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CC/05/2

**COMMITTEE ON THE CARCINOGENICITY OF CHEMICALS IN FOOD,
CONSUMER PRODUCTS AND THE ENVIRONMENT**

**CARCINOGENESIS MODE OF ACTION & HUMAN RELEVANCE
FRAMEWORK**

1. The ILSI human relevance framework (HRF), describes how to use the mode of action (MOA) for a chemical established as a carcinogen in experimental animals to evaluate the human relevance of the animal tumours. This was a topic raised during the CoC 2004 Horizon scanning exercise. It was suggested that the Committee evaluate the applicability of this framework, with a view to building expertise within the Committee and government regulatory bodies.
2. The basis of the HRF framework was work initiated by the International Programme of Chemical Safety (IPCS) in 1994, following recommendations at the United Nations Conference on Environment and Development (UNCED) in 1992. The aim was to harmonize the various approaches currently used in Cancer Risk Assessment by establishing a conceptual framework for evaluating modes of action (MOA) of chemical carcinogens. The culmination of this initial exercise was reported in a paper by Sonich Mullin et al (2001), which details such a framework, based on the Bradford Hill criteria for causality. At this stage no guidance was issued on how to extrapolate these findings and conclusions to humans. It was anticipated that this would be the subject of a further exercise.
3. The COC reviewed the IPCS MOA framework at its meeting on 24th June 1999 and endorsed the approach proposed (COC/MIN/99/2). At that meeting, the Committee recommended that “the IPCS should be encouraged to carry out further work in exploring a framework to cover this area” (i.e. “the extrapolation of animal carcinogenicity to the assessment of potential hazards to humans.”)
4. The case studies undertaken to develop the MOA framework were:
 - Thiazopyr (thyroid tumours in animals; outlined in the paper)
 - Vinclozolin (hormonal disruption/leydig cell tumours)
 - Saccharin (rat bladder cancer)
 - Tamoxifen (liver tumours in rats, endometrial cancer in humans).
5. Since then, the initial framework developed by IPCS has been used as a foundation by a working group under the sponsorship of the US Environmental Protection Agency (EPA) and ILSI Risk Science Institute (RSI) to develop guidance on how to assess the relevance of an animal carcinogen to humans, based on MOA data.

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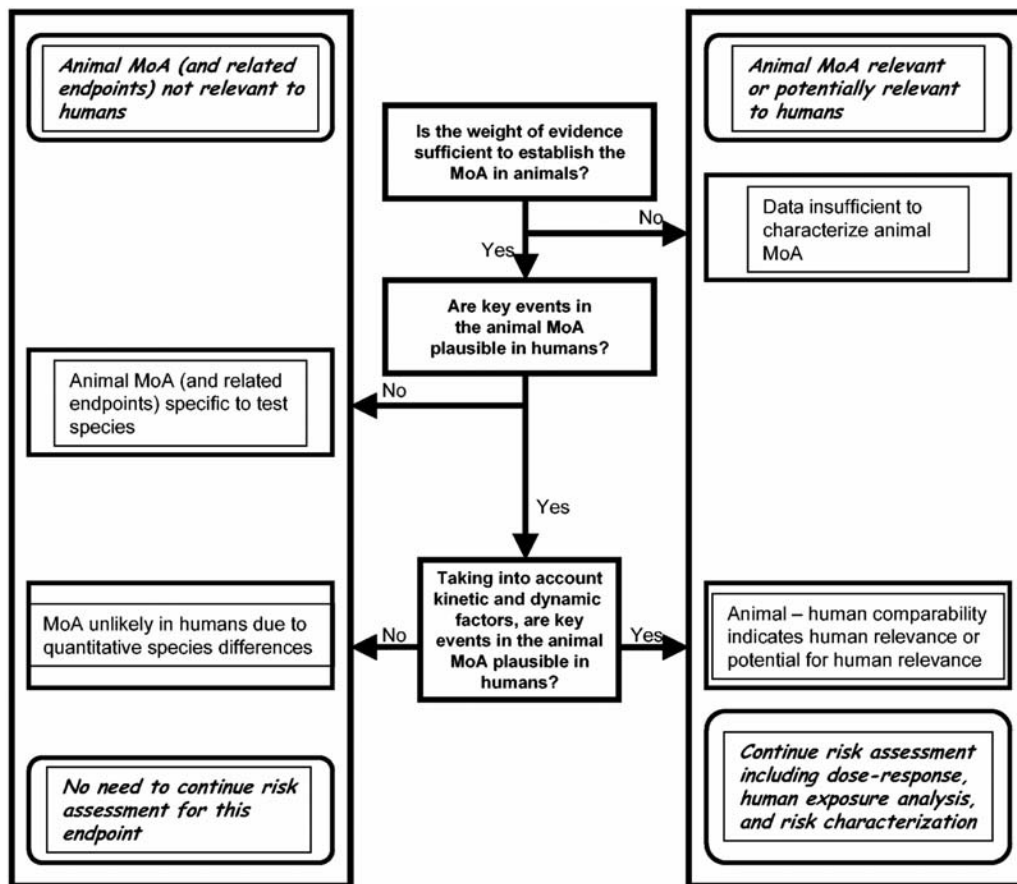
The basis of the HRF is the view that once an MOA has been established and agreed for the tumourigenic effects of a chemical in experimental animals, a systematic analysis of the individual key events comprising that MOA, both qualitatively and quantitatively, should enable the relevance of this MOA to humans to be determined. Hence, the resulting, expanded framework provides a defined procedure whereby assessment is rigorously structured, and provides clear and consistent documentation of the facts and reasoning that includes assessment of inconsistencies and uncertainties in the available data. Details of this process and seven case studies are provided in the appended paper by Meek et al (2003). It is noteworthy that the majority of chemicals which have been evaluated using this process act through non-genotoxic mechanisms. One direct acting genotoxic agent was evaluated by the ILSI group. However, the whole issue of genotoxic chemicals is currently being considered by a working party at the ILSI RSI. Such compounds are also being considered in detail by the IPCS Working Group on Carcinogens, who are currently preparing an extended HRF, which will take into account the work of the ILSI group
www.who.int/entity/ipcs/methods/harmonization/en/CancerWGMarch2004FinalReport.pdf

A summary of the Meek et al paper is provided at the end of this review (Appendix 1).

6. The HRF procedure is summarized as follows and in Figure 1:
 - 1) Is the weight of evidence sufficient to establish a MOA in animals?
 - postulated MOA, identification of key events, animal evidence, application of IPCS MOA guidance.
 - 2) Are the key events in the animal MOA plausible in humans (qualitative assessment)?
 - concordance analysis of animal and human responses, statement of confidence
 - 3) Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans (quantitative assessment)?
 - concordance analysis of animal and human responses, statement of confidence
 - 4) Conclusion: statement of confidence, analysis and implications.

Figure 1: General schematic illustrating human relevance framework and its relationship to human risk assessment.

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Attention should be drawn to a few points which may require clarification:

7. Mode of action can be defined as different from mechanism of action as follows: MOA is defined as a description of key events and processes starting with the interaction of an agent with a cell, through physiological and tissue/organ changes, resulting in tumour development. It is accepted that it is unlikely that complete knowledge of how an agent causes cancer will exist. Mechanism of action implies a more detailed, molecular description of events, which is likely to incorporate MOA in its definition (Wiltse & Dellarco 2000). Thus, whilst mechanism of action allows description of an MOA, the reverse is not true. This is illustrated by considering the carcinogenic peroxisome proliferators; PPAR α activation is a key event for the MOA, but the mechanism of action of tumour formation would involve the characterization of detailed molecular alterations and cellular transformation steps.

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8. The quantity and quality of the data used at each step of the process will be of paramount importance. It can be argued that the critical step is the third (...is the animal MOA plausible in humans?) as the answer to this question will often be 'don't know', unless there is a large amount of supporting human data. As a consequence it is recognised that many chemicals will not be able to clear the high hurdle of demonstrating 'hazard not relevant to humans'.

9. Close examination of the paper by Meek et al (2003; summarised below) reveals that possible variation may occur when different people examine data sets, despite the clear guidelines issued, as individual judgement may be required to evaluate weight of evidence, for example for genotoxicity. It should also be noted that HRF analysis contributes to the hazard identification step of the risk assessment process. In turn this feeds into a full risk assessment in which dose response and exposure data play a greater role.

10. Whilst chemical-specific data often form the basis of the concordance analysis in applying the HRF, this will not always be the case. This is because considerable emphasis is placed on the underlying biology of the processes involved. As an example, a key event in the renal tumours induced by d-limonene in the male rat is irreversible binding to alpha 2U-globulin. Knowledge that humans do not synthesise this protein is sufficient to exclude this key event from the effects of the chemical in humans, without necessarily demonstrating that indeed d-limonene and its metabolites do not bind to a protein in human plasma.

11. It is important to recognise that even if the relevance of an MOA to humans cannot be dismissed, the structured application of the framework should provide a robust basis for risk characterisation, for example by establishing the biological basis of a threshold dose for carcinogenicity and by identifying key events, the evaluation of which should improve the risk assessment.

12. An extremely detailed evaluation of the human relevance of PPAR α - agonist induced rodent tumours was undertaken as part of the same process as described by Meek (Klaunig et al 2003). The thoroughness of this review is evident, and likely to have been time consuming, although it is recognised that this is because of the sheer volume of data generated in this area. However, importantly it is proposed that such an exercise should be required only once for a given MOA. Future assessments of chemicals which share the same MOA and which do not demonstrate additional key events contributing towards other possible MOAs, would require progressively less data, at least for the first half dozen or so compounds. However, the continual clarification of modes of action by filling data gaps is recognised.

13. The process has already been applied by the HSE and the PSD in their chemical evaluations. Three worked examples are presented in Appendix 2 .

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14. In summary, the generation of the guidelines has been an extremely thorough process. It's general applicability is constantly being evaluated and built upon.

Questions for the Committee:

- What are the views of the Committee on the approach outlined in the published HRF
- Under what circumstances would application of the HRF be pertinent?
- Would the Committee wish to use this approach in future if deemed applicable?
- Would the Committee endorse the development of expertise and procedures to perform such evaluations within relevant Government agencies and department?
- Does the Committee wish to be updated on developments in this field, such as the ongoing IPCS activity?

DH Toxicology Unit
April 2005

References

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Appendix 1 to CC/05/2

This appendix is a very brief overview of the paper by Meek et al (2003); although an attempt has been made to include all pertinent information, certain elements may have been omitted or abridged.

Human relevance framework: components and applicability

Background

No matter how well-defined and fully analysed, a data set derived entirely from animal studies will not permit a definitive conclusion on the human relevance to be determined. That this assessment contributes to the hazard identification element of the risk assessment process is noted.

1. Weight of evidence in Animals: The demonstration of a MOA in animals and the adequacy of this data set will form the corner stone of the evaluation. It is acknowledged that once a particular MOA has been defined for one chemical, application to subsequent chemicals demonstrating the same MOA will be significantly reduced. Where data sets inadequately delineate the MOA (case study acrylonitrile), it is likely that an assessor must assume that there is a potential carcinogenic risk in man. In these cases, a full risk assessment for the endpoint is required.

2. Are key events in the animal MOA plausible in humans? Essentially, this encompasses a qualitative assessment of the relevance to human cancer, the default being that the animal tumours will be relevant. Human data corresponding to that used in the animal MOA assessment are evaluated, and can include pertinent generic data from evaluation of different chemicals (with same MOA) as well as that from the chemical under study. This includes (but is not limited to), cancer incidence at the anatomical site, an understanding of the nature of the target site (physiological, biochemical, histological) and human vs animal responses to the chemical (or class of chemicals). Case studies limonene/atrazine).

3. Taking into account kinetic and dynamic factors, is the animal MOA plausible in man? This is considered to be the quantitative analysis of the applicability of the animal MOA. Factors to be taken into account include biotransformation, toxicokinetics and toxicodynamics and how these impact on relative susceptibility of humans to animals. This will often result in establishing numerical differences/similarities in responsiveness; case studies, chloroform, phenobarbitone (thyroid).

4. Statement of confidence, analysis and implications

This should address issues such as quality and quantity of data

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CASE STUDIES:

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Ethylene oxide : MOA =A direct acting alkylating agent (demonstration of the process for genotoxic carcinogens)

- 1 Weight of evidence - In bioassays, induced tumours include; site of injection tumours in female NMRI mice; lung, Harderian gland, uterus, mammary gland tumours and malignant lymphomas in B6C3F1 mice after inhalation exposure; brain gliomas, mononuclear cell leukaemia and peritoneal mesotheliomas in F344 rats after inhalation exposure and forestomach tumours in female Sprague Dawley rats after oral administration. The evidence for a genotoxicity, specifically DNA alkylation, is robust. This includes the formation of DNA adducts, *in vivo* mutations at the hgprt locus and in lacI transgenic mice, and *in vivo* SCE's and chromosomal aberrations. Confidence in the DNA alkylation MOA is high.
- 2 Plausibility of key events in humans - There is evidence of an increase in tumours in man (lymphatic and haematopoietic) following occupational exposure to ethylene oxide. Additionally, alterations in other ethylene-oxide induced genotoxic endpoints have been demonstrated (chromosomal aberrations, micronuclei, hprt mutations, SCE's). Together there is strong evidence that the postulated mode of action in animals is applicable to humans.
- 3 Evaluation of the kinetic and dynamic factors using a physiologically-based pharmacokinetic model indicates qualitatively similar mechanisms (hydrolysis, glutathione conjugation), and quantitative differences of biomarkers of exposure are accounted for by differences in basic physiology rather than MOA.
- 4 Statement of confidence and conclusion- there are adequate data to conclude that the DNA alkylation MOA is applicable in humans.

Acrylonitrile: MOA = metabolically generated active oxygen species causing brain tumours (e.g data inadequate to support hypothesis).

1. Weight of evidence for animal MOA - In bioassays, induced tumours include; brain and nervous system (e.g. astrocytomas), GI tract and mammary gland. Acrylonitrile is metabolised via two principle routes, CYP2E1 oxidation and glutathione conjugation and, the former considered to be the activation pathway. The hypothesized MOA of brain tumour formation is via the generation of reactive oxygen species. This may be associated with oxidation to 2-cyanoethylene oxide and the generation of cyanide. Examining the evidence of key events in animals - Markers of oxidative stress *in vitro* and *in vivo* have been reported following exposure to acrylonitrile. For example, elevated levels of 8-oxodeoxyguanosine adducts in brain, activation of NF- $\kappa$ B and GSH depletion. However, these endpoints do not correlate precisely with results of carcinogenicity bioassays. There is some evidence of direct interaction with DNA; acrylonitrile is weakly mutagenic in bacterial systems and generates 7-(2-oxoethyl)-guanine adducts. However, the statement of confidence observes that, overall, patterns of results demonstrating oxidative damage are often in conflict with those derived from cancer bioassays (e.g relative sensitivities of different rat

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strains do not concord) and thus the weight of evidence for the hypothesised MOA is considered inadequate

2. and 3 : With regards to the key events and kinetic and dynamic factors, the evaluation of the plausibility of the MOA in humans is deemed unnecessary due to the weakness of the MOA in animals.
- 4 Statement of confidence - Although there are negative epidemiological investigations, they are not considered to contribute to the risk assessment in this instance. Overall, the lack of a demonstrable MOA in animals means that further evaluation within the HRF is not feasible.

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d-Limonene –  $\alpha$ 2u-globulin MOA = chemically-induced species and sex specific events.

- 1 Weight of evidence for animal MOA –A significant increase in renal tubular tumours is observed in male F344 rats following lifetime administration. The postulated MOA is that sustained renal cell proliferation, a consequence of necrosis and compensatory proliferation, leads to tumour formation following irreversible binding of a d-Limonene metabolite to  $\alpha$ 2u globulin. There is no evidence of the preliminary renal effects (acute nephropathy) or tumours in females or a strain of rat lacking  $\alpha$ 2u-globulin (NBR rat), providing credence to this hypothesis.
- 2 Are key events plausible in man? The limonene metabolite, which binds to  $\alpha$ 2u globulin is produced in man. However, there is convincing evidence that the protein does not occur in man, nor is there any evidence that the metabolite can bind to structurally similar proteins inducing analogous events.
- 3 With regards to the key events and kinetic and dynamic factors: Not relevant due to the fact that MOA is not plausible in humans.
- 4 Statement of confidence: The list of criteria (data rich determined) which indicate that the MOA is closely defined (binding to protein is key event, protein absent in man), and therefore not relevant to man.

Atrazine – (herbicide) MOA= inhibition of ovulatory LH surge in rat mammary tumours.

1. Weight of evidence for animal MOA - Atrazine causes an earlier onset, and overall augmentation of the spontaneous incidence of mammary tumours in female SD rats. Males, mice and other strains of rat are unaffected. The postulated MOA is that atrazine causes a persistent secretion of estrogens and prolactin which leads to the suppression of the lutenising hormone (LH) surge and ovulation suppression. Consequently mammary glands are hyperstimulated by estrogens arising from follicles, and eventually proliferation and hormonally mediated mammary tumours result. Atrazine is devoid of genotoxic activity. A threshold mechanism is implicated; ‘either the atrazine is sufficient to block ovulation or it is not’. In F344 reproductive senescence is different from that seen in the SD rat and thus the same MOA is not observed.
2. Are key events plausible in man? The estrous cycle is fundamentally different in women compared to rodents; the LH surge is driven by ovarian estrogen

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compared to norepinephrine pathways in rodents. Additionally, reproductive aging in rats is due to the inability of norepinephrine to maintain the LH surge and ovulation, whereas in humans is characterised by the exhaustion of ovarian follicles and consequently estrogen. Some epidemiological studies provide evidence that atrazine is not associated with an increase in cancer in humans.

3. With regards to the key events and kinetic and dynamic factors: Not relevant due to the fact that MOA is not plausible in humans.
4. Statement of confidence: It is considered that there is a detailed and comprehensive evaluation of the MOA underlying the tumours in rats indicating LH suppression and estrous cycle disruption, that the mechanism is threshold-based and not applicable to humans due to significant differences in ovulation and endocrinological aging.

### Phenobarbital – MOA thyroid tumours associated with increased hepatic clearance of thyroxin

1. Weight of evidence to establish MOA in animals – the hypothesis is based upon disruption of thyroid-pituitary axis by the induction of hepatic metabolizing enzymes, including UDP-glucuronyltransferase. This increases the conjugation and subsequent clearance of the thyroid hormones T4 and T3. TSH synthesis is stimulated as feedback to the hypothyroid state which in turn leads to follicular stimulation and hyperplasia. Tumours are seen in rats only at doses greater than the ED50 for microsomal enzyme induction (~1 mg/kg/day) and there is evidence that a critical reduction in T4 is needed before an increase in TSH is seen (no absolute figures provided in this review). Compensatory thyroid enlargement is able to bring TSH and T4 levels back to normal but is accompanied by sustained increases in organ activity and hyperplasia. One uncertainty is that not all agents which increase thyroid hormone clearance cause concomitant TSH increases eg 3MC, PCB's. It is not known whether they promote thyroid tumours. However for PB, the evidence for the hypothesised MOA is strong.
2. Are the key events in man plausible? – thyroid –pituitary homeostasis is thought to be similar across species. Thus sustained alterations in T3/T4 also lead to goiter (e.g iodine deficiency). Also evidence that TSH-induced growth is critical to development of thyroid tumours. Thus MOA could be relevant to man .
3. With regards to the key events and kinetic and dynamic factors – The potential for PB to alter thyroid homeostasis in man has been studied. At 100mg/kg/day reduced T4 was seen but no effects on TSH noted. The importance of the dose response relationship is demonstrated, especially with reference to attaining a critical decreases in the level of thyroid hormone. In man differences are associated with a) the half life of T4; 12h in rats, 5-9 days in man; b) increased turnover /clearance of T3+T4 means that the thyroid is more active in rats, and required 10x more T4 than in man and c) TSH levels are 25x higher in rats than humans. These points indicate the particular sensitivity of rats to alterations in the thyroid-pituitary axis. There is also epidemiological evidence that long term

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treatment of patients with PB is not associated with an increased risk of thyroid tumours.

4. Statement of confidence and implications: There is convincing quantitative evidence that the process in animals (particularly the sensitive rat) is not directly applicable to man. Clinical doses, which cause enzyme induction are insufficient to alter thyroid hormone status.

## Chloroform: MOA= sustained cytotoxicity and cellular regeneration

1. Weight of evidence to establish the MOA in animals? The hypothesis for the mode of action is based upon the generation of phosgene by CYP2E1, which binds irreversibly to tissue macromolecules, leading to sustained cytotoxicity, and resultant persistent regenerative hyperplasia. This appears to be in contrast to induced cell proliferation in the absence of necrosis. The extent of chloroform-induced necrosis directly correlates with the degree of covalent binding (in male and female rats and male mice), its histological locality and is dependent on the generation of metabolites (specifically phosgene). Furthermore, depletion of glutathione, binding to which provides a degree of protection, results in increased covalent binding. Regional distribution of lesions linked to CYP2E1 location. However, there is a lack of in vitro metabolism data, in particular the generation of putative reactive species by CYP2E1 in different species.

*Renal tumours, mice-* the covalent binding /renal tubule necrosis correlation is similarly observed with strain and sex-related differences in sensitivity,

*Liver tumours: mice...* dependent on administration of bolus doses, and induction of sustained proliferation (neither seen when administered via drinking water).

Studies in CYP2E1 null mice demonstrated no cytotoxicity or proliferation, thus strengthening the hypothesis for CYP2E1 generated reactive species.

*Renal tumours rat:* Weight of evidence is less than that observed in mice. Less data generated generally (study in Osborne Mendel rats produced tumours but with no supporting proliferation/metabolite type data. Sustained proliferation seen in a short term study, in F344 after gavage but not drinking water administration, but there are no bioassays in this strain).

It is concluded that the weight of evidence for the proposed MOA in animals is considerable.

2. Are key events plausible in humans? Acute toxicities in humans, as a consequence of occupational or medical exposure, manifest in the same target organs as observed in rodents. Evidence of proliferation or carcinogenicity is not available. However some key events, irreversible binding to liver macromolecules requires prior metabolism, are applicable.
3. With regards to the key events and kinetic and dynamic factors: Species differences in the rate of phosgene generation is likely. Based on models, quantitative variations were consistent with species variations in metabolic rate (15-fold greater in rats than in humans).

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4. Statement of confidence: High degree of confidence for the weight of evidence for MOA in animals. As the end-points in animals are histological (rather than traditional biomarkers), clear evidence of the events occurring in man is not similarly robust. A PK model, suggests that quantitative differences between man and animals as regards exposure /metabolism exist, which would affect relative sensitivities. However strong qualitative similarities are evident. Dose response relationships, are likely to be important. No definitive conclusions drawn.

### Melamine – MOA – urinary bladder tumours associated with urinary tract calculi

1. Weight of evidence for animal MOA – dietary administration results in bladder tumours in male F344 rats but not in females or in B6C3F1 mice. Tumours observed together with calculi. Much higher dose were needed to cause calculi in female rats. Calculi observed in male mice in absence of tumours. Correlation between calculi, ulceration, hyperplasia and tumours, although not 100%, is strong and the correlation between calculi and tumours is considerable. Data from other chemicals which cause bladder tumours (eg uracil) support this temporal relationship. This is considered to be a high dose phenomenon.
2. Are key events plausible in humans: there is no information on human toxicity. If a comparable MOA is assumed in humans as in animals a high enough dose for melamine to precipitate in the urine would be required. Urothelium changes are seen in humans and calculi-associated tumours of the urothelium are also evident, although it is believed that calculi do not remain in the human bladder for long periods of time. However , qualitatively the key events of the animal MOA are applicable to humans.
3. With regards to the key events and kinetic and dynamic factors: It is not clear whether calculi alone are a carcinogenic risk or whether other contributing factors are needed. Due to the more rapid clearance of calculi in man the risk is seen to be less than in rodents. Risk is totally dependent on calculi formation which in turn is dependent on exposure.
4. Statement of confidence: Precipitation as calculi will be influenced by other factors such as osmolality, protein and citrate. Cytotoxic lesions will be dependent on size , number and coarseness of calculi. MOA in rodents is convincing , humans appear less susceptible and MOA is likely to be a high dose phenomenon only.

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Appendix 2 to CC/05/2

## Examples worked by PSD

XX

### Chronic toxicity and carcinogenicity

Hepatic and testicular toxicity was also apparent in chronic studies in the rat and mouse. In the rat, liver toxicity was characterised grossly by hepatomegaly, nodules and coloured foci. Histopathologically, findings were characterised by bile stasis and bile duct hyperplasia, hepatocellular hypertrophy and hyperplasia, inflammation and necrosis. Clinical chemistry revealed a perturbation of lipid metabolism: findings are consistent with the known herbicidal mode of action (inhibition of acetyl coenzyme A carboxylase). The incidences of hepatocellular adenoma and carcinoma were raised in both sexes at dose levels of  $\geq 750$  ppm; findings were also apparent at 1500 ppm at the interim sacrifice. Electron microscopy indicated hepatic peroxisome proliferation at  $\geq 750$  ppm. In the mouse, liver toxicity was characterised by hepatomegaly with hepatocellular hypertrophy, Kupffer cell hyperplasia and hepatic pigmentation. Incidences of hepatocellular adenoma and carcinoma were increased in females at 250 ppm. Increased incidences of renal squamous cell carcinoma were seen in both sexes at 1500 ppm.

Testicular toxicity was characterised at 12 months in the rat by decreased testis weights, interstitial hyperplasia, tubule degeneration and epididymal aspermia. At 24 months, testis weights were increased and the incidence of Leydig cell tumours was increased at dose levels of  $\geq 750$  ppm. In the mouse, evidence of testicular toxicity was limited to sperm degeneration and Leydig cell tumours at the top dose level of 250 ppm.

### Relevance of Leydig cell tumours

Leydig (interstitial) cells are the major site for the synthesis of testosterone. Testosterone production is controlled by a number of factors including the major effector, luteinising hormone (LH), secreted by the pituitary. Testosterone acts as a paracrine hormone in supporting tubular spermatogenesis. Leydig cell adenomas occur with relatively high frequency in CD rats (5.3%) and with a much lower frequency in man (~1% of testicular tumours and ~0.01% of all tumours). The incidence of Leydig cell adenomas in rats is also known to be frequently increased by the administration of xenobiotics. A number of physiological differences between rat and man are thought to account for the species differences:

- In man, sex hormone binding globulin (SHBG) is produced by the liver and binds to approximately 95% of testosterone in peripheral blood. SHBG is not produced in rats, consequently the rat testis is more susceptible to xenobiotic-induced disruption of testosterone levels.

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- Raised levels of circulating LH in the ageing rat are also thought to be a major causative factor in the induction of Leydig cell adenomas.
- Human Leydig cells contain about 1500 LH receptors, whereas rat Leydig cells contain approximately 20000 receptors. Rat Leydig cells are therefore more responsive to LH.

The majority of testicular toxicants produce non-specific histopathology consisting of tubular atrophy with depletion of germ cells. Spermatogonia and early spermatocytes may be present in some cases; cells are continuously differentiating and dying on reaching the stage sensitive to the toxic effect. Germ cell depletion is frequently accompanied by Leydig cell hyperplasia, reflecting a hormonal response to disturbed spermatogenesis: impaired testosterone secretion induces the secretion of LH by the pituitary.

In the case of XX, clear evidence of direct testicular toxicity was noted in studies in the rat, mouse and dog. Findings were characterised grossly by reduced organ weight, 'soft' testes and coloured foci. Histopathology revealed tubular necrosis, atrophy and/or degeneration associated with aspermatogenesis or hypospermatogenesis and epididymal cellular debris. Interstitial cell hyperplasia was also noted in the rat following chronic administration. Evidence of increased LH secretion was seen histopathologically as the presence of 'castration cells' in the anterior pituitary in the rat 90-day and multi-generation studies. Findings in the multi-generation study were also associated with reduced fertility at overtly toxic dose levels.

The available evidence therefore indicates that the increased incidence of Leydig cell tumours seen in the rat and mouse chronic studies is associated with chronic testicular toxicity. Toxicity results in reduced testosterone secretion with the consequent stimulation of Leydig cell proliferation by increased LH production by the pituitary. The progression from testicular toxicity to tumorigenesis can be clearly seen in the chronic rat study, where mean testis weights at affected dose levels are significantly decreased at the interim sacrifice but are significantly increased at the terminal sacrifice.

A clear threshold was demonstrated for Leydig cell tumorigenesis; this finding is additionally considered unlikely to be of relevance to the human risk assessment.

### Relevance of liver tumours

A number of structurally diverse chemicals have been shown to produce liver enlargement, peroxisome proliferation and the induction of peroxisomal enzyme activities in rats and mice; some peroxisome proliferators have also been shown to increase the incidence of liver tumours in these species. Some phenoxypropionic acid herbicides (haloxyfop and propaquizafop) are known to be peroxisome proliferators, however fluzafop-butyl and quizalofop-ethyl have not been shown to be peroxisome proliferators.

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Proposed mechanisms of liver tumour formation by peroxisome proliferators include the induction of sustained oxidative stress, enhanced cell replication, inhibition of apoptosis, the promotion of spontaneous mutations or a combination of these processes. Rodent peroxisome proliferators are not considered to be genotoxic agents and thresholds for carcinogenesis have been demonstrated. Xenobiotic-induced peroxisome proliferation is not generally seen in humans, however marginal effects have been reported for some fibrate hypolipidaemic agents. It is therefore considered that peroxisome proliferators do not present a hepatocarcinogenic hazard in humans at low levels of exposure.

In the specific case of XX, liver tumorigenicity in the rat is associated with chronic liver enlargement and hepatotoxicity; evidence of peroxisome proliferation was also seen in the rat. A clear threshold was demonstrated for liver tumorigenesis; this finding is additionally considered unlikely to be of relevance to the human risk assessment. Peroxisome-proliferating compounds are also known to increase the incidence of Leydig cell tumours, however the mechanism for this is unclear as compounds do not induce peroxisome proliferation in the testis.

### Relevance of renal tumours

XX was shown to induce renal squamous cell carcinoma in top dose animals of both sexes in the chronic rat study. This is an unusual tumour type that was not observed in control rats from either the performing laboratory (3 studies initiated in 1990-94) or the breeder/supplier (76 studies performed in a number of laboratories during 1977-1997).

Renal squamous cell carcinoma is a relatively rare tumour of the renal pelvis urothelium that is generally associated with chronic irritation and metaplasia induced by calculi or infection. Evidence of renal and bladder urothelial irritation was noted in female rats at the top dose level in the chronic study; a slight increase in renal urothelial irritation was also noted in top dose males. Urinary erythrocytes were also noted with increased severity and/or frequency at later time points in both sexes at higher dose levels, however there was no consistent association with renal calculi. Further evidence for high dose level renal toxicity is limited, however increased kidney weights were seen in the 90-day mouse study; nephrosis was also noted in the 1-year dog study. In the 90-day dog study, skin irritation/inflammation consistent with urine contamination was noted; urinalysis in this study also revealed lower urine pH. Dilation of the renal pelvis was reported in parental animals in the rat multi-generation study.

Although equivocal results were reported for a number of *in vitro* studies, the results of a mouse micronucleus study indicate that XX is not genotoxic *in vivo*. Renal tumorigenesis as a result of a genotoxic effect is therefore considered to be unlikely.

The excretion of radioactivity in urine in studies with XX was shown to be considerably greater in females than males (36.0% vs 12.3% at 50 mg/kg bw). Major urinary metabolites were identified as quizalofop acid and hydroxy-quizalofop acid, with CHQ as

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a minor metabolite. Hydrolysis of XX also results in the formation of tefuryl alcohol. Little toxicological information is available for tefuryl alcohol, however it is possible that this compound will give rise to irritant metabolites. Similar renal findings have not been reported for quizalofop-ethyl or quizalofop-P-ethyl, further indicating the possible influence of an irritant metabolite such as tefuryl alcohol.

In summary, the renal tumours induced by administration of XX are considered likely to be a consequence of chronic irritation, rather than due to a genotoxic action. Effects in the chronic rat study were seen at the top dose level and a clear NOAEL of 750 ppm (equivalent to ~40 and ~50 mg/kg bw/d in males and females respectively) was seen. The proposed ADI of 0.01 mg/kg bw/d is therefore considered to provide an adequate margin of safety for renal carcinogenicity.

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**ZZ**

## **Temporal Association**

### **Rat Studies**

In the rat (Sprague-Dawley), the induction of liver cell proliferation was observed after both one and four weeks of feeding with 20,000 ppm of ZZ<sup>iv</sup>. The induction of liver enzymes was also measured after four weeks of dietary feeding at the same concentration<sup>v</sup>. Consistent with the observed induction of hepatocyte proliferation and liver enzymes, increases in absolute liver weights were seen in 28-day studies in male and female rats receiving dietary concentrations of ZZ at 5000, 10,000 and 20,000 ppm<sup>(viii)</sup>.

Ninety-day studies were conducted using Sprague-Dawley rats receiving dietary concentrations of ZZ at 1000, 5000, 10,000 and 20,000 ppm<sup>(ix)</sup>. Statistically significant increases in absolute and relative liver weight were observed in males at 1000 ppm and in males and females at 5000 ppm and above. Increased periportal cytoplasmic vacuolation was observed in 5000 ppm and 10,000 ppm females and in 20,000 ppm male and female rats. These changes observed were considered reversible since after a 28-day recovery period in the high dose group, they were largely reversed.

In chronic studies, the first liver tumors were observed in female rats of the 20,000 ppm group at the 12-month interim-sacrifice (2/10 females)<sup>vi</sup>. No other liver tumors were observed at this time point in either the control or treated (500 ppm and 5,000 ppm) male or female rats. At this time point, hepatonecrosis was also observed in 4/10 females of the 5000 ppm group and in 5/10 females of the 20,000 ppm group.

### **Mouse studies**

Twenty-eight day studies were not conducted with mice. In 90 day studies in mice, statistically significant increases in absolute and relative liver weights were noted for males and females at 3500 and 7000 ppm; only slight but insignificant liver weight changes were noted at 1000 ppm<sup>(x)</sup>. Additionally, at 3500 and 7000 ppm, but not 1000 ppm, centrilobular hepatocellular hypertrophy was observed in males and females at 3500 and 7000 ppm. These changes are not proof of, but are suggestive of hepatocellular proliferation in the mice at this time point. Some slight increases in serum cholesterol levels were seen in the 90-day study that were also not clearly dose-dependent, but could be suggestive for liver enzyme induction.

## **Strength, Consistency, and Specificity of Association of Tumor Response with Key Events**

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**Apparent threshold levels can be established, and an association between preneoplastic key events and neoplastic lesions can be observed in female rats and male mice.**

In female rats treated on a chronic basis, there were dose-related increases in liver weights (with a NOAEL of 500 ppm) as well as preneoplastic histopathological changes of hepatocellular necrosis and polyploidy, hepatocellular hypertrophy, and eosinophilic and basophilic foci of cellular alteration (all with a NOAEL of 500 ppm). Similarly, the increased incidence of hepatocellular adenomas in female rats was dose-related (with a NOAEL of 500 ppm). Therefore, for female rats, the apparent threshold level for the progression from preneoplastic events to neoplastic lesions can be established between 500 ppm (25 mg/kg b.w./day) and 5000 ppm (290 mg/kg b.w./day).

Furthermore, in male mice treated on a chronic basis, there were dose-related increased incidences and increased grades of severity of preneoplastic hepatocellular hypertrophy (both centrilobular and diffuse) at 1000 and 7000 ppm (with a NOAEL of 250 ppm). For instance, centrilobular hepatocellular hypertrophy was observed in 0/65, 0/65, 15/65, and 34/66 for males at 0, 250, 1000, and 7000 ppm, respectively. Whereas the severity of this change was only minimal to slight/mild at 1000 ppm, more severe changes were observed at 7000 ppm, namely moderate to moderately severe centrilobular hepatocellular hypertrophy was observed in 14 males at 7000 ppm. Importantly, a significantly increased incidence of treatment-related liver tumors occurred only in this dose group (7000 ppm) that demonstrated the most marked severity of centrilobular hepatocellular hypertrophy (graded moderate or moderately severe). A significantly increased incidence of liver tumors was not associated with centrilobular hypertrophy of a milder (or lower) severity.

Similarly, diffuse hepatocellular hypertrophy was observed in 0/65, 0/65, 6/65, and 20/66 for male mice at 0, 250, 1000, and 7000 ppm, respectively. Whereas the severity of this change was only minimal to slight/mild at 1000 ppm, more severe changes were observed at 7000 ppm. Namely, moderate diffuse hepatocellular hypertrophy was observed in 9 males at 7000 ppm. Importantly, an increased incidence of treatment-related liver tumors occurred only in the dose group (7000 ppm) that demonstrated the most marked severity of diffuse hepatocellular hypertrophy (graded moderate). No increased incidence of liver tumors was seen with diffuse hepatocellular hypertrophy of mild (or less) severity.

Therefore, for male mice, the apparent threshold level for the progression from moderate to moderately severe preneoplastic change of hepatocellular hyperplasia to neoplastic lesions can be established between 1000 ppm (190 mg/kg b.w./day) and 7000 ppm (1300 mg/kg b.w./day).

## **Biological Plausibility and Coherence of the Database**

Based on the weight-of-the-evidence presented above, the mechanism of action for tumor formation in rats results from prolonged oral exposure to ZZ which is accompanied by

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the induction of cytochrome P450 liver enzymes, as well as by chronic induction of liver cellular proliferation (stimulation) which is reversible and has a threshold. The existing data for mice indicate that this mechanism is also plausible for mice; however, the definitive mechanistic studies were only conducted in rats. Never the less, changes such as hepatocellular hypertrophy and liver weight increases in the mouse strongly suggest that this mechanism is reasonably anticipated to also be the mechanism for liver tumor induction in mice.

The apparent threshold for the development of tumors in rats is between 25 and 290 mg/kg b.w./day. The threshold for the development of tumors in mice is between 1000 ppm (190 mg/kg b.w./day) and 7000 ppm (1300 mg/kg b.w./day).

Although the mechanism of tumor formation could theoretically be extrapolated to humans, in practice this would be irrelevant. The liver tumors observed in female rats and male mice only follow prolonged oral exposure to such high doses of ZZ that they are, thus, of limited relevance to human risk evaluation, since humans are never likely to be exposed *firstly*, to these extreme doses of ZZ, and *secondly*, are never likely to be exposed for such a prolonged period of time to such high doses. Thus, based on the available data, it is unforeseeable that humans would ever be exposed to sufficiently high concentrations of ZZ on a chronic basis that would achieve a dietary intake level to the magnitude necessary to present a carcinogenic risk for man.

### Appendix

#### **Other modes of action**

ZZ did not show any indication of genotoxicity in a battery of assays. Specifically, it was negative in the Ames Mutagenicity Assay<sup>(xi)</sup>, in the CHO/HPRT Mutagenicity Test<sup>(xii)</sup>, in the Chromosome Aberration Test in CHO Cells<sup>(xiii)</sup> and in the Mouse Micronucleus Test<sup>(xiv)</sup>. ZZ was also tested for tumor-initiating potential in Sprague-Dawley rats by measuring for the development of GST-positive foci of cellular alteration in the liver<sup>(xv)</sup>. It was found to be negative as a tumor initiator. Furthermore, it was not associated with an increased incidence of neoplastic findings in any other organ or tissue. No effects suggesting hormonal modulation were identified. The main target for systemic toxicity was the liver. Some minor indications of toxicity occurred in the kidney.

Overall, the data available indicate that ZZ is not working through secondary toxicity in one organ indirectly affecting the liver. It appears to operate via liver enzyme induction and liver cell proliferation, which only results in liver tumorigenesis after long-term treatment at very high dose levels.

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## **IPCS Framework for QQ**

### **1. Introduction**

QQ increased the incidence of liver tumours in male mice exposed via the diet to >850 ppm (108 mg/kg bw/d). Data are available on the similarity with phenobarbital (PB) and the relationship to non-neoplastic liver effects. This analysis addresses the relevance of these liver tumours to the human risk assessment.

### **2. Postulated mode of action**

QQ produces an initial increase in hepatocyte mitogenesis (BRDU incorporation) and induction of xenobiotic metabolising enzymes (PB like). This is followed by an increase in liver weight (evident within the first week and subsequently), hypertrophy, necrosis and tumours after chronic exposure. Genotoxicity data are adequate and negative.

A non-genotoxic phenobarbital like mechanism is proposed

### **3. Key events**

The metabolism of QQ in male mice is different to male rats and female mice, with a higher level of cleavage of the dioxolane ring.

Mechanistic studies have shown a mitogenic response, increased liver weight and increased xenobiotic metabolising enzyme activities. The pattern of these shows similarity to equivalent doses of phenobarbital.

Increased liver weight, hepatocyte hypertrophy, vacuolation and necrosis were increased within 13 – 17 weeks at 850 ppm with marginal effects at 500 ppm. Tumours of the liver were increased in male mice at 850 ppm in one study and at 2500 ppm in another. There was an equivocal increase in liver tumours at 500 ppm in one study but not at 500ppm in another study.

Increased liver weights and hepatotoxicity were also seen in female mice and rats, but no increases in tumours were found.

### **4. Dose-response relationship**

The lowest dose level producing a significant increase in tumours after 2 years (850 ppm; 108 mg/kg bw per day) was that which produced clear hepatotoxicity in 13 and 17 week studies. The dose level producing a marginal response in the 13 / 17 week studies (500 ppm) produced a slight, non-significant increase in liver tumours relative to the

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concurrent control, but towards the bottom of the historic control range in one study but no increase in another.

Increases in microsomal xenobiotic metabolising enzyme activities, mitogenic effects and liver weight were seen at 850 ppm and to a greater extent at 2500 ppm.

The tumours were seen at doses producing other hepatic changes.

### **5. Temporal association**

The progression from mitogenic response to liver hypertrophy and tumours is consistent with a progression over time. However, there have been no studies of recovery or outcomes following short-term dosing followed over the normal life-span.

### **6. Strength, consistency, specificity of association of tumour response with key events.**

Within the database available the findings are consistent with the key events.

### **7. Biological plausibility & coherence**

The proposed mechanism is plausible and coherent. The mechanism would not be expected to produce findings at tissues other than the liver.

Liver tumours in mice are a relatively frequent finding with compounds that are enzyme inducers and following liver hypertrophy.

An outstanding question is why female mice and rats do not also have liver tumours despite showing similar non-neoplastic hepatic effects. The difference in the metabolism of QQ in male mice (more dioxolane ring cleavage) might be an explanation.

### **8. Other modes of action**

An adequate range of genotoxicity studies, including a modern *in vivo* mouse micronucleus assay indicate there is unlikely to be a genotoxic mode of action.

Alternative non-genotoxic modes of action have not been investigated to any great extent. CYP enzymes typically increased by peroxisome proliferators and 3-methylcholanthrene were not increased by QQ.

### **9. Assessment of postulated mode of action**

The overall weight of evidence indicates with a moderate to high level of confidence that a phenobarbital type mechanism is responsible for the male mice liver tumours.