RSC Expert Panel Proposal on Determining Potential Responses and Adverse Effects at Very Low Dose Exposure to Endocrine Disrupting Chemicals

1. Background

Currently, there is a wide range of toxicologically contentious issues at the boundary between ***Scientific Knowledge*** *and* ***Policy*** with ramifications for health and economics that are interdependent not mutually exclusive. Most of these are concerned with estimating the risks from very low levels of exposures to types of chemicals that frequently also have 'natural' or endogenous analogues with normal physiological functions but might also differ subtly in receptor binding and their mode of action. For instance, very low exposures to dioxin-like ligands of the aryl hydrocarbon receptor are considered of great health concern but operating in an in vivo environment of natural ligands that are probably beneficial, rather than detrimental, to human health[1]. Similarly, the debate about endocrine disruption by synthetic chemicals has been hugely contentious for over two decades with many issues still unclear such as definition and terminology, potency, timing, thresholds for responses and what might be considered adverse outcomes for health. In particular, the proposal that adverse effects for people may occur at exposure levels orders of magnitude far lower (low dose/non monotonic) than traditional No Observed Adverse Effect Levels (NOAEL) identified in standard toxicity studies has proved challenging for regulatory purposes[2-4]. Epidemiological and experimental studies have not been decisive in resolving the issues [5]. Recently, the evidence in the scientific literature for potential non-monotonic dose-responses for food safety has been rigorously evaluated with systematic review methodology of *in vivo*, *in vitro* and epidemiological studies without clear findings so far [6]. Clear regulatory decisions, with potential widespread health and economic implications should be taken on the basis of unequivocal scientific evidence which is still greatly needed in this field and must be based on recognised standards of experimental design and analytical practice [7].

The Expert Panel on Endocrine Disruptor Low Dose Effects was constituted to formulate an approach using established endpoints of endocrine disruption in vivo defining with key events for Adverse Outcome Pathways (AOPs) with a well understood chemical, to clarify the issues and their likelihood for chemical regulation that would arise from adverse responses at exposure doses considerably lower than currently studied. In addition, in anticipation of funding requirements estimates of

potential costs were acquired from commercial organisations thought to be the most appropriate to conduct such studies. This initiative arose from preliminary ECETOC discussions but was independent to avoid perceptions of conflict of interest.

2. Overall purpose

To provide data that describes the key events in endocrine modes of action leading to adverse effects in an intact mammalian organism following exposure to an endocrine active chemical as defined by IPCS/WHO and to clarify the existence and relevance of low dose (LD) and non-monotonic dose responses (NMDR). It is important that this includes generation of a mode of action basis for the dose responses at exposure levels relevant for humans.

Objectives

- Develop and agree an experimental approach to understand how defined key events in one endocrine mode of action fit one with another and lead to an adverse outcome.
- Develop an experimental approach that will allow a description of the quantitative dose response relationship for defined key events in order to deepen our understanding of the relationship between key events.
- To translate the experimental approaches into a protocol, secure funding and generate a database.

Outputs

Authoritative published contributions to the science that provide new insight into the existence of thresholds, the shape of the dose responses for key events and the relevance of these features to adverse outcomes (toxicity).

Outcomes

- A body of science based knowledge that is used for the appropriate protection goals in human and environmental safety.
- The knowledge to put low dose or non-monotonic findings into their proper context and relevance while ensuring human and environmental safety.

3. Selection of the male pituitary-gonadal axis as an endocrine axis to be studied

Taking into account the present scientific and EU policy debates (LD effects and NMDR) and in order to gain a basic understanding of the mechanistic (mode of action) basis of dose-response, the Panel concluded that the focus should be on a single axis and that effort should be concentrated on the **pituitary-gonadal axis** as a first priority. The Panel believed that a highly relevant window of exposure to study was during **perinatal life** since this represents the most vulnerable life stage to

chemical perturbation and that a short-term (developmental) toxicity study on the outcome on males was the most amenable for investigation[8-13]. As male reproductive disorders are/have been a key concern in the EDC area, addressing whether or not such disorders can be induced by dose levels of a compound at or below its NOEL or NOAEL would be an important demonstration of principle.

Knowledge gained through studying a single axis could serve as a template for how to build knowledge and address questions arising from perturbation of other endocrine axes.

It would be important to develop or employ already developed AOPs that could be used to link early key events to underpin Mode of Actions (MoAs) at tissue levels of chemicals leading to toxicity. This would be key to understanding the significance of any changes observed at low doses [14-15]. Many agents of concern are likely to have multiple AOP's or MOA's leading to adverse outcomes. This will therefore be an important consideration for using a single selected agent as a model endocrine – disrupting chemical selection to try and simplify and refine the approach.

With respect to the male pituitary-gonadal axis a number of AOPs resulting in abnormal male sexual development are already being developed (such as the OECD AOP for anti-androgenicity). A series of critical steps were proposed to complement the time line of adverse effects observed:

- Identify existing AOPs and the state of their development including all those that are considered pertinent to the phenotypic endpoint.
- Focus on the gaps in describing the quantitative dose response relationships for each key event
- Understand and define the degree of biological change (natural variability and perturbation above normal homeostatic control) that is needed to trigger the next key event and how this relates to the adverse event; the low dose effects and the 'threshold'
- Understand the shapes of the dose responses for each key event and their relationship to the adverse effect
- Construct PBPK dose response models in order to understand what has been observed in real studies and to predict what may be observed across a range of doses/exposures
- Develop, parameterise and validate in vitro models in order to predict effects at the level of human exposure

It was proposed to develop a greater understanding of the still incomplete AOPs starting from the antagonism of the androgen receptor and (as the molecular initiating event) in male offspring of adult females exposed during gestation. The initiating event usually result in a decrease in the size/weight of the male reproductive tissues, penile length and anogenital distance (AGD) as well as resulting male puberty, in addition to any specific malformation such as hypospadia. Before deciding

on the final details of the experimental protocol, it would be wise to start drafting the androgen disruption AOP including all currently existing knowledge. This should include the sequence of key events as well as their quantitative interrelationships as far as possible. Based on that knowledge and identified data gaps, the details of the experimental protocol and necessary parameters to be studied can be fine-tuned. If it is possible to identify transition points in the dose-response curve for sensitive events, such as gene or protein changes of a prototype AR antagonist, these could be used in subsequent comparator studies with other anti-androgens.

4. Choice of a model endocrine –disrupting chemical

Insight into the AOPs contributing to abnormal male sexual development can be gained by using agents acting via different initiating events (steroidogenesis inhibition / AR antagonism). The chemicals that might be investigated are the "pure" steroidogenesis inhibitors such as certain phthalates (e.g. DEHP), ketoconazole, paracetamol (no AR activity), anti-androgens such as the "pure" AR antagonist flutamide, and some chemicals that may interfere through both molecular initiating events (inhibition of steroidogenesis & AR activity) such as prochloraz and linuron [9, 11-12].

Flutamide. Flutamide is known to induce early postnatal changes in AGD and nipple retention in the rat which consequently induces adverse reproductive developmental effects[11, 13, 16]. It was proposed as one of the best candidates as a model endocrine-disrupting chemical due to the already well described responses as an androgen receptor antagonist, its ADME and its ability to be detected and quantified. Recent studies have also prioritised flutamide for such investigations [17-18]. Any differences in low dose effects (threshold) and in the shape of the dose response (NMDR) to flutamide for key events would be identified as well as the relationship to the ultimate adverse outcome. AGD can be permanent and the pattern of malformation induction in different tissues shows a dose dependency – some of the malformations in the tract are an interference with testosterone binding to the androgen receptor, while others (the more common ones with flutamide) are interference with dihydrotestosterone mediated development. An additional recommendation is to examine in these detailed dose–response studies any changes in fetal gene expression. This is likely to be challenging but ultimately more sensitive and informative of the degree of molecular response required to overcome homeostatic conditions. In the dam, besides reproductive organs, hepatic xenobiotic metabolism could be examined since this is under the regulation of other nuclear receptors (i.e. CAR, PXR, PPAR, etc.) which are important for the metabolism and clearance of chemicals from the exposed animals.

Alternatives. It was recognised that there may be a perception that choice of an 'environmental' chemical would be better than a pharmacological agent. For example, procymidone (a pesticide), a weak AR-antagonist has shown effects for anti-androgenicity at low doses, NMDR for relative testis weight (increased at low doses and decreased at higher doses) and monotonic dose response for AGD, nipple retention etc., which could make it a good candidate. Other possible chemicals that might be used such as DEHP are, however, associated with a greater number of a mixture of effects and mechanisms and possible confounders. Further wider discussion may be required on the choice.

It is anticipated that in order to understand quantitative dose response relationships for key events a combination of in vitro and in vivo approaches will be required. Additional studies using human cells in vitro could give an additional insight into human variability in order to reduce the uncertainty when using such data in human health risk assessment. The standard regulatory testing approaches applied to agrochemicals (for example) will identify endocrine activity and the adverse events that may arise as a consequence. Any supplemental testing including evaluating effects initiated in defined periods of sensitivity will be triggered by the need to know this to inform key events and dose responses leading to the adverse effects.

Studies should use the concept of environmental equivalent dose levels (based on relative potency at the AR for example) in order to provide relevant information on low dose human exposures to EDCs. One drawback of the great potency of flutamide might be that it does not represent those EDCs with much lower AR antagonistic activity and present at much higher absolute levels for a given response. Eventually such a comparison would have to be made.

5. Proposed study design for evaluating potential low dose effects on the reproductive tissues of male rats following in utero exposure

A specific fetal time-window for induction of key events and adverse effects will be established for flutamide (if confirmed) over the currently published dose response range as well as at dose levels orders of magnitude below the current accepted NOAEL. This is a crude metric that can be applied to any other compound that interferes with either androgen production or action. However, it was recognised that a Benchmark Dose (BMD) approach would be required to assess the true dose-response relationship and this would need to be incorporated into the final study design (including adequate sizes of animals and number of doses) and ultimate assessment of findings using appropriate software.

Outline protocol

- Time mated female Sprague-Dawley rats from an accredited breeder will be obtained at least 2 weeks before the study. Rats should be maintained on low EDC bedding and low EDC diet before and during the study in a temperature and light cycle controlled environment. A non-treated group of rats on a diet containing standard background endocrine activity (e.g. through phytoestrogens) could be included to compare any diet-related 'normal' variation in endocrine-related parameters with the flutamide dose-response on a low EDC diet to illustrate changes on ED parameters within normal variation. Animals will be fed and watered ad libitum. Samples of feed and water will be retained and stored at -20 °C for checking possible confounder EDCs. It was recognised that the strain of rat to be used might require further consideration of any possible advantages over confounders.
- At 9-10 weeks of age rats will be randomised and mated. Gestation day (GD) 0.5 will be established on the morning when a copulatory plug has been observed or vaginal semen detected.
- Female rats will be administered flutamide in 1ml /kg of corn oil by oral gavage in the mornings on GDs 15.5 to 18.5 at dose levels of 0, 0.00625, 0.0625, 0.625, 6.25, 12.5, 25 and 50 mg/kg/day) [11]. [However, a toxicokinetic protocol that has also been produced has a proposed range of 0.0001 to 100 mg/kg (7 groups) differing by an order of magnitude and so that a greater number of low doses may be required]. An alternative is to continue dosing until sacrifice which might detect changes in expression that are reversible but are key events for an AOP.
- Dam numbers should be at least 20 per dose group and housed singly as it is likely that at lower dose levels large numbers will be required to detect an interpretable response depending on control variability. Dams will require examination for signs of overt toxicity and behavioural changes as well as daily weighing throughout the dosing regimen and during pregnancy and lactation.
- After birth termed PND1 the number of pups per litter to be recorded and standardized on PND4 to 4 males plus 4 females to minimize litter size effects on growth and development. Pups to be fed by mother until weaning (PND21). There was an alternative view that this may produce greater variation between litters rather than less.

Examinations

- Male pups to be examined for AGD on PND1 or 2, nipple retention on PND 13 (number), and testes descent on PND 16. At PND 25 pup weight, sex, size, penile urethral opening (i.e.

presence or not of hypospadias and severity) to be recorded. AGD of female pups may also be recorded but should not be affected by flutamide exposure although will act as a further control.

- Pups will be culled and blood collected for estimation of hormones in serum such as luteinizing hormone, follicle stimulating hormone and testosterone.
- Penis length, testis position, testis weight, appearance and integrity; presence and length of gubernaculum, size of vas deferens; prostate weight will all be measured as a detailed recording of male parameters. Testes, seminal vesicles and epididymis may be fixed in Modified Davidson's fluid for histopathological examination or when histochemical studies such as cell proliferation (PCNA) or cell death markers are required samples may be quick frozen in liquid nitrogen for frozen sectioning.
- Samples of testes and other relevant tissues from fetuses (such as prostate, epididymis and seminal vesicles) at the end of exposure may be snap-frozen for gene transcript analysis including genes Insl3 or Scarb1, implicated in cryptorchidism, and likely to be part of the toxicity pathways that lead to but prior to the adverse effects. It should be possible after termination at GD19.5 to isolate prioritized tissues from dams and fetuses, i.e. HPG axis related tissues, and analyse tissues separately. Other biomarkers of key events sensitive to anti-androgen effects and leading to adverse effects will be identified to aid construct of time lines and thresholds in vivo for AOPs but will require careful consideration of based line variation unrelated to flutamide exposure. This needs to be carefully discussed as it will be challenging with respect to both appropriate timing and dose and to the expertise required to identify and process small amounts of pertinent tissues.
- Gene expression analysis could be extended to whole genome analysis. Analysis of gene expression could be both general, from the statistical perspective, as well as specific, focusing on relevant endocrine related pathways and gene ontology terms. Samples for gene expression will need to be taken at precise time points relative to last exposure. For instance, 6 hours (end of last exposure day) or 24 hours (next day) are practical, the optimum appears inside that time window in many gene expression studies.
- Dams to be culled. Expression parameters in dams as for fetuses (after delivery or at pre-delivery necropsy) could provide important information on endocrine disruption, and for comparison with dose-response information in their pups.

6. Additional studies to aid AOPs

Toxicokinetics of flutamide in dams and fetuses

Development of AOPs would benefit from flutamide toxicokinetic data of dams and fetal tissues during the dosing period and would be compared with associated key events and adverse endpoints over the range of dose response. This would include maximum exposure to both flutamide and its major active metabolite 2-hydroxyflutamide within a few hours of each oral dosing. Excretion of flutamide reportedly is complete after 3 days with no evidence of accumulation to give a stable body burden [19-20]. Lowest detectable levels will be an important parameter for risk assessment to assess critically an apparent very low dose or monotonic response and to develop PBK modelling scenarios.

A detailed complementary experimental outline for such a study and a formal study protocol have also been prepared by Sue Marty but are not included here although it is anticipated that they would be included.

In vitro approaches

Observations of low-dose responses or non-monotonic dose responses to EDCs have often been made from in vitro models. Their outcomes (including existing knowledge) could be useful, both for designing the draft AOP before finalizing in vivo study design, as well as after the in vivo study has been analysed, to be applied for addressing individual key event relationships to further build the AOP. Fetal testis or the steroidogenic cell line H295R may also be used to investigate the shape of the dose-response curve and correlation could be investigated between the in vitro dose-response and the in vivo dose-response. This would assume that the internal dose of the parent and active metabolites can be monitored in the target tissues from the toxicokinetic investigations. Findings might be used iteratively to feed back into consideration of the key events of in vivo responses being careful to use an appropriate cell in which androgens are proven to regulate expression of a specific mRNA/protein (e.g. PSA in prostatic cell lines).

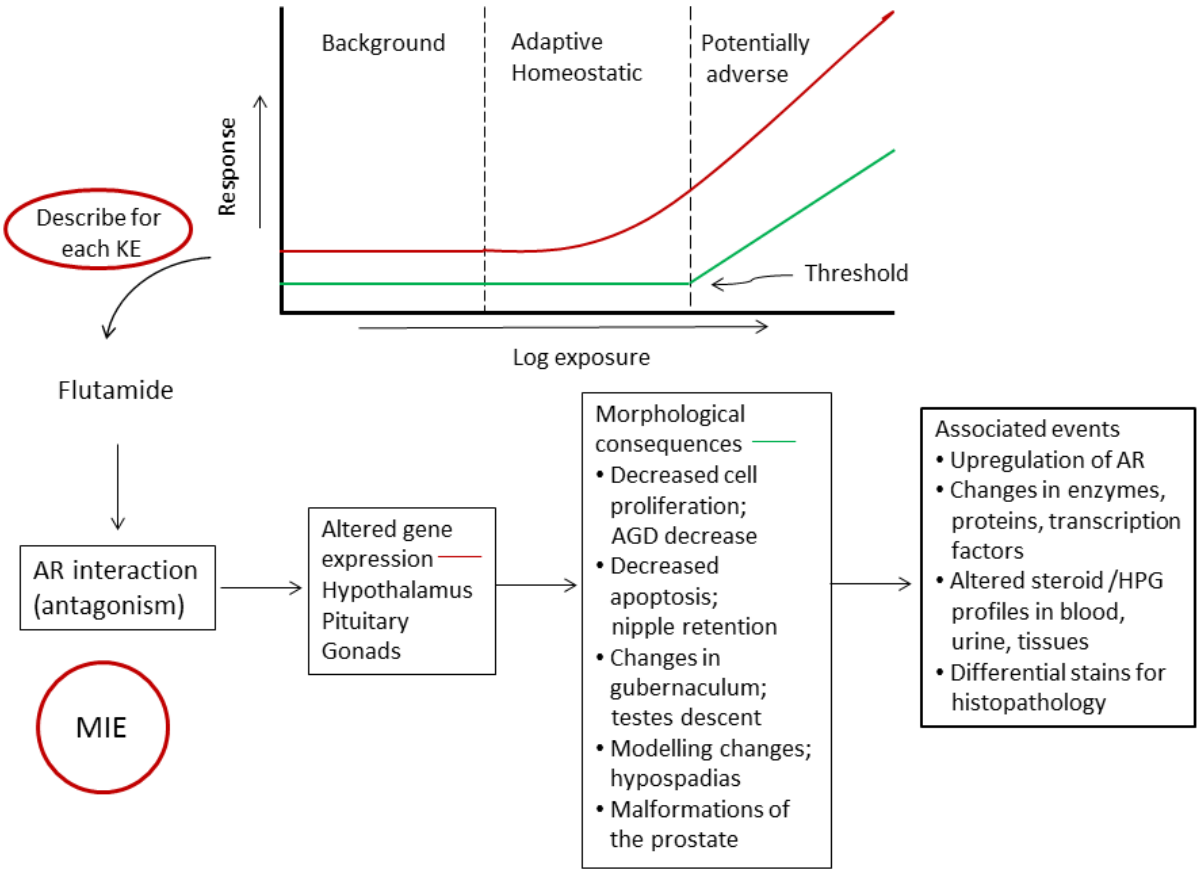
As with in vivo studies, benchmark doses need to be established in these studies with dose levels ranging from effective dose level to hundreds/thousands fold lower and comparison made with other EDCs and realistic human exposure.

7. Summary of approach

Delineation of key events in MIE and subsequent molecular changes in expression of hormones e.g. steroid levels, transcription factors and their targets, down-stream proteins and enzymes for cell processing resulting from disturbances of homeostasis are important goals to construct AOPs for the low dose effects on the reproductive tissues of male rats following in utero exposure to the candidate EDC flutamide.

At low doses these may be adaptive responses i.e. reversible but with greater levels of endocrine disruption, perhaps with a threshold, they may represent pathological outcomes that are non-adaptive and irreversible leading to morphological consequences (see Scheme). It will be important to determine from TK studies both in vivo (including target tissues) and in in vitro models the levels of flutamide/active metabolite (or other EDC) at which cellular homeostasis can be disturbed and crucially in this in vivo model the timing. It is unlikely that this will occur at drug levels below the limits of detection by mass spectrometric techniques. Construction of AOPs from MIE to morphological endpoints should allow comparison against EDC exposure especially at very low levels and extrapolation to humans. In addition, it should enable the recognition of NMDR phenomena and their occurrence and relevance to both the adaptive and morphological endpoints of the in vivo scenario. A significant challenge will be defining intermediate events between gene expression changes and morphological consequences, e.g. posttranscriptional mediators of cellular effects such as proliferation and differentiation leading to organ malformations, which could be aided by knowledge from the field of developmental biology of reproductive organ development.

Scheme


Background
Adaptive Homeostatic
Potentially adverse
Response
Describe for each KE
Threshold
Log exposure
Flutamide
AR interaction (antagonism)
MIE
Altered gene expression
Hypothalamus
Pituitary
Gonads
Morphological consequences
• Decreased cell proliferation; AGD decrease
• Decreased apoptosis; nipple retention
• Changes in gubernaculum; testes descent
• Modelling changes; hypospadias
• Malformations of the prostate
Associated events
• Upregulation of AR
• Changes in enzymes, proteins, transcription factors
• Altered steroid /HPG profiles in blood, urine, tissues
• Differential stains for histopathology

8. Estimated resources required

The above protocol has been sent out to 3 companies for estimates of potential costs. Three proposals have been received (***A***, ***B*** and ***C***). The basic proposal is similar between the companies with the cost of the study ranging from ~€233 000 to ~€295 000In vitro investigations are also proposed, mainly to investigate the MIE (ie AR antagonism) and the cost for these investigations range from ~€10 000 to ~€54 000.

Each proposal is in-line with the experimental protocol proposed by the RSC Expert Panel. Consequently, much information concerning the morphological changes (e.g. AGD, testis descent, hypospadias, penis length…) due to anti-androgen exposure will be gained as well as extensive dose response information concerning the morphological changes. These data should allow the question concerning low dose adverse effects and/or the existence of NMDRs to be addressed for the AOP in question. Determination and/or interrogation of the Key Events leading to the morphological changes however, are not addressed in the basic proposals. Furthermore, the optional extras proposed by the different labs will provide only limited information concerning the KEs, which is likely a reflection of the limited knowledge we currently have concerning the AOP:

- Effects on circulating hormones are offered as an additional measurement by ***B*** (PND 4 and PND 21) and ***A*** (PND4 only).
- Microscopy is offered as a possible measurement by each lab; however no detail is given so the assumption is that standard microscopy will be performed. ***C*** do offer measurement of cell proliferation by PCNA but not sure that this would be the best marker to use for proliferation.
- Gene transcript measurements are offered by ***B*** either as full microarray or on 2 genes (*Insl3* and *Scarb1*). However, no information would be gained concerning dose response curves, NMDR etc. as molecular investigations would be only conducted on 2 groups and 1 male/litter and only at final sacrifice (PND 21). Furthermore no evaluation of other key genes (for example involved in steroidogenesis and the control of this pathway) are offered nor is evaluation of pertinent genes in the pituitary or hypothalamus.

No relevant information concerning exposure (maternal and/or fetal) will be gained. ***B*** offer TK measurements at 3 time points on GD 18 as an optional extra but TK evaluations should be investigated more thoroughly perhaps using subgroups of dams so that a full kinetic evaluation can be discerned. This will include measurements during gestation and after dosing has stopped as well fetal measurements. The male fetuses from these subgroups could also be used for molecular investigations in the relevant tissues to supplement the data at final sacrifice (PND 21).

Overall, each proposal will provide a good starting point to address the question concerning whether or not low dose adverse effects and/or NMDR exist. The inclusion of some refined parameters such as those noted above would add further weight to the eventual findings.

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