Australian Government

Australian Pesticides and Veterinary Medicines Authority

ATRAZINE TOXICITY: ANALYSIS OF POTENTIAL MODES OF ACTION

Prepared for the APVMA by the Office of Chemical Safety and Environmental Health, Office of Health Protection, Department of Health and Ageing, Canberra

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CONTENTS

LIST OF FIGURES AND TABLES

FOREWORD

The International Programme on Chemical Safety (IPCS) has published conceptual frameworks to analyse the relevance to humans of modes of action (MOA) for both cancer and non-cancer endpoints observed in toxicity studies. This MOA analysis utilises all of the available animal data and aims firstly to determine whether it is possible to establish a plausible MOA through the identification of key events along the causal pathway to either a cancer or non-cancer endpoint. If an MOA can be established as plausible in a test animal species, the data are further analysed to determine whether the MOA is relevant to human physiology.

According to the conceptual framework, where it is considered that a plausible MOA may be relevant to human physiology, the MOA can then be examined with regard to its potential to result in effects on human health, which are not covered by existing health standards. Accordingly, fulfilment of the steps of the conceptual framework would then form a credible case for further regulatory consideration of the chemical.

This report is an analysis of available data on atrazine within the IPCS conceptual framework, and includes consideration of both published and non-published reports, as well as assessments prepared by international agencies. The main emphasis is on published data indicating that atrazine may have endocrine MOA which may be responsible for observed adverse effects in animals. The Office of Chemical Safety and Environmental Health (OCSEH) has sought to determine firstly whether the weight-of-evidence is sufficient to support the postulated MOAs, and secondly, if the MOAs are relevant to humans.

The report focuses on the assessment of the postulated MOA for the induction of mammary tumours in female Sprague Dawley rats and the postulated MOAs for the observed reproductive and developmental effects following atrazine administration to animals, possibly as a result of effects on luteinising hormone (LH), gonadotrophin releasing hormone (GnRH), and/or the enzyme, aromatase.

In addition to previously assessed studies, a search of the available literature was performed to locate any recent additional relevant studies. A PubMed search was conducted using the following terms: atrazine and aromatase; sperm; developmental toxicology; reproductive toxicology; testicular; resorption; LH; and GnRH. Searches were limited to studies from 2004 onwards (as previously published data was evaluated as part of the APMVA Review of Atrazine in March 2008). The reports prepared by the US EPA in 1993 and the WHO/FAO Joint Meeting on Pesticide Residues (JMPR) in 2007 to assess the MOA for atrazine-induced mammary tumours were also considered. A SciFinder search for ‘atrazine’ was also undertaken to identify any additional literature available and which was relevant to an MOA analysis (published after 2004 and prior to March 2009).

In preparing this report, minor differences were identified in the data sets used by various regulatory agencies to evaluate the MOAs. The OCSEH did not have access to several unpublished toxicology studies which were evaluated by the JMPR and/or the US EPA, and in these cases, the OCSEH used the assessments in those reports.

ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
APVMA	Australian Pesticides and Veterinary Medicines Authority
cAMP	cyclic 3'-5'-adenosine monophosphate
cGMP	cyclic guanosine 5'-monophosphate
CH	Chorionic gonadotrophin
CRH	Corticotrophin releasing hormone
DACT	Diamino-s-chlorotriazine
E2	Estradiol
ER	Estrogen Receptor
F344	Fischer (rat)
FAO	Food and Agriculture Organization of the UN
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FSH	Follicle simulating hormone
GNRH	Gonadotrophin releasing hormone
HPA	hypothalamus-pituitary-adrenal
HPG	hypothalamus-pituitary-gonadal
IC_{50}	The concentration at which 50% inhibition is observed
IPCS	International Programme on Chemical Safety
JMPR	WHO/FAO Joint Meeting on Pesticide Residues
LE	Long-Evans (rat)
LH	Luteinising hormone
LOEL	Lowest observed effect level
LOAEL	Lowest observed adverse effect level
NOEL	No observed effect level
NOAEL	No observed adverse effect level
MOA	Mode(s) of action
MRNA	Messenger ribonucleic acid
OCSEH	Office of Chemical Safety and Environmental Health
PII	Promoter II
PDE	Phosphodiesterase
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PRL	Prolactin
SAP	Scientific Advisory Panel of the US EPA
SD	Sprague Dawley (rats)
SUSDP	Standard for the Uniform Scheduling of Drugs and Poisons
TSH	Thyroid stimulating hormone
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

1 EXECUTIVE SUMMARY

Atrazine is a selective systemic triazine herbicide which is used widely throughout Australia as a pre- and post-emergence herbicide for grass and broadleaf weeds in sorghum, maize, and sugarcane crops. Atrazine is also used in the establishment of pine and eucalypt plantations and on triazine-tolerant canola crops. Studies published since 2001 have suggested that atrazine may have effects on the endocrine/reproductive system(s) which were not previously considered.

This report examines three potential modes of action (MOA) for atrazine according to the criteria identified in the *IPCS Framework for Analysing the Relevance of a Cancer Mode of Action for Humans*. It was updated in June 2010, prior to publication, to include preliminary information regarding a possible fourth MOA (see Addendum).

The first postulated MOA considered in this report is generally accepted to be plausible in one animal strain i.e. the effect of atrazine on the regulation of luteinising hormone (LH) and subsequent proliferative changes mediated by prolactin in mammary tissues of female Sprague-Dawley (SD) rats. There is also a consensus that the end-point of mammary tumour formation in rats by this MOA is not relevant to humans. The other two potential MOAs [depression of gonadotrophin-releasing hormone (GnRH) and increased aromatase expression], together with the emerging MOA considered in the June 2010 Addendum to this report, reflect developments from available research findings but are more speculative.

The second postulated MOA considered in this report is that atrazine disrupts hypothalamic function and depresses production of GnRH, which in turn affects reproductive and developmental endpoints and ultimately may lead to full litter resorptions in a test animal species. However, this terminal effect is only observed at high doses. The MOA analysis for the reproductive effects observed in a number of species concluded that although this MOA was also potentially plausible, significant data gaps existed and there is presently insufficient weight-of-evidence to enable this MOA to be accepted as plausible in animals. Therefore, it was not possible to examine the next step in the framework regarding human relevance.

The third postulated MOA considered in this report is that atrazine increases aromatase enzyme activity, increasing the aromatisation of testosterone and conversion to oestrogen. It is further postulated that an increased oestrogenic environment may favour i) induction of cancers and/or proliferation of pre-existing oestogen-dependent cancers and/or ii) altered relative sex hormone levels, which in turn may have an adverse effect on a terminal or downstream end-point of reproduction and/or development. This area of research is expanding rapidly but currently available data were largely restricted to *in vitro* evidence that examined the effects of exposure to continuous concentrations of atrazine on aromatase. The absence of relevant benchmark studies which establish the occurrence of the proposed key events, any dose responsiveness or any temporal associations, precludes the further examination of this postulated MOA or its human relevance.

In conclusion, adequate information to establish a link between atrazine exposure and an identified toxicological endpoint was found for only one MOA but the MOA was considered not to be relevant to human health risk assessment. With respect to the other postulated MOAs, further studies are needed to explore whether the MOAs can be established, and then any relevance to human risk assessment.

The results of this MOA analysis do not suggest that at this stage a further scientific review of atrazine toxicity is warranted or provide a basis to undertake a re-evaluation of the existing health values. The ADI in place in Australia was established on the no-observed-effect-level (NOEL) for the most sensitive end-point in repeat-dose toxicity studies and is currently considered by the OCSEH to be protective against adverse effects that may be caused by exposure to atrazine.

2 JUNE 2010 ADDENDUM

Following the submission of this report to the APVMA in January 2010, the APVMA requested peer review from experts in the US EPA. However, their consideration of the report was impeded by the decision of the US EPA to 're-review' atrazine (see http://www.epa.gov/pesticides/reregistration/atrazine/atrazine_update.htm#atrazine). The OCSEH and the APVMA decided to await the outcome of the Federal Insecticide, Fungicide and Rodenticide Act Scientific Advisory Panel (FIFRA SAP) meeting on the *'Re-evaluation of the Human Health Effects of Atrazine: Review of Experimental Animal and In Vitro Studies and Drinking Water Monitoring Frequency'*, which was held in Washington D.C. from 26-29 April 2010. In addition to considerations related to those MOAs covered in the January 2010 MOA report, a possible new MOA was presented to the FIFRA SAP by the Office of Pesticide Programs, US EPA (US EPA, 2010). The OCSEH and the APVMA have obtained copies of the meeting transcripts and the draft background white paper (US EPA, 2010). Both organisations consider that this evolving MOA and an update on the aromatase-based MOA should be acknowledged in this report's June 2010 Addendum.

The possible new MOA involves the hypothalamus-pituitary-adrenal (HPA) axis, which has the capacity to influence the hypothalamus-pituitary-gonadal (HPG) axis. The HPG axis is considered by most experts (see FIFRA SAP transcript, US EPA, 2010) to be the most plausible MOA by which the endocrine system and/or reproductive system could be affected by atrazine, leading to the endocrine and/or reproductive toxicity end points seen in animal studies.

The second matter (in addition to the emerging HPA axis MOA) which arose during the SAP discussions, was that the weight-of-evidence for a major role of aromatase (the enzyme which converts androgens to oestrogens) in mediating the effects of atrazine has not been substantiated by *in vivo* observations. Moreover, there is some evidence which indicates that atrazine may be influencing a number of steroid pathways rather than specifically the conversion of testosterone to oestradiol by aromatase.

However, it is important to note that the exhaustive US EPA examination of available studies performed prior to the FIFRA SAP meeting (see US EPA, 2010) did not identify a sound basis (at this stage) to consider any change to the existing toxicological end-point for atrazine. The new studies examined did not provide a basis from which to extrapolate any risk to human health which would warrant a reconsideration of the established US EPA health values.

The HPA MOA for atrazine is based primarily on the recent publications of Fraites *et al.* (2009) and Laws *et al.* (2009) supporting earlier studies by Modic (2004) and Pruett *et al.* (2003) in which treatment of rats with atrazine activated the HPA axis, leading to increased circulating levels of adrenocorticotrophic hormone (ACTH) and corticosterone which further suggested the possibility of central control or modulation of the gonadal axis. Consistent with this possibility were reports in the early neuroendocrine literature (see Fraites *et al.*, 2009) of the inhibition of reproductive indices and outcomes by various stimuli of the HPA, in particular interactions between the central stress response corticotrophin-releasing hormone (CRH), feedback from the adrenal glands via corticosteroids and progesterone and modulation of GnRH and luteinising hormone (LH). Collectively, there was considered to be sufficient evidence to support the proposal that atrazine may be able to activate the adrenal axis leading to an attenuation of LH secretion and potentially to the development of related reproductive toxicity and/or carcinogenic sequelae in rats.

The key events (as described in the FIFRA SAP Background paper, US EPA, 2010) of the apparent cross-over of the HPA axis with the HPG axis include: hypothalamic changes resulting in an increased release of CRH; increased release of ACTH from the pituitary; increased production of corticosterone and progesterone by the adrenal glands; and consequently decreased secretion of GnRH from the hypothalamus, which may be the result of the aforementioned changes and provide a connection with the well established neuroendocrine mediated modulation of the LH surge (in rodents). The treatment of female Long-Evans rats with 75 mg/kg bw (gavage) atrazine, or molar-equivalent doses of metabolites DIA or DEA (but not DACT) led to increases in ACTH, corticosterone and progesterone, within 15 min (Fraites *et al.* (2009); Laws *et al.* (2009)). Moreover, decreased secretion of hypothalamic GnRH has been observed in rats which received atrazine at 25 mg/kg bw/day for 4 days (Cooper *et al.* 2007) although there is no direct evidence that this GnRH response by the hypothalamus following atrazine treatment in these animals is initiated by central nervous system (CNS) signalling or that it results in any effect on reproduction or development. Therefore this postulated MOA has yet to be established.

A range of factors has been shown to influence the HPA axis. Restraint stress increases ACTH, corticosterone and progesterone to similar levels to those achieved with high doses of atrazine (Fraites *et al.*, 2009). In addition, other studies indicate that increased corticosterone release from the adrenal glands has been shown to modulate neurons which control GnRH, gonadotrophin inhibiting factor, CRH, and norepinephrine. Collectively, these agents are also known to decrease GnRH pulses (FIFRA SAP background paper, US EPA, 2010). With respect to CNS control, slight changes in neurotransmitter levels (decreased striatal dopamine, decreased prefrontal cortex norepinephrine, and decreased hypothalamic serotonin) have been observed in rats which received atrazine at 10 mg/kg bw/day for six months and these observations were considered to be consistent with the known effects of atrazine on the dopaminergic system (US EPA, 2010).

As noted in the FIFRA SAP Background paper, the doses used to discern these effects and explore the MOA pathways were considerably higher than the current NOEL used to establish health values such as the Acceptable Daily Intake (in Australia) or the Reference Dose (in the US) for atrazine. Further research would be needed to fully characterise this postulated HPA axis MOA for atrazine and enable further consideration using the established MOA framework.

The FIFRA SAP discussion also considered recent studies and the weight-of-evidence for the MOA involving the induction of aromatase activity. There has been an increase in the breadth of studies examining the effects of atrazine on aromatase activity as indicated by testing of *in vitro* cell culture systems, with the lowest observed effect concentrations being found to be in the range of 0.3-1.0 μM (Breckenridge, 2009; US EPA, 2010). These concentrations are within the plasma concentration range of atrazine that have been investigated in animal studies (Fraites *et al.*, 2009; Laws *et al.*, 2009) but to date, no evidence of an effect by atrazine on aromatase has been reported following *in vivo* testing at these levels of exposure. Also, the upregulation of other steroidogenic genes (StAR, inhibin-α, P450scc, and 11βHSD in JEG3 cells) has been reported recently and indicates that there is likely to be a broader effect of atrazine on steroidogenesis generally rather than a specific effect on aromatase activity only (Breckenridge, 2009; FIFRA SAP background paper, US EPA, 2010). Another recent insight has been the finding that the assessment of aromatase activity using too few *in vitro* end-points can lead to misleading conclusions on the role of aromatase *in vivo* in this postulated MOA (Higley *et al.*, 2010).

2.1 REFERENCES FOR ADDENDUM

Breckenridge CB (2009) Evaluation of the potential effects of atrazine on aromatase gene expression and/or aromatase *in vitro* and *in vivo*. Syngenta Crop Protection, Inc. Report Number T001579-08, unpublished.

Cooper RL, Laws S, Das PC, Narotsky MG, Goldman JM, Tyrey EL, & Stoker TE (2007). Atrazine and reproductive function: mode and mechanism of action studies. Birth Defects Res. B Dev. Reprod. Toxicol. 80: 98-112. (cited from US EPA, 2010)

Fraites MPJ, Cooper RL, Buckalew A, Jayaraman S, Mills L, & Laws SC (2009) Characterisation of the hypothalamic-pituitary-adrenal axis response to atrazine and metabolites in the female rat. Toxicological Sci. 112: 88-99.

Higley EB, Newsted JL, Zhang X, Giesy JP & Hecker M (2010) Assessment of chemical effects on aromatase activity using the H295R cell line. Environ. Sci. Res. 17: 1137-48.

Laws SC, Hotchkiss M, Ferrell J, Jayaraman S, Mills L, Modic W, Tinfo N, Fraites M, Stoker T, & Cooper R (2009) Chlorotriazine herbicides and metabolites activate an ACTH-dependent release of corticosterone in male Wistar rats. Toxicological Sci. Advance Access published on August 18, 2009, doi:0.1093/toxsci/kfp190.

Modic MW (2004) The role of testicular aromatase in the atrazine mediated changes of estrone and estradiol in the male wistar rat. In Biochemistry, Vol. Master of Science. North Carolina Sate University, Raleigh, NC, USA, (cited from Fraites *et al.*, 2009).

Pruett SB, Fan R, Zheng Q, Myers LP & Herbert P (2003) Modeling and predicting immunological effects of chemical stressors: Characterisation of a quantitative biomarker for immunological changes caused by atrazine and ethanol. Toxicol. Sci 75: 343-354 (cited from Fraites *et al.*, 2009).

US EPA (2010) Federal Insecticide, Fungicide and Rodenticide Act Scientific Advisory Panel (FIFRA SAP) meeting on the Re-evaluation of the Human Health Effects of Atrazine: Review of Experimental Animal and *In vitro* Studies and Drinking Water Monitoring Frequency, Washington D.C. 26-29 April 2010, FIFRA SAP draft Background paper. (http://www.epa.gov/scipoly/sap/meetings/2010/042610meeting.html#transcripts), (http://www.epa.gov/pesticides/reregistration/atrazine/atrazine_update.htm#atrazine).

ATRAZINE TOXICITY: ANALYSIS OF POTENTIAL MODES OF ACTION

3 BACKGROUND

In March 2008, the APVMA published the Final Review Report and Regulatory Decisions on Atrazine. The current report was undertaken to assess the data published and/or provided to the OCSEH since 2004 in relation to subsequent findings suggesting that atrazine may be responsible for adverse reproductive and developmental effects through an endocrine mode of action. In assessing this data, the OCSEH considered firstly whether there was sufficient weight-of-evidence to establish a plausible mode of action (MOA) for the effects reported in animals and, secondly, examined the relevance of the MOA for human health.

3.1 SOURCES OF INTERNATIONAL REGULATORY DATA

During the conduct of this MOA analysis, previously published reports from the OCSEH/APVMA on atrazine (APVMA, 2002, 2004, and 2008) were considered. Reports from the US EPA (1993) and the JMPR (2007) were provided to the OCSEH and used in this analysis.

3.2 CURRENT PUBLIC HEALTH STANDARDS IN AUSTRALIA

Atrazine is listed in Schedule 5 of the Standard for the Uniform Scheduling of Drugs and Poisons (SUSDP). The acceptable daily intake (ADI) of 0.005 mg/kg bw/d was established in 1990 and was reconfirmed in 1996 (APVMA, 1997). The ADI is based on a 2-year dietary study in rats in which a NOEL of 0.5 mg/kg bw/d was observed for mammary tumours in one strain (Sprague Dawley, SD) of female rats. Mammary tumours were not observed in male SD rats, male or female Wistar rats, nor in male or female CD-1 mice. Safety factors of 10 fold for species differences and 10-fold for uncertainty were applied to the NOEL to derive the ADI. In addition to the evidence that the mammary tumours were strain-specific, the assigned NOEL is also conservative because the tumours that were observed in the female SD rats were considered not to be relevant to human health because of clear differences between SD rat and human endocrine systems. The ADI assigned in Australia is also four-fold lower than the group ADI of 0.02 mg/kg bw/d established by the JMPR (2007) on the basis of the NOAEL for atrazine of 1.8 mg/kg bw/d for the suppression of the luteinising hormone surge and the subsequent disruption of the oestrous cycle in rats receiving 3.6 mg/kg bw/d for six months (with safety factors of 10-fold for species differences and 10-fold for human inter-individual variability then applied to the NOAEL). The JMPR (2007) considered that this ADI was protective of potential neuroendocrine effects and other adverse effects which might be caused by prolonged exposure to atrazine and its chloro-*s*-triazine metabolites.

The JMPR (2007) established an ARfD of 12.5 mg/kg bw on the basis of a NOAEL for impaired (suckling-induced) prolactin secretion in dams and subsequent alterations in development of the central nervous system and prolactin regulation in male offspring. An acute reference dose (ARfD) for atrazine has not been established in Australia because of its low acute toxicity after single or a few high doses.

3.3 REGULATORY ACTION ON ATRAZINE IN AUSTRALIA

Several assessments of the toxicology of atrazine have been conducted in Australia which have led to the regulatory actions described in chronological order below:

- **1990**: Evaluation of unpublished data mitigated earlier concerns of a higher incidence of mammary tumours in rats and concluded that the rodent studies showed no evidence of carcinogenic potential. Available epidemiological data showed no association between atrazine exposure and cancer. An ADI of 0.005 mg/kg bw/d was set, based on the NOEL of 0.5 mg/kg bw/d in a 2-year rat study and using a 100-fold safety factor. Atrazine remained exempt at that time from poisons scheduling.

- **1994**: The Advisory Committee on Pesticides and Health (ACPH) considered atrazine use and water contamination issues. The ACPH recommended the development of forestry guidelines to reduce the possibility of water contamination with pesticides and agreed to review the ADI and the drinking water Health Guideline Value. The ACPH concluded that rat mammary tumours were not relevant to the human risk assessment of atrazine and confirmed the NOEL of 0.5 mg/kg bw/d, the ADI of 0.005 mg/kg bw/d, and the Australian Drinking Water Health Guideline Value of 0.02 mg/L. Atrazine was rescheduled from 'exempt' to Schedule 5 of the Standard for the Uniform Scheduling of Drugs and Poisons (SUSDP) ('Caution' signal heading on atrazine products).

- **1996**: The OCSEH completed a report entitled *Review of the Mammalian Toxicology and Metabolism - Toxicokinetics of Atrazine*. This report evaluated new data on the toxicology of atrazine, including all previously submitted data and made the following conclusions:

    1. No change to the current NOEL for atrazine was warranted. The observed mammary tumours were not considered to be relevant to human health, but the response was considered to reflect a hormonal interaction and an appropriately conservative endpoint on which to establish the ADI.

    2. The ADI for atrazine of 0.005 mg/kg bw/d (based on the NOEL of 0.5 mg/kg bw/d and using a safety factor of 100) was confirmed.

    3. The Australian Drinking Water Health Guideline Value for atrazine of 0.02 mg/L should be reconsidered by the National Health and Medical Research Council (NHMRC).

    4. No change to the poisons schedule (Schedule 5 of the SUSDP) was warranted.

    5. Significantly stricter controls over uses in riparian zones should be considered.

    6. There was no objection to the continued approval of atrazine.

- **2001**: The NHMRC revised the Australian Drinking Water Guidelines and revised the Health Value upwards to 0.04 mg/L; this took into account possible metabolites of atrazine which can occur in water and have a similar toxicity profile to atrazine. It also specified a Guideline Value (an action level) equivalent to a practical, reliable limit of detection (currently 0.0001 mg/L).

- **2006**: The OCSEH evaluated recently-published reports relating to the carcinogenicity, developmental effects and endocrine-disrupting potential of atrazine, and considered whether these reports would (i) change the recommendations made in the review performed in 1996, and (ii) warrant a cumulative risk assessment be undertaken. The report noted that:

    1. Atrazine treatment was associated with altered neuroendocrine homeostasis in one strain of rats (SD), but atrazine does not bind to the oestrogen receptor and has no intrinsic oestrogenic activity.

    2. The level of exposure to mixtures of triazine compounds *via* food and drinking water was not of concern, although a cumulative risk assessment may need to be considered in the future, if the level of exposure increased.

    3. No changes to the existing health standards for atrazine were recommended.

- **2007**: The OCSEH revisited the above report (June 2006) and made minor amendments. No alterations to the conclusions were made nor did the OCSEH recommend any changes to the existing health standards.

[A more detailed history of the assessment of atrazine may be found at http://www.apvma.gov.au/chemrev/downloads/atrazine_tox.pdf (pages 104-106)]

3.4 OTHER NATIONAL AND INTERNATIONAL ASSESSMENTS

The European Commission (EC, 2003) removed atrazine from Annex I of Directive 91/414/EEC with the following conclusion:

> The information available is insufficient to satisfy the requirements set out in Annex II and Annex III Directive 91/414/EEC. In particular the available monitoring data were insufficient to demonstrate that in large areas concentrations of the active substance and its breakdown products will not exceed 0.1 µg/L in groundwater. Moreover it cannot be assured that continued use in other areas will permit a satisfactory recovery of groundwater quality where concentrations already exceed 0.1 µg/L in groundwater.

It may be noted that this 0.1 µg/L value was an arbitrary limit set for any pesticide in water, regardless of its safety profile, and it is likely that water in Europe could have met this standard (had monitoring been conducted) following the controls and use restrictions placed on atrazine in most jurisdictions in the mid-1990s.

The US EPA IRED (US EPA, 2003) risk assessment indicated human health risks of concern, particularly regarding the predicted levels of atrazine present in drinking water in the USA, and for occupational and home garden use. The IRED recommended increased monitoring of atrazine levels in water supplies and changes to the use pattern of atrazine products to limit exposure during and post-application. Prior to this, the US EPA completed a draft hazard and dose response assessment and characterisation of atrazine (US EPA, unpublished, 2000), which was made available to the OCSEH and used for this report.

The JMPR also completed a review of atrazine in 2007 and a pre-publication copy was also provided to the OCSEH and was referenced in this review (JMPR, 2007). A summary report of the assessment has been published at

(http://www.fao.org/ag/AGP/AGPP/Pesticid/JMPR/DOWNLOAD/2007_rep/report2007jmpr.pdf). The WHO have advised that a full version of the 2007 monographs (including a 100-page atrazine review) has been published at http://whqlibdoc.who.int/publications/2009/9789241665230_eng.pdf].

3.5 SUMMARY OF ATRAZINE TOXICITY

The following text summarises the toxicity of atrazine covering studies available up to the conclusion of the 1997 Australian atrazine review (available on the APVMA website at http://www.apvma.gov.au/chemrev/downloads/atrazine_tox.pdf). More recent studies are considered in the following sections which analyse different atrazine MOAs.

Atrazine has low acute toxicity, however somewhat higher acute toxicity has been ascribed to some metabolites of atrazine, especially in female animals. The acute toxicity of desethyl- and desisopropylatrazine was twice that of atrazine in some studies, whilst the acute toxicity of diaminochlorotriazine (DACT) and hydroxyatrazine was similar or marginally lower.

In the guinea-pig sensitisation test, atrazine was shown to be a strong skin-sensitising agent but was negative in a human skin sensitivity (patch) test in fifty subjects.

Repeat-dose administration of atrazine to mice, rats and dogs resulted in relatively nonspecific toxicity endpoints across different species and included reduced bodyweight gain, reduced food consumption and some liver enlargement at high doses. Anaemia was noted in short-term repeat-dose studies (rabbits), subchronic studies (rats, dogs) and chronic studies (mice, rats, dogs). Other frequently observed effects in more than one study included: small and/or cyanotic testes; cardiovascular effects; and renal toxicity. Isolated observations included (one study) reduced thymus weights, increased pituitary weight, retinal degeneration and muscle tremors.

The carcinogenicity of atrazine in mammals has been studied extensively. In one strain of female rats, atrazine was shown to induce either an increase in the overall incidence or an earlier onset of mammary tumours and in one study an earlier onset of pituitary tumours was observed. It is likely that these responses, observed in female SD rats only, occur above a certain threshold. Further, these effects were not seen in male SD rats, female Fischer rats, or male or female CD-1 mice.

In SD rats, analysis of oestrogen levels and oestrous cycling show an increased number of days in oestrus or under oestrogen dominance and suggest that the earlier onset of mammary tumours in female SD rats probably relates to an accelerated ageing of the neuro-endocrine system. In contrast, treatment of female Fischer rats did not exhibit any effects on the length of the oestrous cycle, oestradiol or progesterone levels with atrazine dosing, at doses from well below to levels which caused other toxic effects.

Neither atrazine, simazine nor the common metabolite, diamino-s-chlorotriazine (DACT), at concentrations up to 100 mM, competed with radioactive oestradiol binding (5 nM) to oestrogen receptors (ER) in extracted uterine tissue under equilibrium conditions (at 4°C). Collectively, the triazines were about 105 times less

potent than oestradiol itself in causing a fifty percent reduction in labelled oestradiol binding to ER (at 25°C). Results from three types of *in vivo* studies in ovariectomised rats suggested that none of these three chloro s triazines possessed any significant intrinsic oestrogenic activity but that they are capable of weak inhibition of oestrogen stimulated responses in the rat uterus. This weak inhibitory activity may play a role in the changes in reproductive endocrine function which have only been observed in female SD rats.

No teratogenic effects were noted in two- and three-generation reproduction studies with atrazine or in developmental studies with atrazine and each of its four metabolites. In a developmental study in rats which received water resembling groundwater contaminated with pesticide/fertiliser mixtures at 0.5, 5 and 50 ng/mL atrazine (1x, 10x and 100x actual concentrations found in US corn-growing states), no significant adverse effects were reported. No reproductive or developmental effects were observed in the two- and three-generation dietary studies in rats at the highest doses tested, even though some maternal toxicity[1] was noted in the two-generation study.

Modulation of endocrine homeostasis is a known effect of many chemicals if the exposures overwhelm the normal inactivation, metabolism, and/or excretion pathways (OCSEH, 2007). Thus, endocrine modulation *per se* is not considered to be an adverse end-point but rather a possible mechanism of action which could lead to other toxicological outcomes e.g. reproductive, developmental, or carcinogenic effects. Previous studies evaluated by the OCSEH (APVMA, 1997) suggested that the neuroendocrine system is susceptible to modulation by atrazine, which was associated with earlier onset of reproductive ageing and possibly earlier onset or increased incidence of mammary tumours in female SD rats. The weight-of-evidence suggests that these effects are peculiar to this strain of rat and are unlikely to be applicable to all rodents or all mammals (OCSEH, 2007).

The weight-of-evidence suggesting that it is unlikely that the response seen in SD rats is relevant to the risk of mammary tumour development in humans is based on the following:

1. The earlier onset in mammary tumours was not seen in male SD rats, in female Fischer 344 rats, or male or female CD-1 mice;
2. The response observed in female SD rats was only observed to occur above a certain threshold;
3. The background incidence of mammary tumours in SD rats is significantly higher than in female Fischer 344 rats;
4. Neither atrazine nor its metabolites are genotoxic in animal cells;
5. Menopausal women develop episodes of declining oestrogen secretion and longer periods of low oestrogen levels, in contrast to the situation in ageing SD rats.

The above data were considered by the Advisory Committee on Pesticides and Health (ACPH, February, 1997). The Committee concluded that the benign mammary tumours observed in SD rats were not relevant to human health risk assessment. However, the response reflected a hormonal interaction and was triggered

[1] The observed lack of effect on immature rats at all doses up to exposures sufficient to induce signs of maternal toxicity, are apical results which have not been contradicted by any *in vitro* studies.

at a lower exposure than any other effect. Overall, the observation of hormonal interaction was considered to be an appropriately conservative endpoint on which to establish the ADI. The ACPH concluded that the existing ADI for atrazine of 0.005 mg/kg bw/d was protective of human health.

3.6 EFFECTS IN AMPHIBIANS AND THEIR HUMAN RELEVANCE

Atrazine has been shown to disrupt sex differentiation and organogenesis in amphibians, although there is insufficient experimental evidence to formulate a consensus on trigger levels for these effects in frogs. At high doses, atrazine has been shown to disrupt organogenesis in *Xenopus laevis* tadpoles (Lenkowski, 2008) while at low doses, gonadal development and testicular oogenesis was delayed or retarded in leopard frogs (*Rana pipiens*) (Hayes *et al.*, 2003). These observations were consistent with an earlier investigation which showed that atrazine concentrations less than 0.1 ppb induced hermaphroditism and demasculinised the larynges of male *Xenopus laevis*, whereas at a concentration of 25 ppb atrazine exposure led to a ten-fold decrease in testosterone levels in this species (Hayes *et al.*, 2002). The authors hypothesized that atrazine induced increased expression/activity of aromatase, which catalysed the conversion of testosterone to oestrogen (Hayes *et al.*, 2002). Consistent with this hypothesis, the postulated aromatase MOA may offer an explanation for an observed increase in the proportion of female *Rana Pipiens* larvae following exposure to atrazine (Orton, 2006) and an increase in intersex gonads in male frogs (*Rana pipiens*) where the latter was attributed to the presence of atrazine at more than 0.1 µg/L (0.1 ppb) (Haynes *et al.*, (2003).

In contrast to the above observations, some of the effects have not been able to be repeated by other research groups (Renner, 2008; Oka, 2008; DuPreez, 2008) and do not appear to be observed in the field. For example, in a field study conducted in a rice irrigation area in Australia where a range of pesticides including atrazine are used, no evidence of gonadal malformations in late-stage tadpole or juvenile *Limnodynastes sp.*, nor in *Litoria raniformis* frogs when relatively high field concentrations of atrazine (0.16 to 1.67 µg/L) were detected during this developmental period (Hyne *et al.*, 2009).

Therefore, the relevance to mammals including humans, of any effects observed in amphibians is unclear at this stage because of the lack of robust data.

[Note that recent investigations on the role of aromatase have suggested that the weight of evidence to support this MOA is currently considered to be less plausible compared with when the January 2010 part of this report was prepared based on earlier studies, see June 2010 Addendum for details].

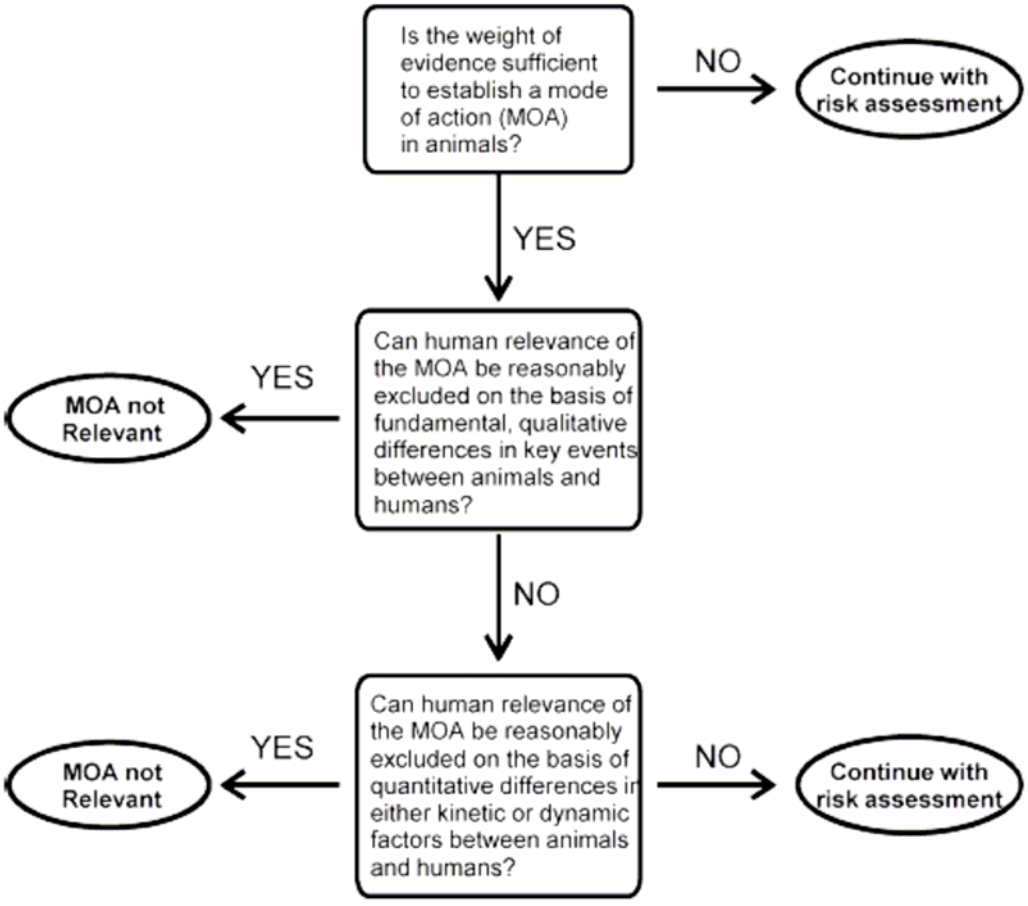
Presently, the use of data for the assessment of human health risks by regulatory agencies does not extend to data derived from non-mammalian species. The OECD publishes a series of test guidelines, which are a recognised international standard for chemical testing used for regulatory purposes. The methods described in these guidelines cover tests for physical and chemical properties, effects on human health and wildlife, and accumulation and degradation in the environment. There is currently no validated test method for the use of amphibians (or reptiles) in assessing the risk to human health from chemical exposure.

Therefore, the OCSEH considers that although there is some evidence of adverse effects in amphibians and reptiles that may impact on the environmental assessment of atrazine, it is not possible at this time to relate those findings to the assessment of human health risks.

4 THE MODE OF ACTION FRAMEWORK

The International Programme on Chemical Safety (IPCS) developed a framework for analysing the human relevance of cancer and non-cancer modes of action (MOA) of chemicals in order to promote the harmonisation of human health risk assessment (Boobis *et al.*, 2006; 2008, Sonich-Mullin *et al.*, 2001). A diagrammatic representation of the framework is shown in Figure 1.

Figure 1: IPCS general scheme illustrating the main steps in evaluating the human relevance of an animal MOA



Application of the IPCS framework to analyse the human relevance of an MOA requires firstly an assessment of whether it is possible to establish the MOA in a test animal species. To achieve this, the key events of the postulated MOA are identified using a weight-of-evidence approach based on the Bradford Hill criteria. Once an MOA is established as plausible in an animal species, the key events are then compared both qualitatively and quantitatively between experimental animals and humans in order to make a clear statement of confidence, analysis and implications of the data (Boobis *et al.*, 2006). The application of the IPCS framework enables a transparent evaluation of the data, the identification of key data gaps and facilitates the presentation of the data in a structured manner.

Presently, three separate MOAs are postulated to explain the toxicity findings associated with exposure to atrazine, including carcinogenic, reproductive, and developmental endpoints.

The postulated MOAs considered in this report are:

1. MOA1: Atrazine putatively alters neurotransmitter and neuropeptide function regulating luteinising hormone (LH) and secretion of prolactin, leading to anovulation, persistently raised oestrogen levels and prolactin stimulation of the mammary gland, proliferative changes in the mammary gland and an earlier onset and/or higher incidence of mammary adenocarcinomas and fibroadenomas.
2. MOA2: Atrazine putatively inhibits the release of gonadotrophin-releasing hormone (GnRH) resulting in a decrease in the secretion of chorionic gonadotrophin (CG), which in turn leads to full litter resorptions. Evidence in support of this postulated MOA is available from both *in vitro* and *in vivo* studies.
3. MOA3: Atrazine putatively increases aromatase expression which results in an earlier onset and/or higher incidence of mammary adenocarcinomas and fibroadenomas, and induces adverse effects during critical periods of reproduction and development. Evidence in support of this postulated MOA is available from *in vitro* studies only.

5 MOA1: MAMMARY GLAND TUMOURS / LUTEINISING HORMONE

5.1 THE WEIGHT-OF-EVIDENCE FOR AN MOA IN A TEST ANIMAL SPECIES

5.1.1 INTRODUCTION

Administration of atrazine induces mammary tumours in female SD rats, but not in male SD rats or in female Fischer rats or in male or female CD-1 mice. The postulated MOA for the induction of tumours in female SD rats is that atrazine disrupts the luteinising hormone surge, leading to increased incidence and earlier onset of mammary tumours in SD rats (APVMA, 1997).

Evidence in support of this MOA has been published previously (APVMA, 1997, Cohen *et al.*, 2004). Other documents also contain more detailed evaluations of individual studies (JMPR, 2007; US EPA, 2000). Some of the pivotal data contained in these reports have not been evaluated by the OCSEH, and in these cases, the OCSEH has used the JMPR and the US EPA reports.

This postulated MOA is discussed below under the headings proposed in the IPCS conceptual framework (Sonich-Mullin *et al.*, 2001).

5.1.2 POSTULATED MOA

The postulated MOA for the induction of mammary tumours by atrazine in female SD rats involves the disruption of the hypothalamic-pituitary-ovary axis. It is proposed that atrazine indirectly modulates this axis by modifying catecholamine (dopamine and/or noradrenaline) and/or peptide hormone (somatostatin) function which in turn would modulate the release of gonadotrophin-releasing hormone-II (GnRH-II) from the hypothalamus. The consequence of this is that the surge of luteinising hormone (LH) released from the pituitary gland is of insufficient amplitude or duration to trigger ovulation. The result of failed ovulation is persistent secretion of oestrogen which provides feedback to the pituitary gland, which causes the increased secretion of prolactin. The resultant change in the hormonal environment leads to a proliferative response of the mammary gland, characterised by an earlier onset of appearance and/or a higher incidence of adenocarcinomas (high oestrogen, moderate prolactin levels) or fibroadenomas (high prolactin with a background level of oestrogen) (McConnell, 1989; O'Connor *et al.*, 2000; Simpkins, 2000; Simpkins *et al.*, 2000; Cooper *et al.*, 2007).

5.1.1 KEY EVENTS IN THE MOA

- Atrazine affects the hypothalamus, leading to a decrease in catecholamine and/or peptide hormone levels, and a decrease in GnRH-II secretion;
- Decreased GnRH-II release from the hypothalamus leads to an attenuation of LH released from the pituitary gland; the subsequent LH surge is of insufficient amplitude or duration to trigger ovulation;
- Failure to ovulate results in persistent secretion of oestrogen/oestradiol from the ovarian follicles, which provides a feedback to the pituitary leading to increased secretion of prolactin;

- Prolonged exposure to endogenous oestrogen and prolactin causes hyperstimulation of the mammary gland leading to an earlier appearance and/or a higher incidence of mammary tumours;
- The progression of mammary (and pituitary) neoplasia is expedited by trophic effects associated with decreased activation of the GnRH-II receptor in these mammary cells.

5.1.2 DOSE-RESPONSE RELATIONSHIPS

The reports prepared by the US EPA and JMPR report a number of studies with no-observed-adverse-effect-levels (NOAELs) and/or lowest-observed-adverse-effect-levels (LOAELs) for the key events identified in the MOA (refer Table 1 overleaf).

Attenuation of the LH surge was noted at doses of 3.65 mg/kg bw/d in a 26-week mechanistic study and at higher doses (up to 300 mg/kg bw/d) in shorter and/or single dose studies. At a dose of 29.4 mg/kg bw/d the LH surge was suppressed completely (Morseth, 1996b). In 26 week and three-week studies, doses of 3.65 mg/kg bw/d and 75 mg/kg bw/d, respectively, resulted in disruption of the oestrous cycle (increased number of days spent in oestrus). Histomorphological changes (increased mammary gland acinar-lobular development, secretory activity and galactocele formation), noted in mammary tissue at doses of 4.2-20 mg/kg bw/d, were indicative of increased exposure of mammary tissue to prolactin and oestrogen. Consistent with the previous key events, an increase in mammary tumours was noted at doses of 3.1 mg/kg bw/d and greater.

The above information, together with the data presented in Table 1, illustrates that there is a strong association between the doses causing attenuation of the LH surge in female SD rats and the increased incidence/earlier onset of mammary tumours.

Table 1: Summary of responses to atrazine in female SD rats

EFFECT	NOAEL/LOAEL	REFERENCE
Altered GnRH and dopamine	< 25/25 mg/kg bw/d (4-day mechanistic study)	Cooper *et al.*, 2007
Attenuation of LH surge	< 300/300 mg/kg bw/d (1-day mechanistic study)	Cooper *et al.*, 2000
	< 50/50 mg/kg bw/d (3-day mechanistic study)	Cooper *et al.*, 2000
	< 6.25/6.25 mg/kg bw/d (4-day mechanistic study)	Cooper *et al.*, 2007
	1.8/3.65 mg/kg bw/d (26-week mechanistic study)	Morseth, 1996c

Disruption of oestrous cycle	< 300/300 mg/kg bw/d (1-day mechanistic study)	Cooper *et al.*, 2000
	< 75/75 mg/kg bw/d (21-day mechanistic study)	Cooper *et al.*, 1996
	1.8/3.65 mg/kg bw/d (26-week mechanistic study)	Morseth, 1996c
Increased pituitary weight	<4.23/4.23 mg/kg bw/d (9 months)	Thakur, 1991
Pituitary adenomas (decreased latency)	4.23/26.23 mg/kg bw/d (9 months)	Thakur, 1991
Increase in mammary gland acinar-lobular development, secretory activity and galactocele formation	3.5/20 mg/kg bw/d (2-year study, 1-year interim kill)	McConnell, 1995
Mammary galactoceles	LOAEL = 4.23 mg/kg bw/d (9 month)	Thakur, 1991
Mammary carcinomas (decreased latency)	LOAEL = 3.79 mg/kg bw/d (12 month)	Thakur, 1992
Increase in incidence of palpable mammary masses	0.5/20 mg/kg bw/d (2-year study, 1-year interim kill)	Thakur, 1991, 1992
Mammary fibroadenomas (decreased latency)	<4.23/4.23 mg/kg bw/d (15 months)	Thakur, 1991
Increase in incidence of mammary tumours	0.5/3.5 mg/kg bw/d (2-year study)	Mayhew, 1986
	1.5/3.1 mg/kg bw/d (2-year study)	Morseth, 1996d, 1998

5.1.3 TEMPORAL ASSOCIATION

The temporal dependence of the biochemical events which occur prior to the detection of mammary tumours in e.g., SD rats that have been treated with atrazine, has been outlined in the US EPA (2003) and JMPR (2007) reports. However, a significant difficulty with using the available toxicity study reports to narrow the effective dose range and time interval of interim observations/sacrifices, is that the doses tested and timing of investigations are often too broad to be particularly informative. In a long-term toxicity study, female rats that received 3.65 mg/kg bw/d atrazine for 21-22 weeks developed the clinical sign of increased oestrus duration and at 26 weeks, attenuation of the LH surge. In rats which received a slightly (15%) higher dose

(4.2 mg/kg bw/d) for 39-40 weeks, increased pituitary alterations and prolactin-associated mammary gland histology (observed at necropsy) show that the findings can be modulated by dose and duration of exposure. The mean onset for mammary adenocarcinomas/adenomas in these rats at this dose was 65 weeks, compared with 73 weeks for the development of mammary fibroadenomas and 76 weeks for carcinomas in control rats.

Dissection of earlier key events has been approached using single-dose studies with disruption of oestrus and attenuation of the LH surge being demonstrated at doses up to 300 mg/kg bw/d or with lower (and short term) repeat-dose treatments (50 and 75 mg/kg bw/d for three days or three weeks, respectively) compared with the NOAEL for mammary tumours of 1.8 mg/kg/d in a 2-year carcinogenicity study. Mechanistic studies described in the reports explored the upstream biochemical events related to the capacity of the pituitary to release LH and showed that LH or PRL secretion from the pituitary are unaffected by atrazine and that the primary target tissue for atrazine is likely to be the hypothalamus. Evidence also indicates that a decrease in catecholamine and/or peptide hormone levels and a decrease in GnRH secretion from the hypothalamus lead to the attenuation of pituitary LH release, persistent oestrus and has been described to be similar to premature female (rodent) reproductive senescence.

Persistent oestrus would facilitate continued secretion of oestrogens from ovarian follicles and lead to increased secretion of prolactin. In rodents, this combination of extended exposure to oestrogens and prolactin is known to lead to hyperstimulation of the mammary glands and earlier appearance and/or increased incidence of mammary tumours which are seen in available carcinogenicity studies using daily dietary treatment of animals for durations of 18 24 months to represent life-time exposures. Currently, the relationship between the appearance of clinical signs (e.g. irregular oestrus to persistent oestrus), biochemical signs (e.g. LH surge attenuation) and the development of rat mammary tumours has not been studied in detail.

Collectively, these observations show that the development of an increased rate of mammary masses in female SD rats that received atrazine was dose and time-dependent within those studies. Findings from the toxicity studies which are consistent with the temporal association described above include the development of mammary tumours with the observations of increased oestrus duration, pituitary alterations and prolactin-associated mammary gland histopathology as interim observations.

5.1.4 STRENGTH, CONSISTENCY, SPECIFICITY OF ASSOCIATION OF TUMOUR RESPONSE WITH KEY EVENTS

Both the US EPA (2003) and the JMPR (2007) reviews concluded that the key events occurred consistently in multiple studies. As stated in the JMPR review (2007, Appendix 1, p86):

> “The key events were observed consistently in a number of studies with differing experimental designs. On the basis of information from the studies described in the monograph, there is sufficient weight-of-evidence that the key events (attenuation of the LH surge, disruption of the oestrous cycle) are linked to the morphological changes in the mammary gland indicative of stimulation of oestrogen and prolactin (increase in acinar-lobular development, increase in secretory activity and galactocele formation) which precede the occurrence of tumours. In addition, there is a substantial independent literature on the role of oestrogen and prolactin in the pathogenesis of mammary tumours in rats. There are no significant contradictory data.”

The available reviews conclude that there is sufficient weight-of-evidence that the key events are linked to the morphological changes in rats which precede the occurrence of mammary tumours in female SD rats.

5.1.5 BIOLOGICAL PLAUSIBILITY AND COHERENCE

The development of mammary tumours in SD rats through the disruption of hypothalamic pituitary axis with subsequent attenuation of the LH surge, disruption of the oestrous cycle and prolonged exposure/stimulation of the mammary gland(s) to endogenous oestrogen and prolactin, is plausible. It is well established that prolonged stimulation of the mammary glands with natural or synthetic oestrogen can lead to the development of adenocarcinomas, and that prolonged exposure to prolactin results in the development of fibroadenomas.

The importance of the role of ovarian oestrogen has been highlighted by studies assessed by Australia (APVMA, 1997), the JMPR and the US EPA. Ovariectomised SD rats did not develop mammary tumours above the background incidence.

5.1.6 OTHER POSSIBLE MODES OF ACTION LEADING TO MAMMARY TUMOURS

5.1.6.1 GENOTOXICITY

Atrazine and several of its metabolites have been comprehensively tested for genotoxicity in bacteria, *Drosophila*, and mammalian cells *in vitro* and *in vivo*. The available evidence provides no support for the hypothesis that atrazine causes direct effects on DNA, leading to cancer (OCSEH, 1996; APVMA 2008; JMPR 2007).

5.1.6.2 DIRECT OESTROGENIC ACTIVITY OF ATRAZINE

Studies evaluated by the OCSEH indicate that atrazine does not bind to the oestrogen receptor and has no intrinsic oestrogenic activity (Oh *et al.*, 2003; O'Connor *et al.*, 2000; Roberge *et al.*, 2004). Although McMullin *et al.* (2004) showed that at high concentrations atrazine can interact directly with oestrogen receptors, and postulated that atrazine can inhibit binding of oestradiol to hypothalamic oestrogen receptors, available evidence indicates that this effect occurs only at concentrations above those known to suppress the LH surge.

5.1.6.3 ANOREXIA

In a study designed to compare the effects of food deprivation with the effects of atrazine, the effects seen after atrazine administration to rats could not be distinguished from the effects of restricted food consumption. Effects observed included reduced serum testosterone and LH and decreased ventral prostate and seminal vesicle weights (Trentacoste, 2001).

Consequently, a panel member on the US EPA SAP meeting in 2000 suggested that the attenuation of the LH surge could result from decreased body weight associated with reduced food intake reported in all moderate and high-dose atrazine studies. The panel member suggested that at high doses atrazine may act on the brain and cause appetite suppression, weight loss and reduced adiposity. Reduced adiposity (body fat) is known to result in reduced leptin levels which are associated with amenorrhea in women (Kopp *et al.*,

1997). The decreased leptin levels in SD rats are thought to result in reduced LH, leading to inhibition of ovulation and sustained oestrous. More recently, GnRH-II has been reported to modulate food intake (Kauffmann, 2004) and more generally, GnRH-II mRNA and peptide levels in the mid-brain and other target areas have been shown to be linked to food intake (Kauffman, 2006).

While the above studies indicate that this MOA is plausible, there is insufficient evidence to determine whether this MOA is relevant to humans because the evidence available suggests that it only occurs at high levels of exposure. Furthermore, women on severe dietary restriction/appetite suppression may develop amenorrhea (compared with the attenuated LH surge seen in SD rats).

5.1.7 UNCERTAINTIES, INCONSISTENCIES, AND DATA GAPS

The JMPR (2007) did not identify any inconsistencies in the database concerning the postulated LH surge MOA, however, the exact mechanism by which atrazine disrupts GnRH secretion in SD rats and any possible inter-relationships with human endocrine mechanisms are not completely understood.

Literature reports propose that atrazine may interfere with somatostatin in SD rats, which is proposed to alter GnRH signalling. Other reports suggest that atrazine may affect GnRH signalling by altering cAMP-dependent protein kinase A (PKA), with a subsequent reduction in GnRH receptor mRNA (in aT3-1 pituitary gonadotrope cells) (Roberge *et al.*, 2004).

In general, although there are similar signaling systems in SD rats and other mammals, there are likely to be complex mechanisms underlying GnRH signalling, with both agonists and antagonists of GnRH-I and -II able to cause anti-proliferative and/or pro-apoptotic effects in breast, endometrial (Fister *et al.*, 2007), ovarian (Kim *et al.*, 2004), and prostate cancers (Kraus, 2004), and cancer in other tissues (Grundker *et al.*, 2002; Gunthert *et al.*, 2005). It has also been suggested that GnRH may influence cancer cells in reproductive tissues and signal terminal differentiation of cells, cell cycle arrest or apoptosis (White *et al.*, 2008). The biological actions of GnRH on cell growth and proliferation are the subject of ongoing research, for example GnRH-II is claimed to be more potent than GnRH-I in inhibiting cell growth in HEK 293 cell lines which express both rat and human GnRH receptors (López de Maturana *et al.*, 2008). The anti-proliferative effects of GnRH are an active field of research.

The above claims of possible anti-proliferative effects of increased GnRH have mostly been achieved using agonists and antagonists of GnRH-I and/or GnRH-II. There is little robust evidence to support the converse case, that is, that a decrease in GnRH levels below normal endogenous or clinical levels [as might be theoretically attributable to high doses of atrazine, or inferred from some epidemiology studies which examined the association of prostate and ovarian cancers with atrazine exposure (APVMA, 2007)] would support proliferative responses in *in vitro* or *in vivo* studies.

It is clear that further research is needed to address these gaps in knowledge concerning the tissue distribution of the GnRH receptors in animals and humans and the role of these receptors and hormones in normal human ageing and in human pathologies.

5.1.8 ASSESSMENT OF POSTULATED MOA

The reports prepared by the JMPR and the US EPA, together with the literature assessed by the OCSEH, provide sufficient evidence that atrazine exposure leads to early development and higher incidence of mammary tumours in female SD rats through disruption of the hypothalamic-pituitary-ovarian axis. The concordance of the dose-response and temporal association of the key events leading to mammary tumours in SD rats further supports this postulated MOA. Hence this is considered by the OCSEH to be a plausible MOA in a test animal and the next stage of the framework can be applied.

5.2 HUMAN RELEVANCE - FUNDAMENTAL, QUALITATIVE DIFFERENCES IN KEY EVENTS

The human relevance of the SD rat mammary cancer MOA has been considered in detail by the JMPR (2007).

The JMPR wrote:

> "The MOA for the formation of mammary tumours in female Sprague-Dawley rats after exposure to atrazine depends on the rat-specific nature of the reproductive cycle and reproductive senescence. Because of the fundamental differences between female Sprague-Dawley rats and humans with regard to both the normal regulation of the pre ovulatory LH surge and reproductive senescence, the mammary tumorigenic effect of atrazine in female Sprague-Dawley rats is not expected to occur in humans."

Further information regarding the qualitative differences in the key events between experimental animals and humans, including tabulated differences between different strains of rats, is available in the JMPR atrazine report (JMPR, 2007).

On the basis of the JMPR report, it is accepted by the OCSEH that this MOA, which leads to mammary tumours in female SD rats, is not relevant to humans.

5.3 HUMAN RELEVANCE - QUANTITATIVE DIFFERENCES IN KINETIC OR DYNAMIC FACTORS

It is not necessary to consider this step in the MOA framework, since the postulated MOA for mammary gland tumours in female SD rats is considered to be strain-specific and therefore not relevant to humans (see Figure 1).

6 MOA2: REPRODUCTIVE EFFECTS / HYPOTHALAMIC FUNCTION

6.1 THE WEIGHT-OF-EVIDENCE FOR AN MOA IN A TEST ANIMAL SPECIES

6.1.1 INTRODUCTION

Atrazine has been shown in a number of animal studies to produce adverse reproductive effects. A list a representative studies which demonstrate these effects is shown in Table 2 (sourced from the review by JMPR, 2007).

Table 2: Observed effects of atrazine on reproductive parameters

EFFECT	LOEL[B] (MG/KG BW/D)	STRAIN/SPECIES	REFERENCE
Altered mammary gland development	0.09[A]	LE rats	Enoch *et al.,* 2007
Delayed preputial separation	12.5	Wistar rats	Stoker *et al.,* 2000
Delayed vaginal opening	30, 100	SD, Wistar rats	Ashby *et al.,* 2002
Full litter resorptions	50	F344	Narotsky *et al.,* 2001
Delayed vaginal opening	50	Wistar rats	Laws *et al.,* 2000
Suppressed suckling-induced prolactin release	50	Wistar rat	Stoker *et al.,* 1999
Delayed mammary gland development Increased oestrogen receptor staining	100	LE rats	Moon *et al.,* 2007
Delayed puberty(mammary gland development, vaginal opening)	100	LE rats	Rayner *et al.,* 2004
Delayed preputial separation	100	SD rats	Trentacoste *et al.,* 2001
Increased pre implantation loss Increased post implantation loss	100 100	F344 rats Holtzman rats	Cummings *et al.,* 2000

[A] mixture of atrazine and metabolites; [B] current NOEL = 0.05 mg/kg bw/d.

The postulated MOA for these effects is that atrazine disrupts hypothalamic function and that this may be responsible for the subsequent reproductive and developmental observations in laboratory animals. The evidence does not suggest that the reproductive and embryogenic developmental effects are the result of inhibition of the LH surge (discussed in the previous section), but rather that they may be due to direct effects on GnRH. This MOA is focused on the linkages between biochemical effects and the one single endpoint of full litter resorptions, being a clinical sign of failed implantation and early embrogenesis.

GnRH is essential to ensure that reproductive tissues are provided with adequate energy sources and to ensure tissues develop appropriately to facilitate reproduction. There are various major forms of GnRH in humans, each with separate receptors. GnRH-II is believed to be the ancestral form of GnRH, has high affinity for GnRH-I & GnRH-II receptors and it is considered to be crucial in a variety of reproductive and non-reproductive functions. GnRH-I and II lead to the secretion of chorionic gonadotrophin (CG) from human and rat trophoblast cells, respectively (Palmon *et al.*, 1994). Continual secretion of CG is needed to maintain *corpora lutea* function post ovum release and throughout the first trimester of a pregnancy. Some of the major regulators of GnRH release; oestrogen, prolactin, and somatostatin, are all known to be affected by atrazine at levels of exposure in excess of the existing NOEL. It has been hypothesised that atrazine exposure may lead to interference in CG levels and theoretically could lead to an adverse effect on the reproductive endpoint of full litter resorptions.

6.1.2 POSTULATED MOA

Atrazine is postulated to inhibit the release, or the signalling of maternal GnRH-II, which results in a decrease in the secretion of CG. If levels of CG become insufficient for the maintenance of the *corpora lutea*, this will result in an inadequate release of progesterone and growth factors (such as prolactin), with the potential consequences of an increase in the number of full litter resorptions.

Application of this postulated MOA to developmental effects in laboratory animals via a similar mechanism has not been previously explored, because all evidence indicates any such effect would only begin to occur at levels of atrazine exposure well above those which are sufficient to trigger mammary tumours in female SD rats (which is the basis of the existing Australian NOEL).

6.1.3 KEY EVENTS IN THE MOA

Listed below are the key events identified in the postulated MOA for reproductive effects. The reproductive endpoint chosen for this MOA analysis was full litter resorptions.

- Changes in somatostatin mRNA;
- Decreased secretion of GnRH-II;
- Decreased CG;
- Decreased ovarian function /affected corpora lutea;
- Decreased progesterone and/or progesterone receptors on endometrium, leading to suboptimal endometrial response;
- Inhibited secretion of prolactin from endometrium and pituitary/increased prolactin in maternal pituitary tissue;
- Full litter resorptions.

6.1.4 DOSE-RESPONSE RELATIONSHIPS

The key events identified in the MOA for reproductive effects listed Table 3 (below) indicate the association between atrazine exposure and alterations in GnRH release and/or reproductive effects.

Table 3: Summary of studies on atrazine relating to the postulated GnRH MOA in tested animal species

EFFECT	NOAEL/LOAEL	SPECIES/STRAIN/SYSTEM	REFERENCE
Changes in somatostatin mRNA	0.01/0.1 mg/kg bw/d GD day 14 to day 21	CD-1 mouse	Giuisi *et al.*, 2006
Altered GnRH and dopamine in cytosol	25 mg/kg bw/d (4 day mechanistic study)	LE rat	Cooper *et al.*, 2007
	5/10 mg/kg bw/d (6 month feeding study)	LE rat	Rodriguez *et al.*, 2005
	100 umol/L (*in vitro*, 12-24 h)	SD PC12 line	Das *et al.*, *2003*
Altered ovaries/*corpora lutea*	2 mg/kg bw/d (19 day feeding study)	Swedish/German Landrace pig	Gojmerac *et al.*, 1996
Decreased progesterone and/or progesterone receptors on endometrium, leading to suboptimal endometrial response	100/300 mg/kg bw/d (2 day feeding study)	SD rat	Tennant *et al.*, 1994
	150/300 mg/kg bw/d (3 day feeding studies)	SD rat	Connor *et al.*, 1996.
Inhibited secretion of prolactin from endometrium & pituitary/increased prolactin in maternal pituitary tissue	200/300 mg/kg bw/d (acute oral treatment)	LE rat	Cooper *et al.*, 2000
	0/50 (3 day oral study)	LE rat	Cooper *et al.*, (2000)
	0/50 (3 day oral study)	LE & SD rat	Cooper *et al.*, 2000
	12/25 mg/kg bw/d (gavage study on PND 1-4)	Wistar rat	Stoker *et al.,* 1999
Full litter resorptions	≥ 50 mg/kg, 200 mg/kg (GD 6-10)	F344 rat SD and LE rat	Narotsky *et al.,* 2001
	5/75 mg/kg bw/d (oral study GD 7-9)	NZ White rabbit	Arthur, 1984

GnRH= gonadotrophin-releasing hormone; mRNA= messenger RNA; GD= gestation day; PND= postnatal day.

Changes in somatostatin mRNA levels have been observed in CD-1 mice treated with 0.1 mg/kg bw/d atrazine and alterations to GnRH levels occurred at doses between 10 mg/kg bw/d in C57Bl/6 mice and

125 mg/kg bw/d in LE rats (acute and sub-chronic studies) and suggest that changes in levels of somatostatin may be a prior signalling event. No information was available on the levels of somatostatin protein levels. Morphological observations of multiple ovarian follicular cysts in various stages of development or regression and/or persistent *corpora lutea* were noted in pigs (2 mg/kg bw/d) and Wistar rats (135 mg/kg bw/d) following treatment with atrazine (Gojmerac *et al.*, 1996). The doses required for subsequent key events (decreased progesterone and prolactin) were generally higher at 300 mg/kg bw/d (SD and LE rats). However, within this dose range, full litter resorptions were observed at 50 mg/kg bw/d in F344 rats and 200 mg/kg bw/d in SD and LE rats. No robust information is available on the effect of atrazine on CG, but it is likely to be modulated in part by any effects of atrazine on GnRH. The absence of any evidence in support of a dose-response to atrazine for these key events suggests the doses selected in the respective studies do not permit finer resolution of the thresholds of atrazine effects for the respective key events. It is recognised that there is the possibility that there are other reproductive and developmental endpoints which could be explored in order to investigate their relevance to this potential MOA.

6.1.5 TEMPORAL ASSOCIATION

The data regarding the temporal association for this postulated MOA which have been evaluated by the OCSEH were insufficient for an association to be established. The key events reported from different species in different studies and in some cases from *in vitro* studies were not comparable. Therefore the robustness of the postulated MOA is limited, based on this criterion.

6.1.6 STRENGTH, CONSISTENCY AND SPECIFICITY OF ASSOCIATION OF THE OBSERVED REPRODUCTIVE EFFECTS WITH PROPOSED KEY EVENTS

The reproductive effects reported in studies used to support this postulated MOA (refer Table 3, above) have been drawn from a range of strains and species but there is insufficient data from the same animal strain/species to fully evaluate the postulated MOA. The observation that increased resorptions occurred in a number of different species and/or strains following overdose exposure to atrazine gives some plausibility to this MOA. However, humans would be unlikely to be exposed to the effective dose ranges required to cause these pathologies through, for example the contamination of drinking water sources.

6.1.7 BIOLOGICAL PLAUSIBILITY AND COHERENCE

It is established that atrazine alters GnRH function in various rat strains and this supports the plausibility of this postulated MOA. It is also established that GnRH is required for the secretion of CG, which is essential for the maintenance of pregnancy. However collectively, the adverse reproductive effects reported, and the poor association between the doses used and response(s) observed, is insufficient to enable the OSCEH to conclude that the postulated MOA is coherent and biologically plausible in a test animal.

6.1.8 OTHER MODES OF ACTION FOR THE OBSERVED REPRODUCTIVE EFFECTS

6.1.8.1 DIRECT OESTROGENIC ACTIVITY OF ATRAZINE

See Section 5.1.6.2.

6.1.8.2 ANOREXIA

See Section 5.1.6.3.

6.1.9 UNCERTAINTIES, INCONSISTENCIES, AND DATA GAPS

While it is known that the developing embryo requires GnRH for synthesis of CG and subsequent ongoing survival, there remains considerable uncertainty in relation to dose response issues and temporal issues related to the proposed key events in this postulated MOA for any reproductive and/or developmental effects in a test animal species. Additional studies to further evaluate its plausibility would be necessary to proceed with this analysis for an MOA which is very speculative and not well supported by the available studies, and which seeks to explain a finding (full litter resorptions), which is only observed to occur at relatively high doses.

6.1.10 ASSESSMENT OF POSTULATED MOA

The postulated MOA for reproductive effects has been tested in this report against only one specific endpoint (full litter resorptions), although many other endpoints of reproductive and developmental toxicity could be hypothesised following atrazine exposure. In order to fully assess each endpoint, separate postulated MOAs would need to be constructed and tested for each endpoint. The limited data available suggest that the endpoint of full litter resorptions is only likely to occur if atrazine levels are significantly above the current NOEL, used to establish the ADI. Despite the considerable data gaps described above, the specific postulated MOA is considered feasible, but unconfirmed at this stage. There are no data to indicate its relevance or otherwise to human health risk assessment.

7 MOA3: TUMOURS AND REPRODUCTIVE EFFECTS / AROMATASE EXPRESSION

7.1 THE WEIGHT-OF-EVIDENCE FOR AN MOA IN A TEST ANIMAL SPECIES

7.1.1 INTRODUCTION

Many published articles have indicated a possible association between atrazine and altered aromatase activity *in vitro*, but there is a notable lack of *in vivo* data (see June 2010 Addendum). Aromatase, or cytochrome $P450_{arom}$, from the CYP19 gene, catalyses a key step in oestrogen biosynthesis, converting testosterone to oestradiol. A postulated MOA for atrazine-induced tumours and reproductive effects is via stimulation of aromatase activity.

7.1.2 POSTULATED MOA

The postulated MOA is that atrazine increases aromatase expression, increasing the production of estrogens and inducing physiological processes that result in an earlier onset and/or higher incidence of mammary adenocarcinomas and fibroadenomas. The same MOA is postulated to result in adverse reproductive and developmental effects, although these effects are not specified in any detail.

7.1.3 KEY EVENTS IN THE MOA

The proposed events in the postulated MOA are based on the information obtained from *in vitro* studies. It is recognised that not all of these events will be measurable and that the final endpoints (increased cancer incidence and/or reproductive/developmental effects) are broad i.e., do not define a particular type of cancer or reproductive/developmental effect. Further data would allow refinement of this postulated MOA.

The proposed events along the causal path are:

- Atrazine binds to and inhibits phosphodiesterase (PDE);
- Inhibition of PDE results in increased cAMP;
- Increased cAMP results in increased transcription of the aromatase gene CYP19, possibly mediated *via* Promoter II (PII or ArPII) which is dependent on Steroidogenic Factor-1 (SF-1);
- Increased aromatase gene expression leads to increased oestrogen as a result of aromatisation of testosterone into oestrogens;
- An oestrogen-rich environment may lead to the induction of cancers and/or potentiation of pre-existing oestrogen-dependent cancers; and/or
- Alterations in the relative levels of sex hormones may lead to reproductive and/or developmental effects.
- APVMA_Bullet1. This is Bullet1, the style with a hanging indent for the first level of bullets; bullets should be used when the list we recommend that you use bullets rather than numbers or characters (except where they are necessary, of course)

7.1.4 DOSE-RESPONSE RELATIONSHIPS

The proposed key events identified above were obtained from *in vitro* mechanistic studies; some of the effects observed in these studies are listed in Table 4 below. The findings of increased aromatase activity in the presence of atrazine can be best described as concentration-related but not concentration-dependent or proportional. This is evident by the relatively low end-point multiple of aromatase induction with any given concentration of atrazine. It was apparent that aromatase activity in the control incubations was often high relative to the activity following incubation in test samples with atrazine (e.g., after 24 h). The OCSEH recognises that the IPCS framework (see Section 3 of this report) is based on *in vivo* findings, however in the absence of *in vivo* data, the postulated MOA has been considered, *albeit* cautiously.

Table 4: Changes in aromatase expression / activity induced by atrazine *in vitro*

CONCENTRATION	EFFECT	REFERENCE
3-30 µM	At 30 µM increased cAMP levels about 1.5-fold above control in H295R cells observed (lower concentrations not tested); effects possibly mediated through inhibition of PDE activity. Expression of aromatase mRNA increased 1.2-, 1.4- and 1.5-fold at 3, 10 and 30 µM atrazine. At 30 µM atrazine, aromatase activity increased about 2.2-fold	Sanderson *et al.*, 2002
10-30 µM	Induced aromatase (CYP19) activity to an apparent maximum of about 2.5-fold in H295R adrenocortical carcinoma cells. Increased levels of CYP19 messenger ribonucleic acid (mRNA) between 1.5- and 2-fold.	Sanderson *et al.*, 2000
10 µM	Increased aromatase in JEG-3 cells after 24 h exposure to 1.6-fold control levels. No significant increase in expression of CYP19 mRNA.	Laville *et al.,* 2006
10–30 µM	Concentration-related induction of aromatase activity in H295R cells. The induction responses were confirmed by similar increases in CYP19 mRNA levels.	Sanderson *et al.*, 2001

7.1.5 TEMPORAL ASSOCIATION

No *in vivo* studies are available and this precluded assessment of any measurable events. Therefore this postulated MOA cannot be assessed for any temporal association, unless/until additional studies are performed. Almost all *in vitro* methods used up to 30 µM atrazine for 24 hours, before harvesting cells to determine aromatase mRNA or enzyme activity. Further, it is noted that no robust *in vitro* studies have investigated the temporal relationship of inhibition of PDE, elevation of cAMP, induction of aromatase and increased aromatase enzyme activity within the same study and same cell type.

Note: See June 2010 Addendum to this MOA report (refer page 3 above) for further comment on the postulated aromatase MOA; which indicates that the limited *in vivo* results do not provide support for any atrazine-induced change in the activity of aromatase, or its increased gene expression in rat brain, testes or adipose tissue.

7.1.6 STRENGTH, CONSISTENCY, SPECIFICITY OF ASSOCIATION OF TOXICOLOGICAL EFFECTS WITH KEY EVENTS

There is information in the literature which supports the hypothesised key events that can be incorporated into the postulated MOA framework, despite the lack of robust *in vivo* data.

Atrazine and its degradation products have been shown to inhibit phosphodiesterase (PDE), the enzyme responsible for hydrolysing cAMP to 5'-AMP. Using fluorescence polarization, it was found that atrazine inhibited PDE with an IC_{50} value of 1.8 µM lower than the IC_{50} of 4.6 µM for the known PDE inhibitor isobutyl-1-methylxanthine (IBMX) (Roberge *et al.,* 2004). The atrazine degradation products desethylatrazine and desisopropylatrazine produced up to 1000-fold less PDE-inhibitory activity than atrazine itself. Another atrazine degradation product DACT, together with desethyldesisopropyl-atrazine were considered to be inactive against PDE. Atrazine was found to be a competitive inhibitor of PDE with an association constant of 85 µM using competitive binding assays and ^{14}C-cAMP, in conjunction with thin layer chromatography (Roberge *et al.,* 2004). Other studies have reported IC_{50} values for PDE inhibition by atrazine of the same order (Breckenridge, 2009, unpublished).

Atrazine significantly inhibited PDE activity in crude homogenates of porcine heart, brain, and lung, but not liver or kidney tissue. Further examination of PDE activity in cytosolic fractions of those tissues revealed that only heart cytosolic PDE activity was susceptible to inhibition by atrazine and was similar to the non-specific PDE inhibitor, IBMX. Dixon plots of the crude tissue homogenates showed that heart and brain PDE were inhibited *via* competitive and non-competitive inhibition mechanisms or mixed inhibition, suggesting that atrazine may be a semi- or non-specific PDE inhibitor. Atrazine did not inhibit PDE from crude tissue homogenates as effectively as IBMX, which suggested the possibility of atrazine susceptible and atrazine non-susceptible forms of PDE. The association constants of PDE for atrazine have been reported to be 55 µM for heart and 310 µM for brain (Roberge *et al.*, 2006), notably higher than the concentrations of atrazine that have been associated with aromatase induction and increased activity in H295R cells (Sanderson *et al.,* 2000, 2001, 2002, see Table 4 above). This observation decreases the likelihood of PDE inhibition *in vivo*.

Induction of aromatase activity by atrazine (30 µM) has been associated with increased cAMP levels in adrenocortical H295R cells (Sanderson *et al.,* 2002) but not in ovarian KGN cells (Morinaga *et al.,* 2004, Ohno *et al.,* 2004, Breckenridge, 2009). In H295R cells, steroidogenic cytochrome P450 (CYP450) isozymes are induced by cAMP analogues or through stimulation of adenylate cyclase by forskolin, which leads to increased cAMP-dependent protein kinase A and increased aromatase gene transcription (Sanderson *et al.,* 2001). In other cell types, e.g., JEG-3 cells, regulation of aromatase transcription is controlled by other factors such as the retinoic acid receptor and IL-β (Interleukin 1β) which might modulate the sensitivity of these cells to xenobiotics which are able to increase intracellular cAMP (Laville *et al.*, 2006). In the nucleus, atrazine has been suggested to bind directly to the NR5A1 family nuclear receptor Steroidogenic Factor-1 (SF-1), enhancing the binding of SF-1 to the aromatase Promoter II (PII or ArPII) (Laville *et al.*, 2006) and recently was shown to cause phosphylation of NR5A receptor subfamily via MAPKinase, PI3Kinase and amplify cAMP levels in JEG3 cells (Suzawa & Ingraham, 2008).

The effects of atrazine on aromatase expression have only been observed in *in vitro* studies and only in cell and tissue types that use the SF-1-dependent PII promoter (see Table 5, below). This limited information

suggests that at the concentrations anticipated to be achieved following ingestion of e.g., contaminated water supplies, atrazine may have limited biologically discernable effects on the whole organism.

7.1.7 BIOLOGICAL PLAUSIBILITY AND COHERENCE

The results available from the *in vitro* studies indicate that some of the events in this MOA occur in isolated cells, but whether these events occur *in vivo* is not known. Complicating the plausibility of this MOA is the observation that aromatase mRNA induction increased protein/enzyme activity only occurs in a select range of cell types and where this induction is known to be influenced by different promoter(s) in different cell types. There are six tissue and cell-specific aromatase promoters in humans and atrazine apparently affects aromatase expression only in cell and tissue types that use the SF-1-dependent PII promoter (Fan *et al.,* 2007).

Table 5: Summary of mammalian aromatase promoter II-like expression of aromatase

TISSUE/CELL TYPE	REFERENCE
Rat ovary (granulosa)	Carlone & Richards, 1997; Falender *et al.*, 2003; Fitzpatrick &Richards, 1994; Lynch *et al.*, 1993
Rat R2C (Leydig cell carcinoma)	Carlone & Richards, 1997; Falender *et al.*, 2003; Fitzpatrick & Richards. 1994
Rat H540 (Leydig tumour cells)	Young & McPhaul, 1997
Human prostate stroma	Ellem *et al.*, 2004
Human prostate tumour (epithelial cells)	Ellem *et al.*, 2004
Human LNCaP (prostate cancer cells)	Ellem *et al.*, 2004
Human Sertoli cells	Gurates *et al.*, 2002
Human endometrial stroma	Gurates *et al.*, 2003
Human *corpus luteum*	Michael *et al.*, 1995
Human preovulatory follicles	Simpson *et al.*, 1994
Human ovary (granulosa)	Bulun *et al.*, 2005; Sanderson *et al.*, 2000
Human adipose tissue fibroblast	Bulun *et al.*, 2005
Human breast tumour fibroblast	Bulun *et al.*, 2005
Human malignant epithelial cells	Bulun *et al.*, 2005
Human breast cancer adipose tissue	Bulun *et al.*, 2005
Human extra-ovarian endometrium	Bulun *et al.*, 2005
Human ovary-derived endometrial cells	Gurates *et al.*, 2003
Human H295R (adrenal corticocarcinoma)	Sanderson *et al.*, 2000

(Derived from Fan *et al.*, 2007)

The available evidence in the literature is that atrazine affects aromatase expression only in cell and tissue types that use the SF-1-dependent PII promoter. However, there are a large number of cancerous and reproductive tissues that express aromatase *via* this mechanism.

The lack of consistent *in vivo* data was recognised by the JMPR (2007) which reported that results from studies in rats that had been treated with atrazine did not demonstrate up-regulation of aromatase expression in brain, testes, or mammary gland tissues. Further assessment of this MOA is not possible in the absence of robust *in vivo* data that demonstrates increase aromatase expression and/or enzyme activity following exposure to atrazine.

7.1.8 OTHER MODES OF ACTION FOR TUMOURS AND REPRODUCTIVE EFFECTS

7.1.8.1 NON-CAMP MEDIATED INCREASE IN AROMATASE ACTIVITY

Increased aromatase expression may not be exclusively dependent on cAMP. Tributyltin (an organometal pesticide) has been shown to cause a dose-dependent decrease in aromatase activity in H295R and KGN cells whereas in human choriocarcinoma cell lines (JEG-3), it caused a dose related increase in aromatase enzyme activity (Laville *et al.,* 2006). While the mechanism responsible was not elucidated, it was reported to be independent of cAMP and also highlights the observation that regulation of aromatase gene expression is tissue specific and under the control of different promoters and cellular transcription factors. Therefore, as cAMP exclusivity has not been confirmed for aromatase expression, this possibility needs further studies to determine the role of other possible promoters in the postulated aromatase MOA.

7.1.8.2 DIRECT OESTROGENIC EFFECTS

See Section 5.1.6.2

7.1.8.3 ANOREXIA

See Section 5.1.6.3

7.1.9 UNCERTAINTIES, INCONSISTENCIES, AND DATA GAPS

The postulated MOA could not be assessed in any detail because of the lack of *in vivo* data to enable the effects of atrazine exposure on aromatase expression in the living organism to be examined. *In vitro* studies provide a useful tool to guide the likely parameters of prospective MOAs, however, their relevance to the *in vivo* situation is difficult to predict. As discussed in the OECD test guidelines (OECD, 2002), the endocrine disrupting ability of chemicals can only be definitively described from *in vivo* studies because of the complex inter-relationships of endocrine signalling pathways.

Aromatase gene expression is regulated in a tissue-specific fashion by different promoters that are differently regulated by cellular transcription factors. As a consequence, depending on the tissue studied, different results can be found when comparing the effect of the same chemical in several cell lines or microsomal assays (Laville *et al.*, 2006). Future definition of the complete structure and organisation of the human CYP19 gene will facilitate further characterisation of various molecular mechanisms by which the tissue-specific and temporal expression of this gene is regulated (Bulun *et al.*, 2003).

Human aromatase activity has been shown to be modulated by several pesticides in *in vitro* studies, with the most potent of those tested being prochloraz, fenbuconazole and propiconazole as aromatase inhibitors and tributyltin, aldrin and chlordane as aromatase inducers (Laville *et al.*, 2006). Most compounds were effective in the range 1–10 µM (0.1-4 ppm) with the exception of tributyltin which was effective at the nanomolar range. Further studies are needed to elucidate the exact mechanisms by which aromatase expression is regulated, in order to establish the plausibility of this postulated MOA.

Further information on PDE, and possibly adenylate cyclase, is also needed to establish whether the postulated MOA is applicable in all cases. PDE is classified into 11 different families, each of which has multiple isoforms, which may or may not be inhibited by atrazine. Each isoform displays a range in specificity between cAMP and cGMP, turnover numbers, inhibition and localization in tissues (Roberge *et al.,* 2004), all of which are likely to affect *in vivo* toxicological outcomes.

Currently, there is no verified clinical evidence of adverse affects caused by atrazine and therefore it is not possible to attribute any specific effects of atrazine to adverse affects on the human reproductive endocrine system in prepubertal, premenopausal, postmenopausal women or in men, as they relate to aromatase induction at any level of exposure.

7.1.10 ASSESSMENT OF POSTULATED MOA

The JMPR briefly described the postulated aromatase MOA and concluded that due to the tissue-specific manner through which aromatase is regulated, the biological significance of atrazine induced aromatase expression remains unclear (JMPR, 2007). Based on the information presented in this report, there is insufficient data to assess the plausibility of this postulated MOA, or its relevance to humans. Background information on the observed association between atrazine exposure, aromatase expression and human cancers has been collected into an appendix (See Appendix 1)

8 CONCLUSIONS

It was not possible to conclude that any of these MOAs were relevant to human exposure to atrazine. While two plausible MOAs were identified, further mechanistic research is needed to bridge data gaps before these MOAs can be properly considered, as summarised below. Based on the data available, a re-evaluation of the existing health values is currently unwarranted. The ADI in place in Australia was established on the NOEL for the most sensitive endpoint in repeat-dose toxicity studies and is considered to be adequately protective against modulation of neuroendocrine-dependent processes by atrazine.

MOA	PLAUSIBLE MOA?	ESTABLISHED IN ANIMALS?	RELEVANT TO HUMANS?	EXCLUDED FROM HUMAN RELEVANCE?
MOA-1 Atrazine putatively alters neurotransmitter and neuropeptide function which regulates LH. Altered LH leads to prolonged prolactin secretion and subsequent stimulation of the mammary gland proliferative changes and increased incidence of mammary adenocarcinomas and fibroadenomas.	Yes	Yes	No. Findings only in one strain of rat. No findings in other rat strains or in mice.	Yes
MOA-2 Atrazine putatively inhibits the release of GnRH which decreases the secretion of CH that may lead to increased resorptions/ abortions.	Possible	No	Possible. Insufficient data.	No data
MOA-3 Atrazine putatively increases aromatase enzyme activity *via* inhibition of phosphodiesterase, which increases the aromatisation of testosterone to oestrogen. An increased oestrogenic environment may favour i) induction of cancers and proliferation of pre-existing oestrogen-dependent cancers, and/or ii) altered relative sex hormone levels may effect reproduction and /or development.	Possible	No	No *in vivo* data.	No data

9 REFERENCES

References marked 'Submitted to WHO by Syngenta Crop Protection AG' were secondary citations from JMPR (2007).

Australian Pesticides and Veterinary Medicines Authority (1997) The NRA review of Atrazine. http://www.apvma.gov.au/chemrev/downloads/atrazine_prs.pdf.

Australian Pesticides and Veterinary Medicines Authority (2002) Review of Atrazine: Final Report. http://www.apvma.gov.au/chemrev/downloads/atrazine_finalApril02.pdf

Australian Pesticides and Veterinary Medicines Authority (2004) The reconsideration of approvals of the active constituent atrazine, registrations of products containing atrazine, and their associated labels. Second Draft Final Review Report. <<http://www.apvma.gov.au/chemrev/downloads/atrazine_draftfinal2.pdf>>

Australian Pesticides and Veterinary Medicines Authority (2008) Atrazine: Final Review Report and Regulatory Decision. (Vol 1) http://www.apvma.gov.au/chemrev/downloads/atrazine_finalMay08.pdf and

Atrazine: Final Technical Report. (Vol 2) http://www.apvma.gov.au/chemrev/downloads/atrazine_finaltech.pdf

Ashby J, Tinwell H, Stevens J, Pastoor T & Breckenridge CB (2002) The effects of atrazine on the sexual maturation of female rats. *Regul. Toxicol. Pharmacol.* **35**, 468–473.

Arthur A (1984) A supplement to a teratology study of atrazine technical in New Zealand White rabbits. Unpublished study prepared by Ciba-Geigy Corporation. 4 p. 40566301 (from USEPA Atrazine IRED, 2003).

Boobis AR, Cohen SM, Dellarco V, McGregor D, Meek ME, Vickers C, Willcocks D, & Farland W (2006) IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol*. **36**, 781–792.

Boobis AR, Doe JE, Heinrich-Hirsch B, Meek ME, Munn M, Ruchirawat M, Schlatter J, Seed S & Vickers C (2008) IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Crit. Rev. Toxicol*. **38**, 87–96.

Breckenridge CB (2009) Evaluation of the potential effects of atrazine on aromatase gene expression and/or aromatase *in vitro* and *in vivo*. Syngenta Crop Protection, Inc. Report Number T001579-08, unpublished.

Bulun SE, Chen D, Lu M, Zhao H, Cheng Y, Demura M, Yilmaz B, Martin R, Utsunomiya H, Thung S, Su E, Marsh E, Hakim A, Yin P, Ishikawa H, Amin S, Imir G, Gurates B, Attar E, Reierstad S, Innes J & Lin Z (2007) Aromatase excess in cancers of breast, endometrium and ovary. *J. Steroid Biochem. Mol. Biol.* **106**:81-96.

Bulun SE, Imir G, Utsunomiya H, Thung S, Gurates B, Tamura M & Lin Z (2005) Aromatase in endometriosis and uterine leiomyomata. *J. Steroid Biochem. Mol. Biol.* **95**:57-62.

Bulun SE, Sebastion S, Takayama K, Suzuki T, Sasano H & Shozu M (2003) The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organisation of promoters. *J. Steroid Biochem. Mol. Biol.* **86:**219-224.

Bulun SE, Takayama K, Suzuki B & Sebastion S (2004) Organisation of the human aromatase P450 *(CYP19)* gene. *Sem. Reprod. Med.* 22(1):5-9.

Canosa LF, Stacey N & Peter RE (2008) Changes in brain mRNA levels of GnRH, PACAP and SS during ovulatory LH and GH surges in goldfish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* [epub].

Coban A & Filipov NM (2007) Dopaminergic toxicity associated with oral exposure to the herbicide atrazine in juvenile male C57BL/6 mice. *J. Neurochem.* **100:**1177–1187.

Cohen SM, Klaunig J, Meek ME, Hill RN, Pastoor T, Lehman-McKeeman L, Bucher J, Longfellow DG, Seed J, Dellarco V, Fenner-Crisp P & Patton D (2004) Evaluating the Human Relevance of Chemically Induced Animal Tumors *Toxicol. Sci.* **78**, 181-186.

Carlone D & Richards J (1997) Evidence that functional interactions of CREB and SF-1 mediate hormone regulated expression of the aromatase gene in granulosa cells and constitutive expression in R2C cells. *J. Steroid Biochem. Mol. Biol.* **61:**223–231.

Cooper RL, Laws SC, Das PC, Narotsky MG, Goldman JM, Tyrey EL & Stoker TE (2007) Atrazine and reproductive function: mode and mechanism of action studies. *Birth Defects Res. B Dev. Reprod. Toxicol*. **80**, 98–112.

Cooper RL, Stoker TE, Goldman JM, Parrish MB & Tyrey L (1996) Effect of atrazine on ovarian function in the rat. *Reprod. Toxicol*. **10**, 257–264.

Cooper RL, Stoker TE, Tyrey L, Goldman JM & McElroy WK (2000) Atrazine disrupts the hypothalamic control of pituitary-ovarian function. *Toxicol. Sci.* **53**, 297–307.

Cummings AM, Rhodes BE & Cooper R L (2000) Effect of atrazine on implantation and early pregnancy in four strains of rats. *Toxicol. Sci.* **58**, 135–143.

Das PC, McElroy WK & Cooper RL (2000) Differential modulation of catecholamines by chlorotriazine herbicides in pheochromocytoma (PC12) cells *in vitro*. *Toxicol. Sci.* **56**, 324–331.

Das PC, McElroy WK & Cooper RL (2003) Potential mechanisms responsible for chlorotriazine-induced alterations in catecholamines in pheochromocytoma (PC12) cells. *Life Sci.* **73**, 3123–3138.

De Roos AJ, Zahm SH, Cantor KP, Weisenburger DD, Holmes FF, Burmeister LF & Blair A (2003) Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men. *Occup. Environ. Med.* **60**, E11.

Du Preez LH, Kunene N, Everson GE, Carr JA, Giesy JP, Gross TS, Hosmer AJ, Kendall RJ, Smith EE, Solomon KR & van der Kraak GJ (2008) Reproduction, larval growth, and reproductive development in African clawed frogs (*Xenopus laevis*) exposed to atrazine. *Chemosphere,* **71,** 546–552.

Ellem SJ, Schmitt JF, Pedersen JS, Frydenberg M & Risbridger GP (2004) Local aromatase expression in human prostate is altered in malignancy. *J. Clin. Endocrinol. Metab.* **89:**2434-41.

Enoch RR, Stanko JP, Greiner SN, Youngblood GL, Rayner JL & Fenton SE (2007) Mammary gland development as a sensitive end point after acute prenatal exposure to an atrazine metabolite mixture in female Long-Evans rats. *Environ. Health Persp*. **115**, 541–547.

European Commission (2003) Review report for the active substance atrazine. SANCO/10496/2003-final. 10 September 2003. Available at http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_atrazine.pdf

Falender A, Lanz R, Malenfant D, Belanger N & Richards J (2003). Differential expression of steroidogenic factor-1 and FTF/LRH-1 in the rodent ovary. *Endocrinol.* **144:** 3598–3610.

Fan WQ, Yanase T, Morinaga H, Gondo S, Okabe T, Nomura M, Komatsu T, Morohashi K-I, Hayes TB, Takayanagi R & Nawata H (2007) Atrazine-induced aromatase expression is SF-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans. *Environ. Health Persp.* **115**,720-727.

Filipov NM, Stewart MA, Carr RL & Sistrunk SC (2007) Dopaminergic toxicity of the herbicide atrazine in rat striatal slices. *Toxicol*. **232:** 68-78.

Fister S, Günthert AR, Emons G & Gründker C (2007) Gonadotropin-releasing hormone type II antagonists induce apoptotic cell death in human endometrial and ovarian cancer cells *in vitro* and *in vivo*. *Cancer Res.* **67:** 1750-1756.

Fitzpatrick S & Richards J (1994) Identification of a cyclic adenosine 3', 5'-monophosphate-response element in the rat aromatase promoter that is required for transcriptional activation in rat granulosa cells and R2C Leydig cells. *Mol. Endocrinol.* **8:** 1309–1319.

Giusi G, Facciolo RM, Canonaco M, Alleva R, Belloni V, Dessi'-Fulgheri F & Santucci D (2006) The Endocrine Disruptor Atrazine Accounts for a Dimorphic Somatostatinergic Neuronal Expression Pattern in Mice. *Toxicol. Sci.* **89:** 257-264.

Grundker C, Gunthert AR, Millar RP & Emons G (2002) Expression of gonadotropin-releasing hormone II (GnRH-II) receptor in human endometrial and ovarian cancer cells and effects of GnRH-II on tumor cell proliferation. *J Clin. Endocrinol. Metabol.* **87:** 1427-1430.

Gojmerac T, Kartal B, Curic S, Zuric M, Kusevic A & Cvetnic Z (1996) Serum biochemical changes associated with cystic ovarian degeneration in pigs after atrazine treatment. *Toxicol. Lett.* **85:**9-15.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA & Vonk A (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Natl. Acad. Sci. (USA),* **99:** 5476–5480.

Hayes T, Haston K, Tsui M, Hoang A, Haeffele C & Vonk A (2003) Atrazine-Induced Hermaphroditism at 0.1 ppb in American Leopard Frogs (*Rana pipiens*): Laboratory and Field Evidence. *Environ. Health Persp.* **111**, 568 – 575

Hyne RV, Spolyarich N, Wilson SP, Patra RW, Byrne M, Gordon G, Sanchez-Bayo F & Palmer CG (2009) Distribution of frogs in rice bays within an irrigated agricultural area: Links to pesticide usage and farm practices. *Environ. Toxicol. Chem.* **28:** 1255-1265.

JMPR (2007) Atrazine monograph prepared by the WHO/FAO Joint Meeting on Pesticide Residues. Pfeil R, Dellarco V, & Davies L (Pre-publication draft used for January 2010 MOA report. Publication now available at http://whqlibdoc.who.int/publications/2009/9789241665230_eng.pdf).

Kauffman AS, Bojkowska K, Wills A & Rissman EF (2006) Gonadotropin-releasing hormone-II messenger ribonucleic acid and protein content in the mammalian brain are modulated by food intake. *Endocrinol.* **147:** 5069-77.

Kettles MA, Browning SR, Prince TS & Horstman SW (1997) Triazine herbicide exposure and breast cancer incidence: an ecologic study of Kentucky counties. *J. Environ. Health Persp.* **105:** 1222-1227.

Kim KY, Choi KC, Park SH, Chou CS, Auersperg N & Leung PC (2004) Type II gonadotropin-releasing hormone stimulates p38 mitogen-activated protein kinase and apoptosis in ovarian cancer cells. *J. Clin. Endocrinol. Metab.* **89:** 3020-6.

Kopp W, Blum WF, von Prittwitz S, Ziegler A, Lübbert H, Emons G, Herzog W, Herpertz S, Deter HC, Remschmidt H & Hebebrand J (1997) Low leptin levels predict amenorrhea in underweight and eating disordered females. *Mol. Psychiatry,* **2:** 267-9.

Kraus S, Levy G, Hanoch T, Naor Z & Seger R (2004) Gonadotropin-releasing hormone induces apoptosis of prostate cancer cells: role of c-Jun NH2-terminal kinase, protein kinase B, and extracellular signal-regulated kinase pathways. *Cancer Res.* **64:** 5736-44.

Laville N, Balaguer P, Brion F, Hinfray N, Casellas C, Porcher J-M & Ait-Aissa S (2006). Modulation of aromatase activity and mRNA by various selected pesticides in the human choriocarcinoma JEG-3 cell line. *Toxicol.* **228:** 98-108.

Laws SC, Ferrell JM, Stoker TE & Cooper RL (2003) Pubertal development in female Wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-*S*-chlorotriazine and hydroxyatrazine. *Toxicol. Sci.*, **76**: 190–200.

Laws SC, Ferrell JM, Stoker TE, Schmid J & Cooper RL (2000) The effects of atrazine on female wistar rats: an evaluation of the protocol for assessing pubertal development and thyroid function. *Toxicol Sci.* **58:** 366-76.

Lenkowski JR, Reed JM, Deininger L & McLaughlin KA (2008) Perturbation of Organogenesis by the Herbicide Atrazine in the Amphibian *Xenopus laevis. Environ. Health Persp.* **116:** 223 – 230.

López de Maturana R, Pawson AJ, Lu ZL, Davidson L, Maudsley S, Morgan K, Langdon SP & Millar RP (2008) Gonadotropin-releasing hormone analog structural determinants of selectivity for inhibition of cell growth: support for the concept of ligand-induced selective signalling. *Mol. Endocrinol.* **22:** 1711-22.

Lynch JP, Lala DS, Peluso JJ, Luo W, Parker KL & White BA (1993) Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. *Mol. Endocrinol.* **7:** 776–786.

MacLennan PA, Delzell E, Sathiakumar N, Myers SL, Cheng H, Grizzle W, Chen VW & Wu XC (2002) Cancer incidence among triazine herbicide manufacturing workers. *J. Occupat. Environ. Med.* **44:** 1048-58.

Mayhew DA (1986) Two-year chronic feeding/oncogenicity study in rats administered atrazine. Unpublished report No. 410-1102 dated 29 April 1986 from American Biogenics Corporation, Decatur, IL, USA. Submitted to WHO by Syngenta Crop Protection AG.

Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD, Longfellow DG, Pastoor T, Seed J & Patton DE (2003) A framework for human relevance analysis of information on carcinogenic modes of action. *Crit Rev. Toxicol.* **33:** 591-653.

McConnell RF (1989) Comparative aspects of contraceptive steroids: effects observed in rats. *Toxicol. Pathol.* **17:** 385–388.

McConnell RF (1995) A histomorphologic reevaluation of the ovaries, uterus, vagina, mammary gland, and pituitary gland from Sprague-Dawley and Fischer 344 female rats treated with atrazine. Unpublished report Nos 483-278 & 483-279 dated 10 March 1995 from Hazleton Laboratories, Vienna, Virginia, USA. Submitted to WHO by Syngenta Crop Protection AG.

McMullin TS, Andersen ME, Nagahara A, Lund TD, Pak T, Handa RJ & Hanneman WH (2004) Evidence that atrazine and diaminochlorotriazine inhibit the estrogen/progesterone induced surge of luteinizing hormone in female Sprague-Dawley rats without changing estrogen receptor action. *Toxicol. Sci.* **79**: 278–286.

Michael MK, Morohashi K & Simpson E (1995) Ad4BP/DF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. *J. Biol. Chem.* **270:** 13561–13566.

Mills PK (1998) Correlation analysis of pesticide use data and cancer incidence rates in California counties. *Arch. Environ. Health.* **53:** 410–413.

Moon HJ, Han SY, Shin JH, Kang IH, Kim TS, Hong JH, Kim SH & Fenton SE (2007) Gestational exposure to nonylphenol causes precocious mammary gland development in female rat offspring. *J. Reprod. Dev.* **53**: 333–344.

Morinaga H, Yanase T, Nomura M, Okabe T, Goto K, Harada N & Nawata H (2004) A benzimidazole fungicide, benomyl, and its metabolite, carbendazim, induce aromatase activity in a human ovarian granulose-like tumor cell line (KGN). *Endocrinol.* **145:** 1860-1869.

Morseth SL (1996a) Evaluation of the luteinizing hormone (LH) in female Sprague-Dawley rats - pilot study: final report. Unpublished report No. 2386-109:6791 dated 18 January 1996 from Corning Hazleton, Inc., Vienna, Virginia, USA. [*From APVMA, 1997*]

Morseth SL (1996b) Evaluation of the luteinizing hormone (LH) surge in female Sprague-Dawley rats-method validation: final report. Unpublished report No. 2386-110 dated 18 January 1996 from Corning Hazleton, Inc., Vienna, Virginia, USA. [*From APVMA, 1997*]

Morseth SL (1996c) Evaluation of the luteinizing hormone (LH) surge in atrazine-exposed female Sprague-Dawley rats: 6-month report. Unpublished report No. 2386-111 dated 25 October 1996 from Corning Hazleton, Inc., Vienna, Virginia, USA. Submitted to WHO by Syngenta Crop Protection AG.

Morseth SL. (1996d) Chronic (12–24 months) study in rats with atrazine technical: final 12-month report. Unpublished report No. 2386-108 dated 24 October 1996 from Corning Hazleton, Inc., Vienna, Virginia, USA. [*From APVMA, 1997*]]

Morseth SL (1998) Chronic (12–24 months) study in rats with atrazine technical. Unpublished report No. 2386-108 dated 15 April 1998 from Corning Hazleton, Inc., Vienna, Virginia, USA. Submitted to WHO by Syngenta Crop Protection AG.

Narotsky MG, Best DS, Guidici DL & Cooper RL (2001) Strain comparisons of atrazine-induced pregnancy loss in the rat. *Reprod. Toxicol.,* **15**: 61–69.

O'Connor DJ, McCormick GC & Green JD (1987) G 30027 – 52-week oral feeding study in dogs. Unpublished report No. MIN 852008 dated 27 October 1987 from Ciba-Geigy Corp., Research Department, Pharmaceuticals Division, Summit, New Jersey, USA. [*From APVMA, 1997*]

O'Connor, JC, Plowchalk, DR, Van Pelt, CS, Davis, LG & Cook, JC. (2000) Role of prolactin in chloro-*S*-triazine rat mammary tumorigenesis. *Drug Chem. Toxicol.* **23:** 575–601.

OCSEH (1996) *Review of the Mammalian Toxicology and Metabolism -Toxicokinetics of Atrazine*. Office of Chemical Safety and Environmental Health, Australian Department of Health and Ageing.

OCSEH (2007) *A Review of additional information on atrazine.* Office of Chemical Safety and Environmental Health, Australian Department of Health and Ageing.

Oh SM, Shim SH & Chung KH (2003) Antiestrogenic action of atrazine and its major metabolites *in vivo*. *J. Health Sci*. **49**: 65–71.

Ohno K, Araki N, Yanase T, Nawata H & Iida M (2004) A novel nonradioactive method for measuring aromatase activity using a human ovarian granulose-like tumor cell line as an estone ELISA. *Toxicol. Sci.* **82:** 443-450.

Oka T, Tooi O, Mitsui N, Miyahara M, Ohnishi Y, Takase M, Kashiwagi A, Shinkai T, Santo N & Iguchi T (2008) Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis. Aquatic Tox.* **87:** 215-226.

Orton F, Carr JA, & Handy RD (2006) Effects of nitrate and atrazine on larval development and sexual differentiation in the Northern Leopard Frog *Rana Pipiens. Enviro. Tox. Chem.* **25:** 65-71.

Palmon A, Aroya BN, Tel-Or S, Burstein Y, Fridkin M & Koch Y (1994) The gene for the neuropeptide gonadotropin-releasing hormone is expressed in the mammary gland of lactating rats. *Proc. Natl. Acad. Sci. (U S A)* **91:** 4994-6.

Rayner JL, Enoch RR, Wolf DC & Fenton SE (2007) Atrazine induced reproductive tract alterations after transplacental and/or lactational exposure in male Long-Evans rats. *Toxicol. Appl. Pharmacol.* **218:** 238-248.

Rayner JL, Wood C & Fenton SE (2004) Exposure parameters necessary for delayed puberty and mammary gland development in Long-Evans rats exposed in utero to atrazine*. Toxicol. Appl. Pharmacol.* **195:** 23–34.

Renner R (2008) Atrazine effects in *Xenopus* aren't reproducible. *Environ. Sci. Technol.* **42:** 3491-3493.

Roberge M, Hakk H & Larsen G (2004) Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the oestrogen receptor. *Toxicol. Lett.* **154:** 61-68.

Roberge MT, Hakk H & Larsen G (2006). Cytosolic and localised inhibition of phosphodiesterase by atrazine in swine tissue homogenates. *Food Chem. Tox.* 44:885-890.

Rodriguez, VM, Thiruchelvam M & Cory-Slechta DA (2005) Sustained exposure to the widely Used herbicide atrazine: altered function and loss of neurons in brain monoamine systems. *Environ. Health Perspect.* **113**: 708–715.

Rusiecki JA, De Roos A, Lee WJ, Dosemeci M, Lubin JH, Hoppin JA, Blair A & Alavanja MCR (2004) Cancer incidence among pesticide applicators exposed to atrazine in the Agricultural Health Study*. J. Natl. Cancer Inst*. **96**: 1375–1382.

Sanderson JT, Boerma J, Lansbergen GWA & van den Berg M (2002) Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicol. Appl. Pharmacol.* **182:** 44-54.

Sanderson JT, Letcher RJ, Heneweer M, Giesy JP & van den Berg M (2001) Effects of Chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. *Environ. Health Persp.* **109:** 1027-1031 .

Sanderson JT, Seinen W, Giesy JP & van den Berg M (2000) 2-Chloro-*s*-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? *Toxicol. Sci.* **54:** 121-127.

Simpkins JW (2000) Relevance of the female Sprague-Dawley (SD) rat for human risk assessment of chloro-s-triazines. A report to Novartis Crop Protection. Unpublished report dated 20 June 2000 from Center for the Neurobiology of Aging, University of Florida, Gainesville, Florida, USA. Submitted to WHO by Syngenta Crop Protection AG.

Simpkins JW, Andersen ME, Brusick D, Eldridge JC, Delzell E, Lamb JC, McConnell RF, Safe S, Tyrey L & Wilkinson C. (2000) Evaluation of hormonal mechanism for mammary tumorigenesis of the chloro-s-triazine

herbicides: Fourth Consensus Panel report. Unpublished report dated 13 January 2000. Submitted to WHO by Syngenta Crop Protection AG.

Simpson E, Mahendroo M, Means G, Kilgore M, Hinshelwood M, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Micheal MD, Mendelson CR, & Bulan SE (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrinol. Rev.* **15:** 342–355.

Simpson ER, Michael MD, Agarwal VR, Hinshelwood MM, Bulun SE, & Zhao Y (1997) Expression of the *CYP19* (aromatase) gene: an unusual case of alternative promoter usage. *FASEB* **11:** 29-36.

Sonich-Mullin C, Fielder R, Wiltse J, Baetcke K, Dempsey J, Fenner-Crisp P, Grant D, Hartley M, Knaap A, Kroese D, Mangelsdorf I, Meek E, Rice JM & Younes M (2001) IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul. Toxicol. Pharmacol.* **34:** 146-52.

Starcevic V, Milosevic V, Brkic B & Severs WB (2002) Somatostatin affects morphology and secretion of pituitary luteinizing hormone (LH) cells in male rats. *Life Sci.* **70:** 3019-27.

Stoker TE, Robinette CL & Cooper RL (1999) Maternal exposure to atrazine during lactation suppresses suckling-induced prolactin release and results in prostatitis in the adult offspring. *Toxicol. Sci.,* **52:** 68–79.

Stoker TE, Robinette CL & Cooper RL (1999) Maternal exposure to atrazine during lactation suppresses suckling-induced prolactin release and results in prostatitis in the adult offspring. *Toxicol. Sci.,* **52**: 68–79.

Stoker TE, Laws SC, Guidici DL & Cooper RL (2000) The effect of atrazine on puberty in male Wistar rats: an evaluation in the protocol for the assessment of pubertal development and thyroid function. *Toxicol. Sci.,* **58**: 50–59.

Suzawa M, & Ingraham HA (2008) The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. *PLoS ONE* **7:** :e2117.

Swan SH, (2006) Semen quality in fertile US men in relation to geographical area and pesticide exposure. *Int. J. Androl.,* **29:** 62-68.

Tennant MK, Hill DS, Eldridge JC, Wetzel LT, Breckenridge CB, & Stevens JT (1994) Chloro-*s*-triazine antagonism of estrogen action: limited interaction with estrogen receptor binding. *J. Toxicol. Environ. Health* **43:** 197–211.

Thakur AK (1991a) Determination of hormone levels in Sprague-Dawley rats treated with atrazine technical. Unpublished report No. HWA 483-278 dated 17 October 1991 from Hazleton Laboratories, Vienna, Virginia, USA. Submitted to WHO by Syngenta Crop Protection AG.

Thakur AK (1992a) Oncogenicity study in Sprague-Dawley rats with atrazine technical. Unpublished report No. HWA 483-275 dated 27 January 1992 from Hazleton Laboratories, Vienna, Virginia, USA. Submitted to WHO by Syngenta Crop Protection AG.

Trentacoste SV, & Friedmann AS, Youker RT, Breckenridge CB & Zirkin BR (2001) Atrazine effects on testosterone levels and androgen-dependent reproductive organs in peripubertal male rats. *J. Androl.* **22:** 142–148.

US EPA (2003) Interim Reregistration Eligibility Decision (IRED) for Atrazine Case No. 0062.

US EPA (unpublished, 2000) Atrazine: Hazard and dose-response assessment and characterisation, SAP Report No. 2000-05, FIFRA Scientific Advisory Panel Meeting, June 27-29, 2000, Sheraton Crystal City Hotel, Arlington, Virginia, US.

US EPA (unpublished, 2000a) Preliminary draft hazard and dose-response assessment and characterization: atrazine.

White CD, Stewart AJ, Lu ZL, Millar RP & Morgan K (2008) Antiproliferative effects of GnRH agonists: prospects and problems for cancer therapy. *Neuroendocrinol*. **88:** 67-79.

Young M & McPhaul M (1997) Definition of the elements required for the activity of the rat aromatase promoter in steroidogenic cell lines. *J Steroid Biochem. Mol. Biol.* **61:** 341–348.

Young HA, Mills PK, Riordan DG & Cress RD (2005) Triazine herbicides and epithelial ovarian cancer risk in central California. *J. Occup. Environ. Med.,* **47:** 1148–1156.

10 APPENDIX 1

Possible association between atrazine exposure, aromatase expression and human cancers

Breast cancer

Breast cancer has been associated with increased plasma oestrogen levels derived from both ovarian and extra-ovarian sources. Following exposure to high levels of atrazine, several strains of rats displayed increased plasma oestrogen levels and an increased incidence of oestrogen-dependent mammary cancers (oestrogen-receptor positive). Local oestrogen production is also dependent on the promoter PII, which contributes to the hormonal stimulation of breast cancers in mammary fibroblasts and adipose tissue. Extra-ovarian oestrogen has a marked mitogenic role in breast tumours. Oestrogen levels in mammary tumours can be 10-fold higher than normal systemic levels in postmenopausal women affected by breast cancer, due to a shift in the expression of different promoters . Promoter PII is abnormally activated in mammary adipose tissue in which a tumour has developed. Although normal mammary tissue does not typically utilise promoter PII, once transformed, breast cancer cells 'induce' the use of PII in adjacent fibroblasts. In this regard, the ability of atrazine to stimulate PII may be a significant factor (Fan *et al.,* 2007) although this could only be the case where atrazine exposure sufficient to trigger this effect is experienced, which is a highly unlikely scenario. Atrazine increases the incidence of mammary cancer in one strain of rodents. One limited cohort study showed that atrazine was associated with breast cancer in a situation where people were sourcing drinking water from a well contaminated with atrazine (Kettles *et al.,* 1997). Conversely, limited or no association has been concluded in other epidemiology studies (JMPR, 2007).

Additional research is needed to further examine the translation of *in vitro* findings to toxic effects *in vivo*, and the doses required to produce that toxicity.

Breast, endometrial and ovarian cancer

Pathogenesis and growth of three common women's cancers (breast, endometrium and ovaries) are linked to oestrogen. The clinical use of aromatase inhibitors to treat breast cancer is associated with an improved prognosis, whereas similar efficacy is not observed in endometrial and ovarian cancers.

In theory the ovary, testis, adipose tissue, skin, hypothalamus and placental tissues express normally aromatase, whereas breast, endometrial and ovarian cancer tissues over-express aromatase and produce local oestrogen, which may exert paracrine and intracrine effects. However, this is not consistent with the therapeutic efficacy of aromatase inhibitors in the treatment of post-menopausal breast cancer, suggesting a higher level of complexity exists.

In cancers of breast, endometrium and ovary, aromatase expression is primarily regulated by strikingly increased activity of the proximally located promoter I.3/II region. Promoters I.3 and II are present only minimally in normal breast adipose tissue and they are stimulated by a cAMP-PKA-dependent pathway *via* PGE_2. In breast adipose fibroblasts exposed to prostaglandin E_2 (PGE_2) secreted by malignant epithelial cells, Protein Kinase C (PKC) is also activated, and potentiates cAMP-PKA-dependent induction of aromatase. Thus, inflammatory substances such as PGE_2 may play important roles in augmenting the local production of oestrogen that promotes breast tumour growth (Bulun *et al.*, 2007).

Additionally, the endothelial-type promoter I.7 is also expressed in breast cancer. Thus breast tumour tissue may be able to utilize any of four promoters (II, I.3, I.7, and I.4, the latter being the breast adipose promoter) for aromatase expression and oestrogen production.

Regional variations in aromatase expression in breast adipose tissue have been observed, with the highest expression in adipose tissue proximal to a tumour (*cf.* distal). It has been postulated that there is 'cross-talk' between a breast tumour and the surrounding adipose cells in terms of the ability of the latter to synthesise oestrogens, and that factors produced by developing breast tumours may set up local gradients of oestrogen biosynthesis in the surrounding adipose tissue *via* paracrine mechanisms.

In contrast, normal human endometrial tissue does not exhibit aromatase activity (Bulun *et al.*, 2005) whereas aromatase mRNA levels and enzyme activity are readily detectable in endometriosis. PGE_2 stimulates both aromatase expression and activity in endometriotic stromal cells via the promoter II region of the aromatase gene. The resulting increase in the local production of oestradiol induces PGE_2 formation and establishes a positive feedback cycle. This may contribute to continuous production of oestradiol and PGE_2. Abnormal aromatase mRNA levels and enzyme activity are present in uterine leiomyomata, which are oestrogen-dependent benign tumours of the myometrium. Leiomyomata and endometriosis are often treated using aromatase inhibitors. The efficacy of this treatment may indicate the participation of aromatase activity in these clinical conditions, although cause and effect are unclear (i.e. increased aromatase activity may be a result, not a cause of the problem).

Prostate cancer

Aromatase expression and activity are low in normal prostate cells, but in malignant prostatic cells increase to levels comparable with those observed in breast cancer. This aromatase activity is associated exclusively with PII and limits the efficacy of anti-androgen treatments for prostate cancer. Also, low levels of oestrogen, when bound to oestrogen receptor-α (ER-α), result in proliferation of the prostate. Thus, in prostate cancer, induction of aromatase via PII in prostate epithelia results in oestrogen synthesis that affects the prostate epithelia in an autocrine/intracrine fashion *via* binding to ER-α (Fan *et al.*, 2007).

Presently, there is no robust association of atrazine exposure with prostate cancer in any species, although atrazine treatment of lactating rat dams has been shown to induce prostatitis in F1 male pups following maternal exposure to relatively high doses (50-100 mg/kg bw day) (Stoker *et al.,* 1999; Rayner *et al.,* 2007).