Case Study: Weight of Evidence Evaluation of the Human Health Relevance of Thiamethoxam-Related Mouse Liver Tumors

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Thiamethoxam (CGA293343; 3-(2-chloro-thiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene-N-nitroamine) was shown to increase the incidence of mouse liver tumors in an 18-month study; however, thiamethoxam was not hepatocarcinogenic in rats. Thiamethoxam is not genotoxic, and, given the late life generation of mouse liver tumors, suggests a time-related progression of key hepatic events that leads to the tumors. These key events were identified in a series of studies of up to 50 weeks that showed the time-dependent evolution of relatively mild liver dysfunction within 10 weeks of dosing, followed by frank signs of hepatotoxicity after 20 weeks, leading to cellular attrition and regenerative hyperplasia. A metabolite, CGA330050, was identified as generating the mild hepatic toxicity, and another metabolite, CGA265307, exacerbated the initial toxicity by inhibiting inducible nitric oxide synthase. This combination of metabolite-generated hepatotoxicity and increase in cell replication rates is postulated as the mode of action for thiamethoxam-related mouse liver tumors. The relevance of these mouse-specific tumors to human health was assessed by using the framework and decision logic developed by ILSI-RSI. The postulated mode of action was tested against the Hill criteria and found to fulfill the comprehensive requirements of strength, consistency, specificity, temporality, dose-response, and the collective criteria of being a plausible mode of action that fits with known and similar modes of action. Whereas the postulated mode of action could theoretically operate in human liver, quantitation of the key metabolites in vivo and in vitro showed that mice, but not rats or humans, generate sufficient amounts of these metabolites to initiate the hepatic toxicity and consequent tumors. Indeed, rats fed 3000 ppm thiamethoxam for a lifetime did not develop hepatotoxicity or tumors. In conclusion, the coherence and extent of the database clearly demonstrates the mode of action for mouse liver tumorigenesis and also allows for the conclusion that thiamethoxam does not pose a carcinogenic risk to humans.

Key Words: thiamethoxam; hepatocarcinogenesis; mode of action.

Thiamethoxam is a neonicotinoid insecticide that has been extensively tested in animal models for short- and long-term toxicological effects. An increased incidence of liver tumors was seen in male and female Tif:MAGf mice when fed in the diet for 18 months at concentrations up to 2500 ppm. In marked contrast, there were no increases in cancer incidences either in the liver, or at any other site, in rats fed on diets containing up to 3000 ppm thiamethoxam for two years. Furthermore, thiamethoxam was not genotoxic.

Previous articles in this series reported the development of a mode of action for the mouse-specific liver tumorigenesis (Green et al., 2005a), as well as an explanation for the species differences in response and metabolism between the rat and the mouse and the significance of these species differences to human health (Green et al., 2005b). Given that a well-defined mode of action can be described for the thiamethoxam-related mouse liver tumors, the purpose of this article is to describe the systematic evaluation of the relevance of these tumors to human health.

This evaluation has been prepared according to EPA's Guidelines for Cancer Risk Assessment (U.S. EPA, 1999) and according to methods laid out in the International Life Sciences/Risk Sciences Institute (ILSI-RSI) publication (Cohen et al., 2003, 2004; Meek et al., 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-RSI as a framework for determining the human relevance of rodent tumors, 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...
The often-cited “Hill criteria” (Hill, 1965) comprise a list of nine characteristics that help discriminate an association of events from causation. Whereas Hill’s hallmark criteria were directed towards epidemiology, their rigorous nature can be applied as well to the discrimination of cause and effect in molecular toxicology. Faced with well-conducted and reliable data, Hill states: “Our observations reveal an association between two variables, perfectly clear-cut and beyond what we would care to attribute to the play of chance. What aspects of that association should we especially consider before deciding that the most likely interpretation of it is causation?” (Hill, 1965, p. 295)

For the purpose of analyzing the causal relationship of the data that support the key events in the postulated mode of action for mouse liver tumors, the Hill criteria have been organized into groupings as follows:

Strength, consistency, and specificity. In this analysis, obvious and well-defined effects indicate strength, consistency by repetitive correlation, and specificity by the key events that are intimately related to particular outcomes.

In a 50-week mouse study with thiamethoxam, which was designed to examine the progression of hepatic effects, the earliest change, within one week, was a marked reduction (by up to 40%) in plasma cholesterol. This was followed 10 weeks later by evidence of liver toxicity, including increased single-cell necrosis and increased apoptosis. After 20 weeks there was a significant increase in hepatic cell replication rates. All of these changes persisted from the time they were first observed until the end of the study at 50 weeks. This progression of events was consistently seen in several studies of 10, 20, or 50 weeks duration, with the hallmark indicator being a substantial decrease in plasma cholesterol levels (Green et al., 2005a).

Likewise, obvious differences in metabolism between rats and mice suggest a reason why liver tumors were only seen in mice. Three key metabolites were identified and systematically evaluated for toxicological contribution to the sequence of hepatic effects (Fig. 2).

Mice and rats both produce CGA322704 as a major blood metabolite, which suggests that this particular metabolite is not an indicator of a species difference. However, CGA322704 does not cause liver tumors in mice (Federal Register, 2003) nor does it cause any of the hepatic changes seen with thiamethoxam and is thus considered not to be a part of causative chain of hepatic events. CGA265307 and CGA330050, on the other hand, are produced in substantially greater quantity by mice than by rats (up to 140-fold and 15-fold greater, respectively), suggesting that the metabolic pathway through CGA330050 is critical to the mode of action. In studies where these metabolites were fed to mice for at least ten weeks, CGA330050 was found to induce the same hepatic...

**Postulated Mode of Action**

A mode of action has been identified for the development of thiamethoxam-related mouse liver tumors in thiamethoxam-treated mice that includes metabolite CGA330050-induced hepatotoxicity, with exacerbation of this hepatotoxicity by metabolite CGA265307 via inhibition of inducible nitric oxide synthase, which is followed by cell death, both as necrosis and apoptosis, and increased cell replication (Green et al., 2005a). These changes are proposed as the steps that lead to the tumors seen at 18 months in mice. Figure 1 is a pictorial representation of the time-related series of metabolite formation, key hepatic events, and tumor formation.

The following series of three questions follows the decision logic summarized in the introduction to this article and described by Cohen et al. (2004).

**Question 1: Is the Weight of Evidence Sufficient to Establish the Mode of Action in Animals?**

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 to human health.

![Figure 1. Progression of key events in the formation of thiamethoxam-related mouse liver tumors.](https://example.com/figure1.png)
effects, and to the same degree, as thiamethoxam; however, CGA265307 alone induced none of the clinical or histopathological changes seen in the thiamethoxam-treated mice.

The role of CGA265307 was established by comparing its structural similarity to known inhibitors of inducible nitric oxide synthase (iNOS), by verifying the ability of CGA265307 to inhibit iNOS 

in vitro

, and by assessing the ability of CGA265307 to exacerbate the iNOS-dependent hepatic toxicity of carbon tetrachloride 

in vivo

. Based on structure-activity relationships and 

in vitro

 and 

in vivo

 experimentation, CGA265307’s role is thought to enhance the relatively mild hepatotoxicity induced by CGA330050, which leads to an increase in cellular death (via necrosis and apoptosis).

Differences in metabolism between mice and rats, the contributory role of specific metabolites, and the time-dependent progression of hepatic lesions were consistently seen in a series of separate studies, including two strains of mice. Furthermore, the changes noted in the mouse are not trivial, but are significant and sustained. Taken together, these studies strongly support the postulated mode of action.

**Temporality and dose-response.** Studies of 10, 20, and 50 weeks clearly delineated the time- and dose-related progression of hepatic lesions that includes early changes in indices of liver “dysfunction,” most notably decreases in cholesterol, followed (at 10 weeks) with a persistently increased incidence of cellular death (single cell necrosis and apoptosis), and sustained increase in cell replication rates when measured at the 20- to 50-week time points. An increased incidence of thiamethoxam-related tumors are then noted late in the 18-month mouse study, predominantly in animals killed at the end of treatment.

These studies depict a slowly evolving, time-dependent hepatotoxicity that ultimately results in cellular attrition and replacement. Most notably, this chain of time-dependent effects occurs in a dose-response relationship that parallels the dose-related, late-life occurrence of tumors in mouse livers (Green et al., 2005a), giving a no-effect level of 200 ppm in the mouse for tumor incidence as well as the hepatotoxic precursor key events. Therefore, the postulated mode of action fulfills the Hill criteria with regard to temporality and dose-response.

**Plausibility, coherence, experiment, and analogy.** Taken together, these criteria speak about the believable nature of the postulated mode of action. As stated by Hill, “...the cause-and-effect interpretation of our data should not seriously conflict with the generally known facts of the natural history and biology of the disease.” (Hill, 1965, p. 298). The mode of action postulated here follows the known pattern of cellular death and regeneration and is consistent with published examples of chemicals that generate a similar pattern (Butterworth and Eldridge, 1995; Goldsworthy et al., 1993). Non-genotoxic chemicals are known to promote the formation of tumors via various pathways that result in a higher rate of cell turnover, which results in clonal expansion of aberrant cells. Chloroform and phenobarbital are examples of hepatotoxic chemicals that result in cellular death and regeneration that leads to rodent liver tumors (Meek et al., 2003).

Thus, there is ample evidence in the literature to support a mode of action that incorporates regenerative hyperplasia as the fundamental step in tumor etiology. Thiamethoxam is distinguished by the time-dependent steps that lead to generalized regenerative hyperplasia, particularly the formation of sufficient amounts of two key metabolites (CGA330050 and CGA265307), leading to cellular death, regeneration, and tumor formation.

Alternative modes of action, including genotoxicity, cytochrome P-450 induction, peroxisomal beta oxidation, and oxidative stress were considered experimentally and shown not to be viable (Green et al., 2005a).

In summary, application of the “Hill criteria” to the postulated mode of action provides sufficient support to conclude that there is a causal relationship between the proposed “key events” in mouse liver and the formation of hepatic tumors in mice.

Thus, the answer to the question “Is the weight of evidence sufficient to establish a mode of action in animals?” is clearly yes.

**Question 2: Are the Key Events in the Animal Mode of Action Plausible in Humans?**

Given the clearly described mode of action for thiamethoxam-related tumors in mice, 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. Cellular attrition and regenerative hyperplasia is a mode of action that can
occur irrespective of species or, indeed, tissue. Thus, the simple conclusion is that humans could be susceptible to the thiamethoxam-related progression of key events should sufficient amounts of the relevant metabolites be generated. However, as will be described in the next section, sufficient amounts of the relevant metabolites would not be generated in humans, thereby precluding the likelihood of the key events and tumors occurring in humans. Nonetheless, this stage of the ILSI-RSI decision logic asks very simply for the qualitative possibility that the mode of action could occur in human tissue. The answer in this case is “yes,” which then leads to the critical next question that addresses the realistic plausibility of such a mode of action occurring in humans.

### Question 3: Taking into Account Kinetic and Dynamic Factors, Is the Animal Mode of Action Plausible in Humans?

This stage of the ILSI-RSI decision logic calls for a quantitative analysis to determine whether or not a mode of action, seen in animals, would plausibly occur in humans. Kinetic profiles and consequent species-specific responses will frame the relevance of a toxicological finding to human health. To that end, similar duration toxicity studies were conducted in rats and mice with the intention of comparing the degree of toxicological response in the rat versus the mouse and to fully characterize and quantitate the generation of critical metabolites associated with the key events. Furthermore, in vitro studies were conducted in mouse, rat, and human liver preparations to compare the quantity and rate of formation of the critical metabolites (Green et al., 2005b).

In similar duration toxicity studies in rats and mice fed thiamethoxam for up to 50 weeks, rats did not demonstrate any of the hepatotoxic key events noted very clearly in mouse studies. The lack of these key events in rats is consistent with the lack of hepatocarcinogenicity in this species. This obvious difference between species suggests either that rats and mice respond differently to thiamethoxam and its metabolites or that rats and mice differ significantly in the generation of the key metabolites, CGA330050 and CGA265307.

In a comparative metabolism study in rats and mice fed thiamethoxam for high dietary levels of thiamethoxam (3000 and 2500 ppm respectively), plasma concentrations of CGA330050 and CGA265307 were measured at 10-week intervals over 50 weeks of continuous feeding. Two fundamental quantitative conclusions can be drawn from this study: mice generated significantly more of these two critical metabolites than rats and the generation of CGA265307 increased with time in mice but decreased in rats. After prolonged dietary administration of thiamethoxam, the plasma levels of CGA330050 and CGA265307 were 15- and 140-fold greater, respectively, in the mouse than in the rat. Given the hepatotoxicological role of these two metabolites, this in vivo experiment provides an explanation for the lack of response in rats and thus the difference in hepatotoxicity between rats and mice.

The question then becomes whether or not human metabolism of thiamethoxam would be more similar to rats or mice. In vitro comparisons of metabolic rates were conducted by using liver fractions to measure the metabolic rate constants (Km and Vmax) of thiamethoxam’s conversion to either CGA322704 or CGA330050 and the conversion of either of these metabolites to CGA265307. Substantial differences were found that indicated a very low rate of thiamethoxam metabolism in humans. Overall, the intrinsic clearance (Vmax/Km) for the conversion of thiamethoxam to CGA265307 via CGA322704 was 371-fold lower in human liver fractions than in mouse liver and that via CGA330050 was 238-fold lower. Furthermore, human liver Km values were substantially higher (up to 21 mM) than in mice. Given that sustained high-dose feeding studies in mice generated thiamethoxam plasma levels in the range of 0.04–0.06 mM, sufficient levels of thiamethoxam could not be generated in humans to drive the metabolic pathways to any significant extent. Indeed, rats fed for a lifetime at a rate of 3000 ppm thiamethoxam in the diet did not develop tumors and a response in humans would not be expected because metabolic rates are lower than in the rat, and exposures via diet, environment or operator use are many orders of magnitude lower than 3000 ppm.

The concordance table recommended in the ILSI-RSI decision logic (Cohen et al., 2003; Meek et al., 2003) is a method whereby key events can be compared across species, with the ultimate comparison to humans. The concordance table for thiamethoxam (Table 1) shows that the initial key event is generation of the critical metabolites CGA330050 and CGA265307, with subsequent key events dependent on the

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**Table 1: Concordance Table for Hepatic Key Events**

<table>
<thead>
<tr>
<th>Key event</th>
<th>Mouse</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation of critical metabolites: CGA330050 and CGA265307</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes''</td>
</tr>
<tr>
<td>CGA330050-related liver “dysfunctional” changes: cholesterol biosynthesis and more…</td>
<td>Yes</td>
<td>No</td>
<td>(insufficient 330050)</td>
</tr>
<tr>
<td>CGA265307 inhibition of inducible nitric oxide synthase (iNOS)</td>
<td>Yes</td>
<td>No</td>
<td>(insufficient 265307)</td>
</tr>
<tr>
<td>Hepatotoxicity (clinical chemistry; histopathology; apoptosis)</td>
<td>Yes</td>
<td>No</td>
<td>In vivo data not available</td>
</tr>
<tr>
<td>Sustained increase in cell replication</td>
<td>Yes</td>
<td>No</td>
<td>In vivo data not available</td>
</tr>
<tr>
<td>Tumors</td>
<td>Yes</td>
<td>No</td>
<td>Highly unlikely</td>
</tr>
</tbody>
</table>

*Based on in vitro data.
formation of these key metabolites. 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. As a consequence, the clear conclusion can be drawn that humans would not be at risk of developing liver tumors as a result of exposure to thiamethoxam.

In conclusion, a robust mode of action for thiamethoxam-induced mouse liver tumors has been described. The postulated mode of action was tested against the Hill criteria and found to fulfill the comprehensive requirements of strength, consistency, specificity, temporality, dose-response, and the collective criteria of being a plausible mode of action that fits with known and similar modes of action. The coherent and extensive database demonstrates a clear depiction of the mode of action for mouse liver tumorigenesis and also allows for the conclusion that thiamethoxam does not pose a carcinogenic risk to humans.

**REFERENCES**


