Syngenta Position on Mode of Action and Human Relevance of Cyproconazole Induced Liver Tumours in the Mouse

INTRODUCTION:

Cyproconazole is currently not classified for carcinogenicity (Annex of EU Dir. 76/548 (26th ATP)) and no new data investigating carcinogenicity of cyproconazole in long-term bioassays are available. New data since the 26th ATP are limited to investigative studies to support a mode of action (MoA) for liver tumours in the mouse following exposure to cyproconazole as being via constitutive androstane receptor (CAR) and the non-relevance to humans of this established MoA. The new data are included in the CLH report (2014). Syngenta considers the data sufficient to demonstrate the MoA and non-relevance to humans and therefore, questions the proposal for category 2 H351 classification for carcinogenicity based on the following data for cyproconazole:

- Not genotoxic.
- Not carcinogenic in the rat.
- In the mouse, tumours were limited to the liver.
- The MoA for liver tumours has been demonstrated to be consistent with CAR activation in accordance with the internationally accepted framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI) (Sonich-Mullin et al., 2001, Meek *et al.,* 2003, Cowie, 2011).
- Human non-relevance has been confirmed via *in vitro* human hepatocyte studies demonstrating species differences in proliferation compared to the mouse (Elcombe, 2011).

However, the CLH report indicates concerns that the MoA could also involve cytotoxicity and this could be relevant to humans. Syngenta addresses the concerns of cytotoxicity as an alternative MoA in this document.

CYPROCONAZOLE INDUCES LIVER TUMORS IN THE MOUSE VIA A NON-GENOTOXIC CONSTITUTIVE ANDROSTANE RECEPTOR MEDIATED MECHANISM

A number of time- and dose-related key events have been identified that characterize the MoA that leads to mouse liver tumor formation in cyproconazole treated mice. The non-genotoxic MoA for induction of tumors in mice by cyproconazole is initiated via activation of CAR, leading to altered gene expression for CAR-responsive genes (Figure 1). Alteration of gene expression produces a variety of cellular responses, involving:

- Disruption of cell cycle control mechanisms, resulting in increased cell proliferation and suppression of apoptosis (altered transcript levels of *Gadd45β, Tsc22*).
- Induction of metabolising cytochrome P450 (CYP) isoenzymes, particularly *Cyp2b10, Cyp2a5* and also *Cyp3a11*.
- Alteration in liver biochemistry, specifically, cholesterol/bile acid homeostasis, producing a decrease in plasma cholesterol.

In the proposed MoA, the early gene expression and biochemical changes result in liver growth, hepatocyte hypertrophy and fat vacuolation and an increase in single-cell necrosis. A CAR-mediated transient stimulation of DNA synthesis and cell proliferation results in an environment of higher cell replication that can generate a higher rate of spontaneous mutations. Suppression of apoptosis provides an environment that would allow a mutated cell to clonally expand before it could be removed by normal apoptotic control processes. Following prolonged exposure, transformed cells progress to pre-neoplastic foci, and clonal expansion eventually leads to the development of liver tumors. An outline of the key and associative events in the non-genotoxic MoA is provided in Figure 1 below. The MoA and Human Relevance Framework (HRF) document (Cowie, 2011) provide the key data that are supportive of the MoA and human relevance.

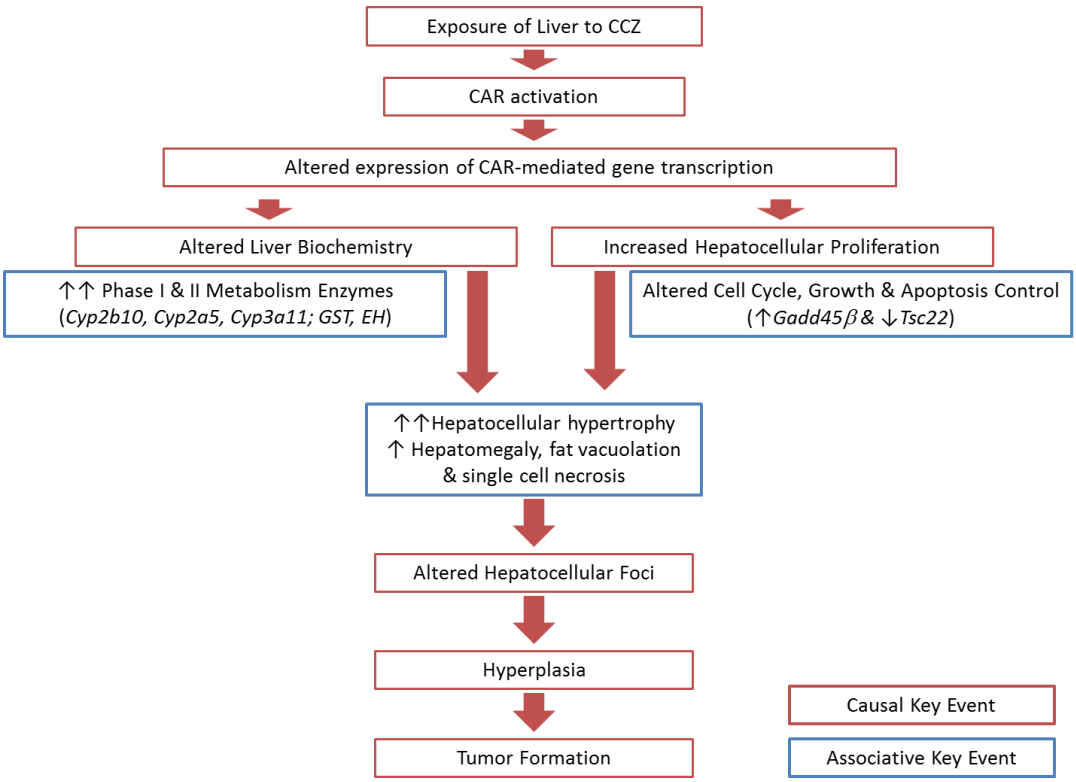

Figure 1: Key and associative events in the CAR-mediated non-genotoxic MoA of liver tumour formation in mice

Recent experiments published by Tamura et al. (2015), used the initiation-promotion model for carcinogenicity and investigated the effects of cyproconazole on hepatocarcinogenicity, at a dose of 200 ppm to wild type (WT) and CAR knockout (KO) male mice on C3H/HeNCrl background. No evidence of increased altered foci or adenoma formation was observed at 27 weeks in the KO animals confirming the crucial role of CAR in liver tumour development following cyproconazole exposure. In addition, the study by Tamura et al. (2015) is essential to the understanding of the MoA for cyproconazole-induced mouse liver tumours, because it demonstrates the absence of causal key events (which are seen in WT mice) when cyproconazole was tested in CAR KO mice. Specifically:

- No increase in alanine aminotransferase (ALT), a marker of liver cytotoxicity
- No increase in cell proliferation at 4 weeks or 13 weeks (by PCNA labeling index)
- No increase in eosinophilic or basophilic altered foci at 27 weeks
- No increase in adenomas at 27 weeks

A small increase in markers of the associative events was observed following 200 ppm cyproconazole treatment to CAR KO mice, compared to control CAR KO mice, including increased relative liver weight, increased hepatocellular hypertrophy, and increase in expression of certain genes such as Cyp2b10 and Cyp3a11. However, the authors of Tamura et al., (2015) rightly concluded that these changes in CAR KO mice treated with cyproconazole were associative events and not causal key events: "These results supported the currently hypothesized MOA that liver hypertrophy is an associative event involved in CAR-mediated liver tumor promotion in rodents (Elcombe et al. 2014)."

The small residual effects of 200 ppm cyproconazole on associative endpoints are postulated by Tamura et al. (2015) to be due to a minor role of activation of another nuclear receptor such as PXR. While the specific nuclear receptor that is involved has not been identified, it is noteworthy that CAR and PXR overlap in the specific CYP isoenzymes that they induce, including Cyp3a and Cyp2b subtypes. Also, phenobarbital, a prototypical CAR activator that produces mouse liver tumours, has been shown to be a weak activator of PXR as well (Lehmann et al., 1998). In an extensive review of the literature, Elcombe et al. (2014) have noted that there is a lack of data indicating that potent PXR activators have any potential to produce liver hepatocellular tumors in rodents. In summary, the initiation-promotion assay of Tamura et al. (2015) tested cyproconazole at the highest tumorigenic dose from the 2-year mouse study, and showed that the causal steps in its MoA for liver tumors are reliant on the activation of a functional CAR receptor.

Several modes of action have been identified for liver carcinogenesis, both in humans and in rodent models (Cohen, 2010, Table 1). Liver carcinogens can be divided into those that are DNA reactive versus those that are non–DNA reactive and both produce their carcinogenic effect by increasing cell proliferation. Some of the key events described for cyproconazole are common to other known modes-of-action. For example, increased hypertrophy and hepatomegaly are not specific surrogate markers for CAR activation because the induction of other CYP P450 isoforms or peroxisome proliferation can also produce these findings. However, these other MoA can be ruled out because the experimental evidence shows that cyproconazole treatment does not result in peroxisome proliferation. The reasons why alternative MoAs can be excluded for cyproconazole are listed in Table 1.

Table 1: Other MOAs

Alternative MoA	Reason for Exclusion
DNA reactivity	CCZ has been evaluated in a wide range of assays for genotoxicity, both in vivo & in vitro, including endpoints for gene mutation, chromosomal damage and DNA repair. Consideration of the full database against currently agreed guidelines for the interpretation of such assays indicates that CCZ is not genotoxic.
Peroxisome Proliferator	CCZ did not increase peroxisomal palmitoyl Co-A oxidase or Cyp4a protein levels and activities in microsomal mouse liver preparations.
Enzyme induction (AHR)	CCZ treatment results in minimal induction of Cyp1a isoenzyme protein level and functional activity in microsomal mouse liver preparations.
Statins	CCZ was not designed to inhibit HMG-CoA reductase, although CCZ treatment does result in reduced plasma cholesterol levels *in vivo* this effect is likely to due to CAR-mediated effects on lipid/cholesterol metabolism & transport.
Infectious	CCZ treatment did not produce signs of infection, cellular inflammatory response or regenerative proliferation.
Increased apoptosis	CCZ did not increase rates of apoptosis *in vivo* as determined by TUNEL staining in samples from three separate strains of mice.
Cytotoxicity	Detailed below

CCZ = cyproconazole

In the cyproconazole CLH report the following statement is cited in Section 4.10.5 Comparison with criteria:

'Overall, the evidence is considered to be limited, i.e., the data suggest a carcinogenic effect but are limited in the context of making a definitive evaluation because (1.) tumours are induced in mouse liver only; and (2.) tumours occur at doses toxic to the liver. The mechanism is not unequivocally demonstrated <u>and could involve cytotoxicity (relevant to humans)</u> and/or species specific CAR/PXR downstream events (with questionable relevance to man). Classification in Category 2 is proposed.'

Cytotoxicity and regenerative hyperplasia is another potential mechanism by which carcinogenesis can occur, however, this is unlikely to be the MoA for cyproconazole as described below.

CYTOTOXICITY AND REGENERATIVE HYPERPLASIA AS AN ALTERNATIVE LIVER TUMOUR MOA

The type of proliferative response the liver undergoes after xenobiotic exposure can be classified as either being mitogenic or cytotoxic (Butterworth *et al*, 1992) and the differences in molecular stimuli that drive both cytotoxicant and mitogenic proliferation in the liver has been

reviewed by Columbano and Shinozuka (1996). Hepatic mitogens generally produce a transient increase in hepatocyte proliferation and a sustained increase in liver weight for the duration of mitogen exposure. However, hepatocellular proliferation rates return to baseline levels once the liver reaches its 'new' increased size. Hepatic mitogens also provide an environment whereby initiated cells have a selective growth advantage, creating advantageous conditions for pre-neoplastic growth, which are commonly defined as foci of cellular alteration. In contrast, hepatic cytotoxicants, under conditions that produce substantial hepatocellular death induce extreme biochemical and pathophysiological alterations in the liver. These alterations, such as inflammatory responses including endonuclease release and oxygen radical generation, can result in DNA damage. A secondary response to cell death is the stimulation of cell division by induction of immediate early genes such as c-fos, c-jun and c-myc (Columbano and Shinozuka, 1996). The association between increased cell division and DNA damage means that DNA replication occurs with less than 100% fidelity and consequently, the spontaneous mutation rate increases and the affected cells progress to malignancy.

The widespread hepatocyte death induced by hepatic cytotoxicants such as chloroform or carbon tetrachloride are characterised biochemically by sustained elevated hepatic clinical chemistry parameters (ALT/AST) at an organ level by gross distortion of lobular shape, increased liver weight and pathologically as a multiple hepatocellular lesions with sustained diffuse necrosis and hepatocellular proliferation which, combined drives subsequent regenerative growth in a non-lobular manner. An outline and comparison of a model hepatic cytotoxicant and a hepatic mitogen (cyproconazole) in mouse liver is presented in Table 2:

Table 2: Comparison of the effects of a cytotoxic and hepatic mitogen in mouse liver.

	Carbon Tetrachloride (CCl_4)	**Cyproconazole**
Mechanism	**Cytotoxicity**	**Mitogen**
Cellular Response	Exposure of the liver to CCl_4 results in metabolism via a Cyp2E1 mediated peroxy free radial pathway, lipid peroxidation is followed by cellular membrane injury, enzymatic leakage, disruption of Ca^{2+} homeostasis and the induction of Ca^{2+} dependent degradative enzymes. Large elevations in markers of hepatic damage are characteristic of this response - which becomes cyclical as regenerated hepatocytes also die and release further degradative enzymes and markers of hepatic damage.	Exposure of the liver to cyproconazole results in activation of CAR leading to induction of Cytochrome P450 enzymes, proliferation of the smooth endoplasmic reticulum. Mild elevations of markers of hepatic damage characterise this response.
Organ Response	Sustained broad spectrum hepatic necrosis characterised by widespread multifocal hepatocyte death, activation of myofibroblasts, extracellular matrix deposition leading to fibrosis, cirrhosis, altered lobular architecture and ultimately a sustained proliferative response leading to regeneration.	Centrilobular hypertrophy, a transient burst of hepatocellular proliferation ultimately leading to foci of altered hepatocytes which does not affect the lobular architecture of the liver. Necrosis is limited to isolated single cells as a consequence of decreased apoptosis.
Long Term Outcome	Hepatocellular adenoma and carcinoma	Hepatocellular adenoma and carcinoma

Several differences between the cytotoxicant carbon tetrachloride and the mitogenic cyproconazole response warrant further discussion and analysis.

Markers of Hepatic Damage

Both carbon tetrachloride and cyproconazole induce pathological changes in the liver resulting in tumours; however, the characteristics of these changes are markedly different. In the liver, carbon tetrachloride induces broad spectrum, non-focal, hepatic necrosis characterised by widespread hepatocyte death. Biochemically, this is characterised by large increases in markers of hepatocellular injury.

Zimmerman (1998) has extensively described xenobiotic induced liver injury and provides a reference range for the expected biochemical ALT response to hepatocellular necrosis which is an 8-500 fold elevation versus control. This is well outside the maximum ~2.6-fold elevation range observed with cyproconazole treatment, supporting the exclusion of cytotoxicity as being a contributing factor in the cyproconazole induced mouse liver tumor response.

A comparison of biochemical markers of liver damage (ALT and AST) recorded following administration of carbon tetrachloride or cyproconazole to mice are summarised in Table 3.

Table 3: A comparison of the effects on liver enzymes in mice following exposure to a cytotoxic compound (CCl_4) and a mitogen (cyproconazole)

Endpoint	Chemical	Study duration					
		Single dose (CCl_4) / 3 day (CCZ)	7/ 8 day	14/15 day	28 day	8 week	90 day
ALT	CCl_4	↑ x244 (Wong et al., 1998)	ND	↑ x48-119 (Hayes et al., 1986)	ND	↑ x30 (Marek et al., 2005)	↑ x6 – 13 (Hayes et al., 1986
	Cyproconazole	No statistical change (Warren et al., 1995)	↑ x2.6 (Milburn, 2006a)	↑ ~x1.7 to 2.5 (Milburn, 2006a, Tamura et al 2013)	No statistical change (Warren et al., 1995) ↑ x1.6 (Tamura et al., 2015)	ND	↑ x2.4 not stats sig (Tamura et al., 2015)
AST	CCl_4	↑ x125 (Wong et al., 1998)	ND	↑ x9-20 (Hayes et al., 1986)	ND	ND	↑ x4 – 13 (Hayes et al., 1986
	Cyproconazole	No statistical change (Warren et al., 1995)	↑ x1.5 not stats sig (Milburn,, 2006a)	No change (Milburn, 2006a)	No statistical change (Warren et al., 1995)	ND	ND

ND – Not determined; CCZ - cyproconazole
Data presented for male CD1 mice.
Cyproconazole data only presented for 200 ppm dose.

The data clearly demonstrate marginal increases in ALT levels in short term studies (up to 14 days) with cyproconazole dosing and no statistical increase in AST, reflecting a gradual leakage of hepatocellular enzymes from the cytosol, rather than the marked increase seen in both enzymes along with widespread cell death following exposure to carbon tetrachloride at all timepoints to 90 days. When cyproconazole was administered to CAR KO mice, effects on enzymatic markers of liver damage (ALT) were reduced, with no increase compared to KO controls at the following timepoints - 7 days (Milburn, 2006b) and weeks 4, 13 and 27 (Tamaru et al., 2015), supporting the role of CAR in these liver changes. This demonstrates a clear difference between the response of a cytotoxicant and cyproconazole to the biochemical hepatocellular injury markers.

Hepatocellular Proliferative Response
In addition to differences in markers of hepatocellular damage, there are marked differences in the hepatocellular proliferative response between a cytotoxicant and a mitogen.

> **Cytotoxicant: sustained hepatocellular proliferative response**
> For example, carbon tetrachloride the proliferative response is still evident at the end of dosing in 90 day (Hayes et al., 1986) and 30 week (Fujii et al., 2010) studies.
>
> **Mitogen: transient early burst of hepatocellular proliferation**
> For example, cyproconazole, at the tumourogenic dose of 200 ppm, hepatocellular proliferation is elevated to a maximum of 1200% compared to controls from day 2 to 7 post dose initiation. After day 7 of dosing, the proliferation rate is equivalent to control levels to day 28 (longest time-point assessed) (Milburn, 2006a; Warren et al., 1995).

In addition, following cyproconazole administration to CAR KO mice, hepatocyte proliferation was not increased on day 7 of treatment at a tumourogenic dose of 200 ppm (Milburn, 2006b) indicating that CAR is essential for the proliferative response. The lack of any cell proliferation by PCNA staining at 4 weeks and 13 weeks of treatment with 200 ppm cyproconazole in a more recent study in CAR KO mice is further confirmation of the lack of response for key events in the absence of the CAR nuclear receptor (Tamura et al., 2015).

SUMMARY

A summary of the key events in a cytotoxicant induced liver response and its comparison to cyproconazole at dose levels used in the carcinogenicity mouse study is outlined below.

Key Event		**Carbon Tetrachloride (CCl_4)**	**Cyproconazole**
Hepatocellular Damage	Multi-focal necrosis	Yes – widespread cell death	No – isolated single cell death
	Biochemical markers	Yes – large, statistical increases	No – small, elevations
Sustained Hepatocellular Proliferation		Yes	No

The classification of Cyproconazole as Carc 2; H351 on the basis that the mechanism is not unequivocally demonstrated and could involve cytotoxicity (relevant to humans) is not appropriate due to the following:

- The histopathological changes observed in the mouse liver are inconsistent with those seen following dosing with cytotoxic agents known to produce tumours as a result of cell damage and regeneration
- the biochemical markers of hepatotoxicity observed with CYPROCONAZOLE are inconsistent with a cytotoxic response in the liver.
- The transient hepatocellular proliferative response observed with cyproconazole is inconsistent with the sustained response following a cytotoxic induced regenerative response in the liver.
- The single cell necrosis observed with cyproconazole is not sufficient to drive a regenerative hyperplastic response.
- A very recent study of 200 ppm cyproconazole treatment in an initiation-promotion study in WT and CAR KO mice demonstrated that the causal key events and the tumours are entirely dependent on the presence of a functional CAR nuclear receptor (Taumura et al., 2015).
- The hepatocellular proliferative response observed in the mouse does not occur in human hepatocytes and is therefore not relevant to man.

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