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Review

Towards a non-animal risk assessment for anti-androgenic effects in humans

Matthew P. Dent [a,*], Paul L. Carmichael [a], Kevin C. Jones [b], Francis L. Martin [b,*]

[a] Safety and Environmental Assurance Centre, Unilever Colworth Science Park, Bedfordshire MK44 1LQ, UK
[b] Lancaster Environment Centre, Lancaster University, Bailrigg, Lancaster LA1 4YQ, UK

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ABSTRACT

Toxicology testing is undergoing a transformation from a system based on high-dose studies in laboratory animals to one founded primarily on *in vitro* methods that evaluate changes in normal cellular signalling pathways using human-relevant cells or tissues. We review the tools and approaches that could be used to develop a non-animal safety assessment for anti-androgenic effects in humans, with a focus on the molecular initiating events (MIEs) that human disorders indicate critical for normal functioning of the hypothalamus–pituitary–testicular (HPT) axis. *In vitro* test systems exist which can be used to characterize the effects of test chemicals on some MIEs such as androgen receptor antagonism, inhibition of steroidogenic enzymes or 5α-reductase inhibition. When used alongside information describing the pharmacokinetics of a specific chemical exposure, these could be used to inform a pathways-based safety assessment. However, some parts of the HPT axis such as events occurring in the hypothalamus or pituitary are not well represented by accepted *in vitro* methods. *In vitro* tools to characterize perturbations in these events need to be developed before a fully integrated model of the HPT axis can be described. Knowledge gaps also exist which prevent us from using *in vitro* data to predict the type and severity of *in vivo* effect(s) that could arise from a given level of *in vitro* anti-androgenic activity. This means that more work is needed to reliably link an MIE with an adverse outcome. However, especially for chemicals with low anti-androgenic activity, human exposure data can be used to put *in vitro* mode of action data into context for risk-based safety decision-making.

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Abbreviations: 5α-R, 5α-reductase type 2; ADME, absorption, distribution, metabolism and excretion; AG, andrographolide; AGD, anogenital distance; AOP, adverse outcome pathway; AR, androgen receptor; BPAD, biological pathway altering dose; CHH, congenital hypogonadotrophic hypogonadism; DHT, dihydrotestosterone; ED, endocrine disrupter; EDSP, Endocrine Disrupter Screening Program; ER, oestrogen receptor; FSH(R), follicle stimulating hormone (receptor); GnRH(R), gonadotropin releasing hormone (receptor); hCG, human chorionic gonadotropin; HPT, hypothalamus–pituitary–testicular; IGD, isolated gonadotropin releasing hormone deficiency; IHD, isolated hypogonadism disease; IL-6, interleukin-6; LH(R), luteinizing hormone (receptor); LOEC, lowest observed effect concentration; MIE, molecular initiating event; MMTV, mouse mammary tumour virus; NRC, National Research Council; Oct1, octamer-binding transcription factor-1; OECD, Organization for Economic Cooperation and Development; PBPK, physiologically-based pharmacokinetic modelling; QIVIVE, quantitative *in vitro* to *in vivo* extrapolation; T, testosterone; TT21C, toxicity testing in the 21st Century; YAS, yeast androgen screen.

* Corresponding authors.
E-mail addresses: matthew.dent@unilever.com (M.P. Dent), f.martin@lancaster.ac.uk (F.L. Martin).

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1. Introduction

There are a number of human disorders demonstrating that impaired androgen signalling can result in severe and irreversible adverse effects in humans (Table 1). Faulty receptors or enzymes that are part of the androgen-signalling pathway can cause a variety of disorders, including malformations of the internal and external male genitalia, impaired fertility, and increased cancer risk. Pharmaceuticals, plant protection products, and industrial chemicals that interfere with testosterone synthesis, action or metabolism have been shown to cause embryo-foetal malformations, impaired fertility and cancer in experimental animals (Table 2), and for some chemicals the critical effect in the risk assessment could be related to their endocrine activity. Since these are health effects that are devastating to individuals, costly to healthcare systems, and even have the potential to impact the reproductive fitness of our species, it is critical that the risk assessments that underpin the safe use of chemicals that have the potential to alter androgen signalling are based on the best techniques possible.

For many years this has involved performing *in vitro* screening tests to prioritize chemicals for subsequent animal testing. The animal tests routinely used to study perturbations in androgen signalling are amongst the most animal intensive, including prenatal developmental toxicity studies and the extended one generation reproduction toxicity study (OECD Test Guidelines 414 and 443 respectively OECD, 2001, 2012a). Although animal-based safety assessments are generally considered protective of human health, there is a growing dissatisfaction with the lack of mechanistic insight that often exists between the level, duration and timing of human exposure to these chemicals and the nature and incidence of adverse effects. This requires the application of conservative assessment factors to the no-observed-adverse-effect levels in animal studies that are orders of magnitude above systemic exposures reached in humans. Coupled with a desire to reduce and ultimately replace the use of animals in experiments, this has triggered the realization that the process of toxicological risk assessment can and should be improved. This proposal was well-articulated in the 2007 National Academy of Sciences report on 'Toxicity Testing in the 21st Century' (TT21C) (Krewski et al., 2010). In addition, the ban on animal testing of cosmetic ingredients sold in the EU further highlights the need to find new ways of assuring safety for chemicals used in cosmetic products.

The TT21C report made an appeal to transform toxicity testing from a system based on high-dose studies in laboratory animals to one founded primarily on *in vitro* methods that evaluate changes in normal cellular signalling pathways using human-relevant cells or tissues. The term 'toxicity pathway' refers to a normal signalling process, which if significantly perturbed, would result in an adverse cellular outcome. More recently, this concept of pathways-based approaches to risk assessment has been expanded by the description of 'Adverse Outcome Pathways' (AOPs). The starting point of an AOP is a molecular initiating event (MIE), which is the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome *via* a pathway (Allen et al., 2014). The AOP is a cascade of events across different levels of biological organization (subcellular, cellular, suborgan, organ, individual and population) which could result in an adverse outcome (Ankley et al., 2010). AOPs therefore provide an opportunity to provide the mechanistic insight that has historically been lacking in many toxicological risk assessments. The OECD has recently used this universal framework based on AOPs to capture and peer review the mechanistic understanding of specific toxic effects and provide a framework for the evaluation of non-animal methods that aim to predict key events in these pathways. This effort includes several AOPs relating to (anti-)androgenic effects. However, there is no clear view on how these individual AOPs may be used together to provide practical tools to those expected to make safety decisions on the use of chemicals. Adverse effects relating to endocrine-sensitive endpoints represent a particular challenge, since the same MIE may result in different adverse outcomes not just at different exposure levels, but also during different windows of development. In addition, although not unique to the endocrine system, MIEs and key events will be shared across multiple AOPs, making it difficult to see how a linear AOP is of any use in safety decision making. We have been trying to address this by providing practical examples of how TT21C methodologies may be combined to inform a risk assessment decision. These case studies have included p53-mediated DNA damage (Adeleye et al., 2014), and oxidative stress (www.TT21C.org). In this review we consider how TT21C principles could be applied to a new case study: perturbations in androgen signalling.

Table 1
Examples of human disorders causally linked with dysfunction of the HPT axis.

Disturbance in HPT axis	Results in	Symptoms in males	Reference(s)
AR gene mutations (inactivating)	Impaired ligand interaction of AR	Androgen insensitivity syndrome (AIS): Spectrum of phenotypes caused by impaired masculinisation of external genitalia, infertility	Hiort et al. (1996)
5α-R gene mutations	Reduced activity of 5α-R enzyme	5α-R deficiency: Spectrum of phenotypes caused by impaired masculinisation of external genitalia	(Brinkmann, 2001; Azzouni et al., 2012)
GnRHR gene mutations	Impaired ligand interaction of GnRHR	Isolated Hypogonadism Disease (also called idiopathic or congenital hypogonadotrophic hypogonadism (CHH) or isolated or congenital gonadotrophin-releasing hormone deficiency (IGD)): Delayed puberty and infertility	Jin and Yang (2014)
LHR gene mutations (activating)	Activation of LHR in absence of hormone	Familial male-limited precocious puberty: Early puberty (<4 y)	Piersma et al. (2007)
LHR gene mutations (inactivating)	Impaired ligand interaction of LHR	Leydig cell hypoplasia: Spectrum of phenotypes caused by impaired masculinisation of external genitalia, infertility	Piersma et al. (2007)
FSHR gene mutation (inactivating)	Impaired ligand interaction of FSHR	Variable suppression of spermatogenesis and fertility. Note causes complete infertility in females.	Tapanainen et al. (1997)
FSHR gene mutation (activating)	Activation of FSHR in absence of hormone	Extremely rare; only 1 male identified so far	Ulloa-Aguirre et al. (2014)