Royal Society of Chemistry

Expert Panel on Endocrine Disrupter Low Dose Effects

RSC London 4th June 2014

Introduction

The RSC held a Workshop on Low Doses Effects of Endocrine Disrupters (ED) on June 4th 2014. The workshop was a follow-up to a previous workshop *"ECETOC Expert Panel to Better Understand Endocrine Disrupter Low Doses Effects" held on* 22-23 April 2013. This introduction provides background and context on low dose and non-monotonic effects: recent reviews and conclusions; for the participants of the 2014 workshop.

The objectives of the RSC workshop were:

1. To recapitulate and discuss the content of the US EPA, the US NAS and the Vandenberg reviews;
2. To determine research priorities and select the endocrine axis to be considered first
3. To develop an outline of a research programme
4. To discuss potential sources of funding for the research programme.

1. Discussion of the US EPA, the US NAS and the Vandenberg reviews

The low dose issue has been debated for about 14 years. A rough timeline of key events is:

- 2000: NIEHS/NTP workshop on low doses of EDs
- 2011: USEPA workshop on low dose effects
- 2012: Vandenberg et al published
- 2012: EFSA workshop on low dose effects.
- 2012: NIH/EC/ANSES/UBA/DK workshop (Berlin)
- 2012: Further papers followed Vandenberg et al (e.g. Birnbaum; Rhomberg and Goodman; Zoeller et al)
- 2012: WHO State of the Science on EDs published
- 2013: USEPA State of the Science on NMDRs published
- 2013: DTU analysis of ED effects for REACH
- 2013: ECETOC workshop on low dose effects; proposals for new studies
- 2014: NRC review of EPA report

Low dose effects have been defined as "a biological change occurring in the range of typical human exposures or at doses lower than those typically used in standard testing protocols." NTP (2001), Melnick et al (2002), USEPA (2013). In practice this means effects occurring at

doses below those tested in traditional toxicology assessments. Non-monotonic dose responses (NMDRs) have been defined as “Measured biological effects with dose response curves that contain a point of inflection where the slope of the curve changes sign at one or more points within the tested range” USEPA (2013). NMDRS may occur at low doses or at high doses but are also not necessarily predicted by traditional toxicology tests. A further consideration is whether thresholds exist for endocrine active chemicals. Figure 1 shows a representation of a possible NMDR and illustrates the problem that it poses for risk assessment. Figure 2 provides an example of a NMDR taken from the USEPA review on NMDRs (USEPA, 2013).

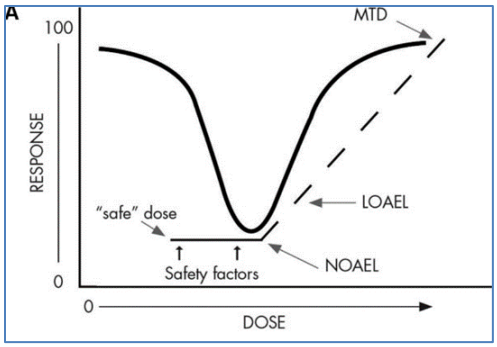

Figure 1. Illustration of NMDR, taken from Vandenberg et al (2013). The dotted line shows a dose response curve that may be obtained from traditional toxicology testing. When the NOAEL (no adverse effect level) is established, several safety factors are then applied to derive a reference dose, i.e. the dose at which exposures are presumed safe. However if the true dose response curve is non-monotonic (solid line) then the reference dose will be in the effect region of the U-shaped curve.

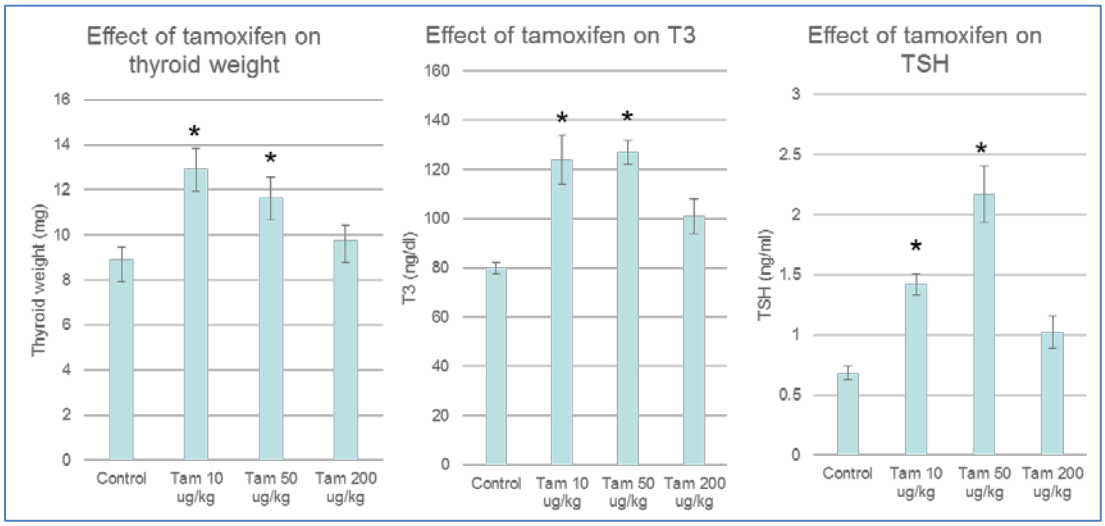

Figure 2. Illustration of NMDRs for Thyroid Endpoints After Exposure to Tamoxifen. Data taken from Kim et al (2002) in USEPA (2013).

These points are all related to the shape of dose-response curves which was the main issue for this RSC workshop to consider.

Before considering prospective experimental approach to address these issues on low dose effects/non-monotonic doses and thresholds the recent reviews on these topics were discussed.

Vandenberg et al (2012), in their wide-ranging review concluded:

- Non-monotonic responses and low-dose effects are remarkably common in studies of natural hormones and EDs.
- Whether low doses of EDs influence certain human disorders is no longer conjecture, because epidemiological studies show that environmental exposures to EDs are associated with human diseases and disabilities.
- When non-monotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses.
- Fundamental changes in chemical testing and safety determination are needed to protect human health.

The Danish Centre for Endocrine Disrupters (DCED, 2013) were more circumspect in their conclusions:

- During development, an assumption of no threshold appears more valid.
- If the mode of action directly involves the receptor, there is likely to be no threshold, but for indirect effects there may be.
- NMDR for EDs exist and have been shown for many ED-mediated *in vitro* and *in vivo* effects.
- There are major limitations in the ability of the current testing requirements to adequately screen for EDs.
- Delayed effects of developmental exposure to EDs are a concern.
- Exposure to EDs during sensitive periods of development may cause effects on developmental programming leading to health effects later in life.

The USEPA (2013) review was the most comprehensive and wide-ranging. They addressed three questions:

1. Do NMDRs exist for chemicals and if so under what conditions do they occur?
2. Do NMDRs capture adverse effects that are not captured using [USEPA's] current chemical testing strategies (i.e., false negatives)?
3. Do NMDRs provide key information that would alter USEPA's current weight of evidence conclusions and risk assessment determinations, either qualitatively or quantitatively?

The findings from the USEPA (2013) review were that NMDRs after exposure to xenobiotics do occur in biological systems but are generally not common. Where NMDRs were observed, biological endpoints closest to the molecular initiating event were more likely to identify a point

of inflection than those effects further downstream. The goal of chemical testing is to identify the potential for hazard after exposure to the xenobiotic of concern, not to identify and describe 100% of all the possible biological effects. They concluded that:

- NMDRs do occur in oestrogen, androgen and thyroid systems in ecological and mammalian studies but are not common.
- NMDRs are not unexpected *in vitro*.
- NMDRs due to compensation may occur.
- NMDRs observed in endocrine endpoints may be biologically relevant and should be evaluated in context with the all of the available data.
- There is currently no reproducible evidence that NMDRs for oestrogen, androgen and thyroid endpoints at low dose are predictive of adverse outcomes.
- Therefore, current testing strategies are unlikely to mischaracterise chemical perturbing these pathways, because of NMDR.

The National Research Council (NRC) recently conducted a peer review of the USEPA (2013) review (NRC, 2014). They applauded the USEPA for undertaking this task but were critical of their approach and claimed it lacked transparency. However, the NRC report does not dispute the main findings of the USEPA review. Their comments were that there was no apparent analytical plan, no criteria for study selection, quality or standard templates for presenting evidence, no criteria for identifying NMDR in advance were presented, more endocrine pathways should have been covered and finally, epidemiological and clinical studies should also have been covered. They suggested that USEPA should consider post-hoc statistical analysis of data to combine evidence from multiple studies, identify resilience and adaptation, distinguish between endpoints that are adverse and those that are adaptive, and indicate how NMDR would be dealt with under current risk assessment guidelines.

The RSC workshop was therefore convened against a background of divergent opinions. The conclusions of each opinion have different consequences for risk assessment procedures. Gaining a greater understanding of dose responses characteristics of EDs (at both low and high ends of the dose-response curve) will advance the science of risk assessment for EDs and will enable adequate protection of human health.

Problem definition

The purpose of the initiative is to develop an understanding of any low dose/non monotonic dose responses (NMDR) following exposure to endocrine active chemicals and the relationship of these to adverse effects seen in intact organisms. Specifically for the protection of human health it is necessary to generate a mechanistic (mode of action) basis for dose responses at human relevant exposure levels. The group endorsed the IPCS/WHO definition of an endocrine disruptor.

2. Endocrine axis to be considered

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 focus should be on a single axis. It was proposed that effort should be concentrated on the pituitary gonadal axis as a first priority although it was recognised that other axes could be investigated at a later stage (e.g. brain (brain development) or pancreas (diabetes)). Knowledge gained through studying a single axis could then serve as a template for how to build knowledge and address questions arising from perturbation of other endocrine axes. The window of exposure to be investigated was considered optimal during perinatal life since it was felt that this represents the most vulnerable life stage to chemical perturbation. In particular, the model system to be used in this research program was discussed at length and it was considered that a short term (developmental) toxicity study was the most appropriate

3. Outline of a research programme

3.1. Potential AOPs that could be used

Considering the male pituitary-gonadal axis a number of AOPs resulting in abnormal male sexual development are currently being developed (such as the OECD AOP for anti-androgenicity). A series of critical steps were proposed to move the issue forward:

- Identifying existing AOPs and the state of their development
- Focus on the gaps and on 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 level of human exposure

It was proposed to develop a greater understanding of the still incomplete AOP starting from the antagonism of the androgen receptor and/or the inhibition of steroidogenesis (as the molecular initiating events) in the male offspring. These initiating events usually result in the decrease in the size/weight of the male reproductive tissues, penile length, ano-genital distance (AGD) in the male offspring that have been exposed to antiandrogens.

3.2. Potential model systems that could be used

Rats would be exposed during Gestation Day 15.5 to 18.5 and the different endpoints would need to be evaluated at different ages when considering the in vivo studies - e.g. Effects on steroidogenesis/steroidogenic enzymes would need to be evaluated during, or at the end of, the treatment period (i.e. measure at either e17.5 or e18.5) whereas AGD cannot be evaluated at this age but can be evaluated at any age beyond (and including) *e*21.5. In addition to the morphological changes (AGD etc..) testicular gene transcripts for a selected number of genes could be investigated (e.g. all the genes involved in steroidogenesis and other genes such as Insl3 or Scarb1 which are likely to be part of the toxicity pathway that lead to the adverse effects). Cellular changes such as cell number in selected tissues could also be investigated using cell proliferation (BrdU) or cell death markers. *In vitro* models using foetal testis or the steroidogenic cell line H295R should also be used to investigate the shape of the dose-response curve for the inhibition of steroidogenesis and correlation could be investigated between the *in vitro* dose-response and the *in vivo* dose-response assuming that the internal dose of the parent and active metabolites can be monitored in the target tissues. Traditional NOAEL and benchmark dose need to be established in these studies with dose levels ranging from effective dose level to hundreds/thousands fold lower than the NOAEL.

3.3. Potential chemicals that could be used

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 recommended to be investigated are the “pure” steroidogenesis inhibitors such as the phthalates (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.

Any differences in low dose effects (threshold), and in the shape of the dose response (NMDR) for key events should be identified as well as the relationship to the ultimate adverse outcome. An additional recommendation was to examine in these detailed dose–response studies in another relevant tissue such as the liver for drug metabolising enzymes since those are under the regulation of other nuclear receptors (i.e. CAR, PXR, PPAR, etc..) which are important for the metabolism and clearance of the chemicals from the exposed animals.

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. 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.

It is anticipated that in order to understand quantitative dose response relationships for key events then a combination of *in vitro* and *in vivo* approaches will be required. 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 should be triggered by the need to know this to inform key events and dose responses leading to the adverse effects.

4. Potential sources of funding:

It was recommended that funding should be diversified and a number of options were discussed including the Cefic LRI and the European Commission.

Workshop Participants

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