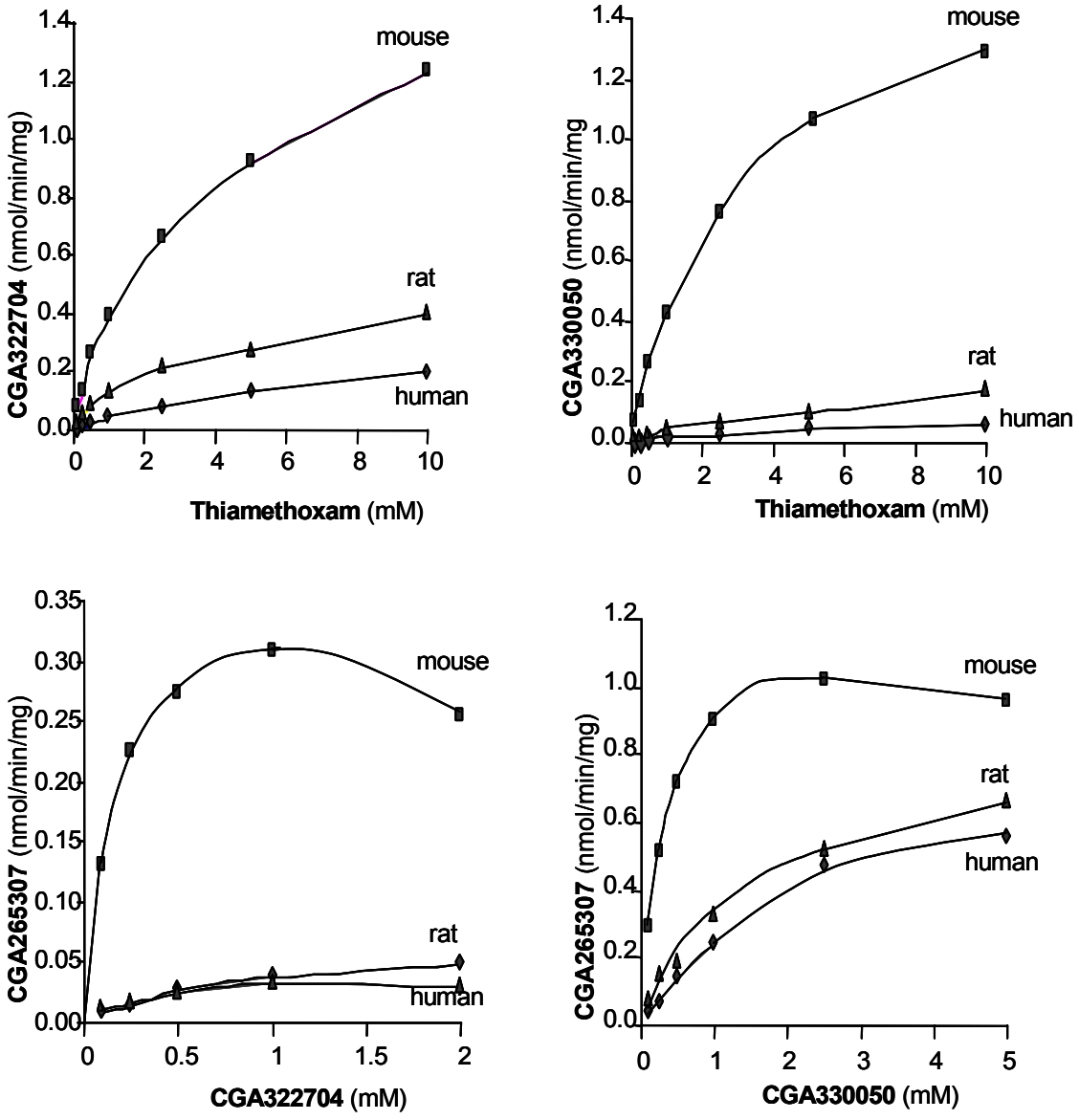


Table 2. Cross-Species Comparison of Thiamethoxam Metabolic Conversion Rates

Metabolic pathway	Relative rate – Mouse:Rat
Thiamethoxam to CGA265307 via CGA322704	54:1
Thiamethoxam to CGA265307 via CGA330050	87:1
	Relative rate – Mouse:Human
Thiamethoxam to CGA265307 via CGA322704	371:1
Thiamethoxam to CGA265307 via CGA330050	238:1

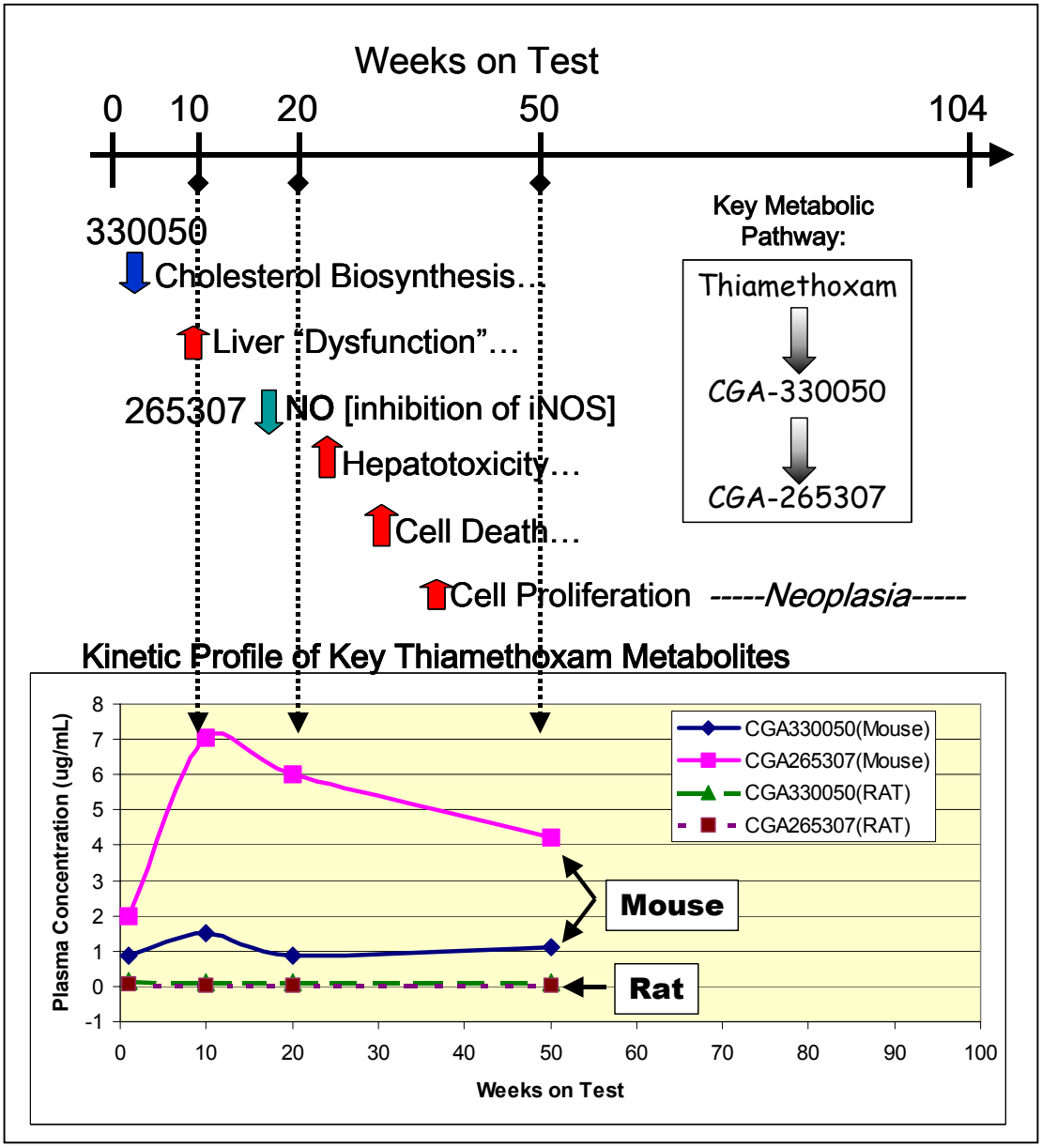
2.6 Summary Of Mode Of Action

A number of key events have been identified which are believed to lead to liver cancer in mice fed on diets containing thiamethoxam. These include a reduction in plasma cholesterol, cytotoxicity in the form of single cell necrosis and apoptosis, and an increase in cell replication rates. The changes occur in a dose-dependent manner and are seen only at the dose levels where cancer incidences were increased. They form a rational temporal sequence with biochemical changes leading to cell death followed by increased cell replication. These events are also highly dependent on the duration of dosing; cholesterol changes are seen within one week, cytotoxicity after 10 weeks of dosing and cell replication increases after 20 weeks. The changes are reproducible between studies and between strains of mice and the early changes are rapidly reversible when the test diets are removed.

None of these changes were seen in rats fed on diets containing thiamethoxam, nor were they seen in mice fed a diet containing CGA322704, a non-carcinogen. The key metabolite responsible for the hepatic changes in mice has been identified as CGA330050, a metabolite that is formed from thiamethoxam but not CGA322704. These hepatic changes are exacerbated by another important metabolite, CGA265307.

The correlation between key events and liver tumors is absolute within these studies. It is consistent with the species differences in carcinogenicity and internally consistent with the known metabolism of thiamethoxam. A clear mode of action has been identified in animals, which is presented graphically in Figure 8.

Figure 8. Hepatic and Metabolic Events Leading to Mouse Liver Tumors



3.0 EPA and ILSI criteria for the evaluation of carcinogenic responses in animal studies

This evaluation has been prepared according to EPA's draft final Guidelines for Cancer Risk Assessment (EPA, 2003) and according to methods laid out in the ILSI-RSI publication "A Framework for Human Relevance Analysis of Information on Carcinogenic Modes of Action" (ILSI, 2003). This methodology provides a decision-logic based approach to determining the relevance to humans of a compound-related increased cancer incidence in animal studies. Both the EPA and the ILSI-RSI documents rely on similar systematic thinking to arrive at a justifiable position.

The process used here and proposed by ILSI (2003), asks a series of questions that guides thinking concerning the significance of findings to human health. The first question is whether or not there are sufficient data to describe a mode of action that is comprised of demonstrable key events. The essence of a full, weight-of-evidence data evaluation relies on a systematic consideration of criteria that either strengthens or weakens a postulated mode of action and the underlying key events. The Hill criteria (Hill, 1965) for causation serve as a general guide. Epidemiologists have used these criteria to rigorously evaluate data, and the IPCS (International Programme on Chemical Safety) have recommended their use as a means to identify causality (IPCS, 2001). This rigorous process is fundamental to the decision as to whether or not a mode of action has been identified.

If there is sufficient evidence for a mode of action in animals, the next question is whether or not it is relevant to humans. To be relevant to humans, the key events that were identified in the test species should be concordant in humans. A concordance table represents this concept and analysis, where each key event is matched across species and directly to humans.

If concordance is established between the test species and humans, the next question is whether or not the mode of action is still relevant to humans when considering the dose levels and kinetic differences between test animals and humans.

The collection of answers to these questions then forms the basis for a weight-of-evidence decision as to the relevance of thiamethoxam's non-genotoxic, mouse-specific hepatic tumorigenesis.

3.1 Is the Weight of Evidence Sufficient to Establish a Mode of Action in Animals?

3.1.1 Specificity, Consistency and Strength

A part of the Hill criteria requires that, for the key events to be causally related to the formation of tumors, they must clearly be shown to be required steps that lead to the cancer and that the findings are reproducible.

The key events observed in mice fed on diets containing thiamethoxam occurred in a logical temporal sequence, in a dose dependent manner, and occurred only at the dose levels where tumor incidences were increased. The key events; hepatotoxicity leading to single cell necrosis, increased apoptosis and increased cell replication rates, are a series of events which are known to lead to cancer in the mouse liver (Goldsworthy *et al*., 1993; Butterworth and Eldridge, 1995). The findings were reproducible in two studies (of 20 and 50 weeks duration) and in two strains of mouse.

None of the key events were seen in rats in a comparable study of 50 weeks duration. CGA322704, a major metabolite of thiamethoxam, which is not a carcinogen, did not induce any of the key events in mice in a 20-week feeding study.

The major metabolites of thiamethoxam have been tested in dietary feeding studies, and CGA330050 was identified as the metabolite that induced changes in the liver comparable to those seen in mice fed on diets containing thiamethoxam. Neither CGA322704 nor CGA265307 had any effect on the mouse liver in 20-week feeding studies. CGA330050 is formed only from thiamethoxam and not from CGA322704. These studies are, therefore, consistent with the known pathways of thiamethoxam metabolism in the mouse. Comparisons of the metabolism of thiamethoxam in rats and mice found that markedly lower amounts of key metabolites CGA330050 and CGA265307 are formed in rats than in mice.

Thus, the metabolism of thiamethoxam in mice and rats is consistent with the observed mouse specific key events and the species difference in hepatocarcinogenicity.

3.1.2 Alternative Modes of Action

There is no plausible sequence of events for liver tumor 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. Therefore, it is highly unlikely that interaction of thiamethoxam with nicotinic acetylcholine receptors plays a role in the mechanism by which this compound causes liver tumors in mice.

Thiamethoxam is not genotoxic in bacteria, eukaryotic cells and mammalian systems. The potential for mouse-specific activation has been tested in bacteria using induced mouse liver S9 for metabolic activation and in mice *in vivo* using the mouse bone marrow micronucleus and liver UDS tests. None of these tests indicate a genotoxic response.

Thiamethoxam did not induce peroxisomal beta-oxidation in mice fed on diets containing thiamethoxam for 14 days and it is concluded, therefore, that thiamethoxam is not a peroxisome proliferator (Trendelenberg, 1998).

There was no evidence of oxidative stress in mice fed on diets containing 0, 2500 and 5000 ppm thiamethoxam for periods up to 50 weeks (Sagelsdorff, 2003).

Induction of xenobiotic-metabolising enzymes has been measured in the livers of mice fed on thiamethoxam diets and although statistically significant induction of several isoforms of cytochrome P-450 did occur, it was considered to be of insufficient magnitude alone to be causally related to the development of liver cancer (Trendelenberg, 1998). As an 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).

Thus the answer to the question "Is the Weight of Evidence Sufficient to Establish a Mode of Action in Animals?" is clearly Yes.

3.2 Is the Animal Mode of Action Plausible in Humans?

Given a clearly described mode of action in animals, which consists of a series of key events that occur in a species-, time-, dose-, and metabolite-dependent fashion, the next question is whether or not this mode of action could be operative in humans. In this step, the key events are matched across species to determine the degree of concordance.

Table 3 shows the initial key event is generation of the critical metabolites CGA330050 and CGA265307. Whereas all three species are clearly *qualitatively* capable of generating these metabolites, kinetic studies have shown that, *quantitatively*, neither rats nor humans would produce enough of these metabolites to begin the progression of hepatic key events.

Table 3. Concordance of key events across species

Key Event	Mouse	Rat	Human
Generation of critical metabolites: CGA330050 and CGA265307	Yes	Yes	Yes*
CGA330050-related liver "dysfunctional" changes: ↓cholesterol biosynthesis; ↓ protein synthesis; ↑P-450; & more…	Yes	No; (insufficient 330050)	No* (insufficient 330050)
CGA265307 inhibition of inducible nitric oxide synthase (iNOS)	Yes	No; (insufficient 265307)	No; (insufficient 265307)
Hepatotoxicity (clinical chemistry; histopathology; apoptosis)	Yes	No	*In vivo* data not available
Sustained increase in cell replication	Yes	No	*In vivo* data not available
Tumors	Yes	No	**Unlikely**

*Based on *in vitro* data

Thus, at this stage in the analysis, the question is whether or not the mode of action is still relevant to humans when considering the dose levels and kinetic differences between test animals and humans. The table shows that the progression of key events in rats and humans does not go beyond the simple generation of CGA330050 and CGA265307. Insufficient

amounts of these metabolites are generated to cause even the first stage of "Liver Dysfunction." Therefore, thiamethoxam is not expected to cause hepatic tumors in humans for the same reasons it does not cause tumors in rats in long-term studies. There is no plausible reason to believe that exposure to thiamethoxam would result in cancer in humans.

3.3 Are Young Animals More Sensitive than Adults?

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 has been 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 key event, within one week, seen in mice fed on diets containing thiamethoxam was a reduction in plasma cholesterol levels (Green 2003b). The correlation between reductions in plasma cholesterol, subsequent changes in liver histopathology and the incidences of liver cancer were absolute over a wide range of studies with thiamethoxam and its metabolites in two species (Green 2003b). Changes in plasma cholesterol can, therefore, be 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.

Groups of 6 male weanling mice (21 days old), and groups of 6 male adult mice (15-17 weeks old), were fed on diets containing 0, 500, 1250 and 2500 ppm thiamethoxam for 7 days (Green, 2003c). Plasma cholesterol was measured at the end of the study. Dietary intake was significantly higher in weanling mice than in adults, approximately double based on the concentrations of the major metabolites at the end of the study. 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. 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.

4.0 Discussion

4.1 Statement of Confidence for Mode of Action in the Mouse

These data confirm the previously demonstrated effects of thiamethoxam on the mouse liver in terms of histopathological changes, induction of single cell necrosis and apoptosis and increased cell proliferative activity at 500, 1250, 2500 and 5000 ppm. In addition, it has been found that thiamethoxam causes a marked reduction in plasma cholesterol levels. The dose response of the key events followed that of the tumor response and was sustained over the duration of all of the studies. The strong correlation between lipid (cholesterol) lowering drugs, both the fibrates and the statins, and the development of liver cancer in rodents (Green, 2003b) suggests that an effect on cholesterol biosynthesis is the earliest key event in the chain of events that lead to liver cancer in mice.

The temporal sequence of events is well defined, starting with perturbation of hepatic homeostasis (hepatocyte enlargement, liver enzyme induction, cholesterol depletion), followed by inflammatory cell infiltration, pigmentation, fatty change and hepatocellular necrosis/apoptosis, which results in compensatory cell proliferation followed by hepatocellular neoplasms observed after 18 months of treatment. These changes have been shown to be reproducible in a number of studies.

A comparison of the dose responses for cholesterol depletion, single cell necrosis, apoptosis and cell proliferation with that for the tumor incidences in mice show a very strong correlation between these precursor events and the tumor incidences (Figures 3 and 4). These changes are consistent with a known and accepted mode of action for the development of liver cancer and are believed to be prerequisites for the development of the liver tumors seen in the mice fed on diets containing thiamethoxam.

4.2 Implications for Human Health

None of the critical events that lead to liver tumors in mice occurred in rats fed on diets containing up to 3000 ppm thiamethoxam. The absence of hepatotoxicity in rats fed on diets containing thiamethoxam may be explained by quantitative differences in the metabolism of thiamethoxam between rats and mice. In humans, *in vitro* studies show that the key metabolites involved in the carcinogenicity of thiamethoxam, CGA330050 and CGA265307, are formed in significantly lower amounts in humans than in rats, the non-responding species (Figure 7). The overall rates for the metabolism of thiamethoxam to CGA265307 via CGA322704 and via CGA330050 (Table 2) are 370 and 240 fold lower, respectively, in humans than in mice (Green, 2002).

Additional factors to consider in evaluating the implications for human health include:

- Thiamethoxam is a non-genotoxic mouse liver specific carcinogen;
- The mouse is a particularly sensitive test species due to its high rates of metabolism and the high background incidence of liver tumors;
- High and sustained doses are required to induce a carcinogenic response with thiamethoxam;
- Tumor incidences were not significantly increased in mice in studies of less than 18 months duration;
- Increased cell replication, a critical event for the development of cancer with non genotoxic carcinogens, is not seen in thiamethoxam treated mice prior to 20 weeks;
- The earliest key event, reduced plasma cholesterol levels, and early liver dysfunction are readily reversible;
- Weanling mice are at least 2-fold less sensitive than adults to the earliest key event (reduced plasma cholesterol) in the mode of action of thiamethoxam.

Taking into account the marked species difference in metabolism and the factors considered above, it is concluded that the thiamethoxam-induced mouse liver tumors have no implications for human health. Furthermore, based on the results of a study in weanling

mice, it is concluded that infants and children would not be more susceptible than adults following exposure to thiamethoxam.

5.0 Conclusion

A plausible mode of action has been elucidated for the development of liver tumors in a mouse bioassay with thiamethoxam. It is concluded that the tumors are specific to the mouse and arise through a non-genotoxic mode of action characterized by a series of key events that include: perturbation of cholesterol biosynthesis, hepatotoxicity, cell death (both as single cell necrosis and apoptosis) and a sustained increase in cell replication rates. Neither the key events nor an increase in liver tumors are seen in rats fed on diets containing up to 3000 ppm thiamethoxam. The key metabolites, CGA330050 and CGA265307, responsible for the key events in the mouse are not formed in sufficient quantities in the rat and explain the lack of a carcinogenic response in this species.

In humans, *in vitro* studies show that the two key metabolites are formed in significantly lower amounts in humans than in rats, the non-responding species. Because humans would not generate sufficient amounts of the key metabolites, the combination of factors leading to the development of liver tumors in mice would <u>not</u> be present in humans. Therefore, it is concluded that humans would not be at risk of developing liver tumors as a result of exposure to thiamethoxam.

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