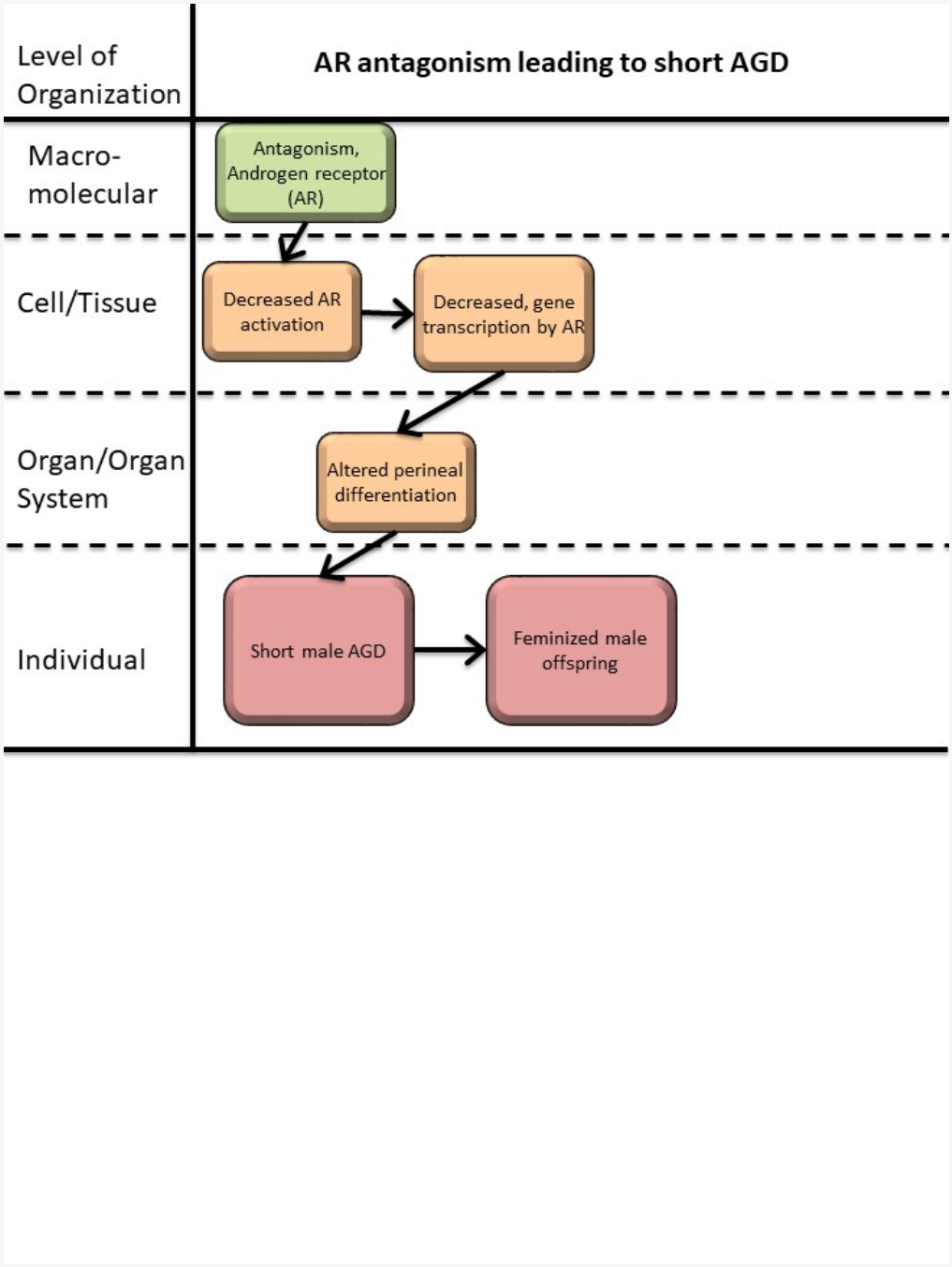
AOP ID and Title:

AOP 306: Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring
Short Title: AR antagonism leading to short AGD

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
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Abstract

This AOP links Androgen receptor antagonism during fetal life with short anogenital distance (AGD) in male offspring. A short AGD around birth is a marker for feminization of male fetuses and is associated with male reproductive disorders, including reduced fertility in adulthood. Although a short AGD is not necessarily 'adverse' from a human health perspective, it is considered an 'adverse outcome' in OECD test guidelines; AGD measurements are mandatory in specific tests for developmental and reproductive toxicity in chemical risk assessment (TG 443, TG 421/422, TG 414).

The AR is a nuclear receptor involved in the transcriptional regulation of various target genes during development and adulthood across species. Its main ligand is testosterone and dihydrotestosterone (DHT). Under normal physiological conditions, testosterone produced mainly by the testicles, is converted in peripheral tissues by 5α-reductase into DHT, which in turn binds AR and activates downstream target genes. AR signaling is necessary for normal masculinization of the developing fetus, including differentiation of the levator ani/bulbocavernosus (LABC) muscle complex in male fetuses. The LABC complex does not develop in the absence, or low levels of, androgen signaling, as in female fetuses.

The key events in this pathway is antagonism of the AR in target cells of the primitive perineal region, which leads to inactivation of the AR and failure to properly masculinize the perineum/LABC complex. In this instance, the local levels of testosterone or DHT may be normal, but prevented from binding the AR.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	26	Antagonism, Androgen receptor	Antagonism, Androgen receptor
	KE	1614	Decrease, androgen receptor activation	Decrease, AR activation
	KE	1687	decrease, transcription of genes by AR	decrease, transcription of genes by AR
	AO	1688	anogenital distance (AGD), decreased	AGD, decreased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Antagonism, Androgen receptor	adjacent	Decrease, androgen receptor activation	High	High
Decrease, androgen receptor activation	adjacent	decrease, transcription of genes by AR	High	Moderate
decrease, transcription of genes by AR	adjacent	anogenital distance (AGD), decreased	Moderate	Low
Antagonism, Androgen receptor	non-adjacent	anogenital distance (AGD), decreased	Moderate	Low
Decrease, androgen receptor activation	non-adjacent	anogenital distance (AGD), decreased		

Stressors

Name	Evidence
Finasteride	High
Flutamide	High

Finasteride

Intrauterine exposure in rats can result in shorter male AGD in male offspring as reported in:

Bowman et al (2003), Toxicol Sci 74:393-406; doi: 10.1093/toxsci/kfg128

Christiansen et al (2009), Environ Health Perspect 117:1839-1846; doi: 10.1289/ehp.0900689

Schwartz et al (2019), Toxicol Sci 169:303-311; doi: 10.1093/toxsci/kfz046

Flutamide

Finasteride is a selective androgen receptor (AR) antagonist (Simard et al 1986) that has been shown to induce shorter male AGD in rats after in utero exposure (Foster & Harris 2005; Hass et al 2007; Kita et al 2016; McIntyre et al 2001; Mylchreest et al 1999; Scott et al 2007; Welsh et al 2007).

References:

Foster & Harris (2005), Toxicol Sci 85:1024-1032; doi: 10.1093/toxsci/kfi159

Hass et al (2007), Environ Health Perspect 115(suppl 1):122-128; doi: 10.1289/ehp.0360

Kita et al (2016), Toxicology 368-369:152-161; doi: 10.1016/j.tox.2016.08.021

McIntyre et al (2001), Toxicol Sci 62:236-249; doi: 10.1093/toxsci/62.2.236

Mylchreest et al (1999), Toxicol Appl Pharmacol 156:81-95; doi: 10.1006/taap.1999.8643

Scott et al (2007), Endocrinology 148:2027-2036; doi: 10.1210/en.2006-1622

Simard et al (1986), Mol Cell Endocrinol 44:261-270; doi: 10.1016/0303-7207(86)90132-2

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Pregnancy	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Male	High

References

1. Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U and **Svingen T** (2019), Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Arch Toxicol* 93: 253-272.

Appendix 1

List of MIEs in this AOP

Event: 26: Antagonism, Androgen receptor

Short Name: Antagonism, Androgen receptor

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	MolecularInitiatingEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	MolecularInitiatingEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male offspring	MolecularInitiatingEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	MolecularInitiatingEvent

Stressors

Name
Mercaptobenzole
Triticonazole
Flusilazole
Epoxiconazole
Prochloraz
Propiconazole
Tebuconazole
Flutamide
Cyproterone acetate
Vinclozolin

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

A large number of drugs and chemicals have been shown to antagonise the AR using various AR reporter gene assays. The AR is specifically targeted in AR-sensitive cancers, for example the use of the anti-androgenic drug flutamide in treating prostate cancer (Alapi & Fischer, 2006). Flutamide has also been used in several rodent in vivo studies showing anti-androgenic effects (feminization of male offspring) evident by e.g. short anogenital distance (AGD) in males (Foster & Harris, 2005; Hass et al, 2007; Kita et al, 2016). QSAR models can predict AR antagonism for a wide range of chemicals, many of which have shown in vitro antagonistic potential (Vinggaard et al, 2008).

Triticonazole

Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.2 uM and an IC50 of 0.3 (±0.01) uM (Draskau et al, 2019)

Flusilazole

Using hAR-EcoScreen Assay, flusilazole showed a LOEC for antagonisms of 0.8 uM and an IC50 of 2.8 (±0.1) uM (Draskau et al, 2019).►

Epoxiconazole

Using transiently AR-transfected CHO cells, epoxiconazole showed a LOEC of 1.6 uM and an IC50 of 10 uM (Kjærstad et al, 2010)

Prochloraz

Using transiently AR-transfected CHO cells, prochloraz showed a LOEC of 6.3 uM and an IC50 of 13 uM (Kjærstad et al, 2010)

Propiconazole

Using transiently AR-transfected CHO cells, propiconazole showed a LOEC of 12.5 uM and an IC50 of 18 uM (Kjærstad et al, 2010)

Tebuconazole

Using transiently AR-transfected CHO cells, tebuconazole showed a LOEC of 3.1 uM and an IC50 of 8.1 uM (Kjærstad et al, 2010)

Flutamide

Using the AR-CALUX reporter assay in antagonism mode, flutamide showed an IC50 of 1.3 uM (Sonneveld et al, 2005).

Cyproterone acetate

Using the AR-CALUX reporter assay in antagonism mode, cyproterone acetate showed an IC50 of 7.1 nM (Sonneveld et al, 2005).

Vinclozolin

Using the AR-CALUX reporter assay in antagonism mode, vinclozolin showed an IC50of 1.0 uM (Sonneveld et al, 2005).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Both the DNA-binding and ligand-binding domains of the AR are highly evolutionary conserved, whereas the transactivation domain show more divergence which may affect AR-mediated gene regulation across species (Davey & Grossmann, 2016). Despite certain inter-species differences, AR function mediated through gene expression is highly conserved, with mutations studies from both humans and rodents showing strong correlation for AR-dependent development and function (Walters et al, 2010). Likewise in fish, androgens are important for development of sexual characteristics (Ogino et al., 2014, 2023). One difference that must be mentioned is that in teleost fish, 11-ketotestosterone is the main androgen in addition to testosterone and DHT and that most teleosts have two *ar* ohnologs, *ara* and *arb*, with arb functioning in a similar manner to the AR in other vertebrates (Ogino et al., 2023).

This KE is applicable for both sexes, across developmental stages into adulthood, in numerous cells and tissues and across

vertebrate taxa

Key Event Description

The androgen receptor (AR) and its function

The AR is a ligand-activated transcription factor belonging to the steroid hormone nuclear receptor family (Davey & Grossmann, 2016). The AR has three domains: the N-terminal domain, the DNA-binding domain and the ligand-binding domain, with the latter being most evolutionary conserved. Testosterone (T) and the more biologically active dihydrotestosterone (DHT) are endogenous ligands for the AR (MacLean et al, 1993; MacLeod et al, 2010; Schwartz et al, 2019). In teleost fishes, 11-ketotestosterone is the second main ligand (Schuppe et al, 2020). Human AR mutations and mouse knock-out models have established a pivotal role for the AR in masculinization and spermatogenesis (Walters et al, 2010). Apart from the essential role for AR in male reproductive development and function (Walters et al, 2010), the AR is also expressed in many other tissues and organs such as bone, muscles, ovaries, and the immune system (Rana et al, 2014).

AR antagonism as Key Event

The main function of the AR is to activate gene transcription in cells. Canonical signaling occurs by ligands (androgens) binding to AR in the cytoplasm which results in translocation to the cell nucleus, receptor dimerization and binding to specific regulatory DNA sequences (Heemers & Tindall, 2007). The gene targets regulated by AR activation depends on cell/tissue type and what stage of development activation occur, and is, for instance, dependent on available co-factors. Apart from the canonical signaling pathway, AR can also initiate cytoplasmic signaling pathways with other functions than the nuclear pathway, for instance rapid change in cell function by ion transport changes (Heinlein & Chang, 2002) and association with Src kinase to activate MAPK/ERK signaling and activation of the PI3K/Akt pathway (Leung & Sadar, 2017).

How it is Measured or Detected

AR antagonism can be measured in vitro by transient or stable transactivation assays to evaluate nuclear receptor activation. There is already a validated assay for AR (ant)agonism adopted by the OECD, Test No. 458: *Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals* (OECD, 2016). The stably transfected AR-EcoScreen^{TM} cells (Satoh et al, 2004) should be used for the assay and are freely available from the Japanese Collection of Research Bioresources (JCRB) Cell Bank under reference number JCRB1328.

Other assays include the AR-CALUX reporter gene assay that is derived from human U2-OS cells stably transfected with the human AR and an AR responsive reporter gene (Sonneveld et al, 2004; van der Burg et al, 2010), various transiently transfected reporter cell lines (Körner et al, 2004), and more.

The recently developed AR dimerization assay provides an assay with an improved ability to measure potential stressor-mediated disruption of dimerization/activation (Lee et al, 2021).

The Rapid Androgen Disruption Activity Reporter (RADAR) assay included in OECD test guideline no. 251 detects AR antagonism in vivo in fish (OECD 2022).

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van der Burg B, Winter R, Man HY, Vangenechten C, Berckmans P, Weimer M, Witters H, van der Linden S (2010) Optimization and prevalidation of the in vitro AR CALUX method to test androgenic and antiandrogenic activity of compounds. *Reprod Toxicol* **30:** 18-24

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List of Key Events in the AOP

Event: 1614: Decrease, androgen receptor activation

Short Name: Decrease, AR activation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:288 - Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male offspring	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

This KE is considered broadly applicable across vertebrate taxa as all vertebrate animals express the AR in numerous cells and tissues where it regulates gene transcription required for developmental processes and functions.

Key Event Description

This KE refers to decreased activation of the androgen receptor (AR) as occurring in complex biological systems such as tissues and organs in vivo. It is thus considered distinct from KEs describing either blocking of AR or decreased androgen synthesis.

The AR is a nuclear transcription factor with canonical AR activation regulated by the binding of the androgens such as testosterone or dihydrotestosterone (DHT). Thus, AR activity can be decreased by reduced levels of steroidal ligands (testosterone, DHT) or the presence of compounds interfering with ligand binding to the receptor (Davey & Grossmann, 2016; Gao et al., 2005).

In the inactive state, AR is sequestered in the cytoplasm of cells by molecular chaperones. In the classical (genomic) AR signaling pathway, AR activation causes dissociation of the chaperones, AR dimerization and translocation to the nucleus to modulate gene expression. AR binds to the androgen response element (Davey & Grossmann, 2016; Gao et al., 2005). AR does not, however, act alone in regulating gene transcription, but together with other co-factors that may differ between cells and tissues and life stages. In this way, the functional consequence of AR activation is cell- and tissue-dependent.

Ligand-bound AR may also associate with cytoplasmic and membrane-bound proteins to initiate cytoplasmic signaling pathways with other functions than the nuclear pathway. Non-genomic AR signaling includes association with Src kinase to activate MAPK/ERK signaling and activation of the PI3K/Akt pathway. Decreased AR activity may therefore be a decrease in the genomic and/or non-genomic AR signaling pathways (Leung & Sadar, 2017).

How it is Measured or Detected

This KE specifically focuses on decreased *in vivo* activation, with most methods that can be used to measure AR activity carried out *in vitro*. They provide indirect information about the KE and are described in lower tier MIE/KEs (see MIE/KE-26 for AR antagonism, KE-1690 for decreased T levels and KE-1613 for decreased dihydrotestosterone levels). In this way, this KE is a placeholder for tissue-specific responses to AR activation or inactivation that will depend on the adverse outcome (AO) for which it is included.

It should be mentioned that the Rapid Androgen Disruption Activity Reporter (RADAR) assay included in OECD test guideline no. 251 detects AR antagonism in vivo in fish (OECD 2022).

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Event: 1687: decrease, transcription of genes by AR

Short Name: decrease, transcription of genes by AR

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent

Biological Context

Level of Biological Organization
Cellular

List of Adverse Outcomes in this AOP

Event: 1688: anogenital distance (AGD), decreased

Short Name: AGD, decreased

Key Event Component

Process	Object	Action
androgen receptor signaling pathway	Musculature of male perineum	disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	AdverseOutcome
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	AdverseOutcome
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	AdverseOutcome
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	AdverseOutcome

Stressors

Name
Butylparaben
p,p'-DDE
Bis(2-ethylhexyl) phthalate
Dexamethasone
Fenitrothion
Finasteride
Flutamide

Name
Ketoconazole
Linuron
Prochloraz
Procymidone
Triticonazole
Vinclozolin
di-n-hexyl phthalate
Dicyclohexyl phthalate
butyl benzyl phthalate
monobenzyl phthalate
di-n-heptyl phthalate

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
perineum

Evidence for Perturbation by Stressor

Butylparaben

Butylparaben has been shown to cause decreased male AGD in rats following intrauterine exposure to 500 and 1000 mg/kg bw/day (Boberg et al, 2016; Zhang et al, 2014). A separate study using 600 mg/kg bw/day did not see an effect on male AGD (Boberg et al, 2008).

p,p'-DDE

p,p,DDE has been shown to cause decreased male AGD in rats following intrauterine exposure to 100-200 mg/kg bw/day (Loeffler & Peterson, 1999; Wolf et al, 1999).

Bis(2-ethylhexyl) phthalate

DEHP has been shown to cause decreased male AGD in rats following intrauterine exposure to 300-1500 mg/kg bw/day (Christiansen et al, 2010; Gray et al, 2000; Howdeshell et al, 2007; Jarfelt et al, 2005; Kita et al, 2016; Li et al, 2013; Lin et al, 2009; Moore et al, 2001; Nardelli et al, 2017; Saillenfait et al, 2009; Wolf et al, 1999).

Dexamethasone

Dexamethasone has been shown to cause decreased male AGD in rats following intrauterine exposure to 0.1 mg/kg bw/day (Van den Driesche et al, 2012).

Fenitrothion

Fenitrothion has been shown to cause decreased male AGD in rats following intrauterine exposure to 25 mg/kg bw/day (Turner et al, 2002).

Finasteride

Finasteride has been shown to cause decreased male AGD in rats following intrauterine exposure to 100 mg/kg bw/day (Bowman et al, 2003).

Flutamide

Flutamide has been shown to cause decreased male AGD in rats following intrauterine exposure to doses between 16-100 mg/kg bw/day (Foster & Harris, 2005; Hass et al, 2007; Kita et al, 2016; McIntyre et al, 2001; Mylchreest et al, 1999; Scott et al, 2007; Welsh et al, 2007).

Ketoconazole

Ketoconazole has been shown to cause decreased male AGD in rats following intrauterine exposure to 50 mg/kg bw/day in one study (Taxvig et al, 2008), but no effect in another study using same dose (Wolf et al, 1999).

Linuron

Linuron has been shown to cause decreased male AGD in rats following intrauterine exposure to 50-100 mg/kg bw/day (Hotchkiss et al, 2004; McIntyre et al, 2002; Wolf et al, 1999).

Prochloraz

Prochloraz has been shown to cause decreased male AGD in rats following intrauterine exposure to 150-250 mg/kg bw/day (Laier et al, 2006; Noriega et al, 2005).

Procymidone

Procymidone has been shown to cause decreased male AGD in rats following intrauterine exposure to doses between 50-150 mg/kg bw/day (Hass et al, 2012; Hass et al, 2007; Wolf et al, 1999).

Triticonazole

Triticonazole has been shown to cause decreased male AGD in rats following intrauterine exposure to 150 and 450 mg/kg bw/day (Draskau et al, 2019).

Vinclozolin

Vinclozolin has been shown to cause decreased male AGD in rats following intrauterine exposure to doses between 50-200 mg/kg bw/day (Christiansen et al, 2009; Gray et al, 1994; Hass et al, 2007; Matsuura et al, 2005; Ostby et al, 1999; Schneider et al, 2011; Wolf et al, 2004).

di-n-hexyl phthalate

DnHP has been shown to cause decreased male AGD in rats following intrauterine exposure to 500-750 mg/kg bw/day (Saillenfait et al, 2009a; Saillenfait et al, 2009b).

Dicyclohexyl phthalate

DCHP has been shown to cause decreased male AGD in rats following intrauterine exposure to 350-750 mg/kg bw/day (Aydoğan Ahbab & Barlas, 2015; Hoshino et al, 2005; Saillenfait et al, 2009a).

butyl benzyl phthalate

BBP has been shown to cause decreased male AGD in rats following intrauterine exposure to 500-1000 mg/kg bw/day (Ema & Miyawaki, 2002; Gray et al, 2000; Hotchkiss et al, 2004; Nagao et al, 2000; Tyl et al, 2004).

monobenzyl phthalate

MBeP has been shown to cause decreased male AGD in rats following intrauterine exposure to 375 mg/kg bw/day (Ema et al, 2003).

di-n-heptyl phthalate

DHPP has been shown to cause decreased male AGD in rats following intrauterine exposure to 1000 mg/kg bw/day (Saillenfait et al, 2011).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

A short AGD in male offspring is a marker of insufficient androgen action during critical fetal developmental stages (Schwartz et al, 2019; Welsh et al, 2008). A short AGD is thus a sign of undervirilization, which is also associated with a series of male reproductive disorders, including genital malformations and infertility in humans (Juul et al, 2014; Skakkebaek et al, 2001).

There are numerous human epidemiological studies showing associations with intrauterine exposure to anti-androgenic chemicals and short AGD in newborn boys alongside other reproductive disorders (Schwartz et al, 2019). This underscores the human relevance of this AO. However, in reproductive toxicity studies and chemical risk assessment, rodents (rats and mice) are what is tested on. The list of chemicals inducing short male AGD in male rat offspring is extensive, as evidenced by the 'stressor' list and reviewed by (Schwartz et al, 2019).

Key Event Description

The anogenital distance (AGD) refers to the distance between anus and the external genitalia. In rodents and humans, the male AGD is approximately twice the length as the female AGD (Salazar-Martinez et al, 2004; Schwartz et al, 2019). This sexual dimorphisms is a consequence of sex hormone-dependent development of secondary sexual characteristics (Schwartz et al, 2019). In males, it is believed that androgens (primarily DHT) activate AR-positive cells in non-myotic cells in the fetal perineum region to initiate differentiation of the perineal *levator ani* and *bulbocavernosus* (LABC) muscle complex (Ipulan et al, 2014). This AR-dependent process occurs within a critical window of development, around gestational days 15-18 in rats (MacLeod et al, 2010). In females, the absence of DHT prevents this masculinization effect from occurring.

The involvement of androgens in masculinization of the male fetus, including the perineum, has been known for a very long time (Jost, 1953), and AGD has historically been used to, for instance, sex newborn kittens. It is now well established that the AGD in newborns is a proxy readout for the intrauterine sex hormone milieu the fetus was developing. Too low androgen levels in XY fetuses makes the male AGD shorter, whereas excess (ectopic) androgen levels in XX fetuses makes the female AGD longer, in humans and rodents (Schwartz et al, 2019).

How it is Measured or Detected

The AGD is a morphometric measurement carried out by trained technicians (rodents) or medical staff (humans).

In rodent studies AGD is assessed as the distance between the genital papilla and the anus, and measured using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) is often calculated by dividing AGD by the cube root of the body weight. It is important in statistical analysis to use litter as the statistical unit. This is done when more than one pup from each litter is examined. Statistical analyses is adjusted using litter as an independent, random and nested factor. AGD are analysed using body weight as covariate as recommended in Guidance Document 151 (OECD, 2013).

Regulatory Significance of the AO

In regulatory toxicology, the AGD is mandatory inclusions in OECD test guidelines used to test for developmental and reproductive toxicity of chemicals. Guidelines include 'TG 443 extended one-generation study', 'TG 421/422 reproductive toxicity screening studies' and 'TG 414 developmental toxicity study'.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2130: Antagonism, Androgen receptor leads to Decrease, AR activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	High
Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to hypospadias in male offspring	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

The AR is expressed throughout vertebrate taxa and its DNA and ligand binding domains are highly conserved (Davey & Grossmann, 2016). AR activity is important for sexual development and reproduction in both males and females (Prizant et al., 2014; Walters et al., 2010). AR function is required during development, puberty and adulthood.

Key Event Relationship Description

The androgen receptor (AR) is a ligand-activated steroid hormone nuclear receptor (Davey & Grossmann, 2016). In its inactive state, the AR locates to the cytoplasm (Roy et al., 2001). When activated, the AR translocates to the nucleus, dimerizes, and, together with co-regulators, binds to specific DNA regulatory sequences to regulate gene transcription (Davey & Grossmann, 2016) (Lamont and Tindall, 2010). This is considered the canonical AR signaling pathway. The AR can also activate non-genomic signalling (Jin et al., 2013). However, this KER focuses on the canonical pathway.

The two main AR ligands are the androgens testosterone (T) and the more potent dihydrotestosterone (DHT), whereas another main androgen in teleost fishes is 11-ketotestosterone (Schuppe et al., 2020). Androgens bind to the AR to mediate downstream androgenic responses, such as male development and masculinization (Rey, 2021; Walters et al., 2010). Antagonism of the AR would decrease AR activation and therefore the downstream AR-mediated effects.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high.

The AR belongs to the steroid hormone nuclear receptor family. The AR has 3 main domains essential for its activity, the N-terminal domain, the ligand binding domain, and the DNA binding domain (Roy et al., 2001). Ligands, such as T and DHT, must bind to the ligand binding domain to activate AR allowing it to fulfill its role as a transcription factor. The binding of the ligand induces a change in AR conformation allowing it to translocate to the nucleus and congregate into a subnuclear compartment (Marcelli et al., 2006; Roy et al., 2001) homodimerize and bind to the DNA target sequences and regulate transcription of target genes. Regulation of AR target genes is greatly facilitated by numerous co-factors. Active AR signaling is essential for male reproduction and sexual development and is also crucial in several other tissues and organs such as ovaries, the immune system, bones, and muscles (Ogino et al., 2011; Prizant et al., 2014; Rey, 2021; William H. Walker, 2021).

AR antagonists can compete with or prevent in different ways AR ligand binding, thereby preventing AR activation. Antagonism of the AR can prevent translocation to the nucleus, compartmentalization, dimerization and DNA binding. Consequently, AR cannot regulate transcription of target genes and androgen signalling is disrupted. This can be observed using different AR activation assays such as AR dimerization, translocation, DNA binding or transcriptional activity assays (Brown et al., 2023; *OECD*, 2020).

Empirical Evidence

The empirical evidence for this KER is considered high

The effects of AR antagonism have been shown in many studies *in vivo* and *in vitro*.

Several stressors can act as antagonists of the AR and lead to decreased AR activation. Some of these are detailed in an AOP key event relationship report by (Pedersen et al., 2022) and shown below, exhibiting evidence of dose-concordance:

Stressors

- Cyproterone acetate: Using the AR-CALUX reporter assay in antagonism mode, cyproterone acetate showed an IC50 of 7.1 nM (Sonneveld, 2005)
- Epoxiconazole: Using transiently AR-transfected CHO cells, epoxiconazole showed a LOEC of 1.6 µM and an IC50 of 10 µM (Kjærstad et al., 2010).
- Flutamide: Using the AR-CALUX reporter assay in antagonism mode, flutamide showed an IC50 of 1.3 µM (Sonneveld, 2005).
- Flusilazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.8 µM and an IC50 of 2.8 (±0.1) µM (Draskau et al., 2019).
- Prochloraz: Using transiently AR-transfected CHO cells, prochloraz showed a LOEC of 6.3 µM and an IC50 of 13 µM (Kjærstad et al., 2010).
- Propiconazole: Using transiently AR-transfected CHO cells, propiconazole showed a LOEC of 12.5 µM and an IC50 of 18 µM (Kjærstad et al., 2010).
- Tebuconazole: Using transiently AR-transfected CHO cells, tebuconazole showed a LOEC of 3.1 µM and an IC50 of 8.1 µM (Kjærstad et al., 2010).
- Triticonazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.2 µM and an IC50 of 0.3 (±0.01) µM (Draskau et al., 2019).
- Vinclozolin: Using the AR-CALUX reporter assay in antagonism mode, vinclozolin showed an IC50of 1.0 µM(Sonneveld, 2005).”(Pedersen et al., 2022)

Other evidence:

Known AR antagonists are used for treatment of AR-sensitive cancers such as flutamide for prostate cancer (Mahler et al., 1998).

Quantitative Understanding of the Linkage

Response-response relationship

The quantitative relationship between AR antagonism and AR activation will depend on the type of antagonist.

Time-scale

Nuclear translocation in HeLa cells transfected with AR-GFP show a response within 2 hours after ligand exposure (Marcelli et al., 2006; Szafran et al., 2008). Another assay focusing on AR binding to promoters in LNCaP cells has shown that after ligand binding, AR is able to translocate and bind to the DNA sequences within 15min showing the speed of AR activation (Kang et al., 2002).

Known Feedforward/Feedback loops influencing this KER

AR antagonism can lead to increased AR transcript stability and levels as a compensatory mechanism in prostate cancer cells (Dart et al., 2020). In turn, in presence of increased AR levels, AR antagonists can exhibit agonistic activity (Chen et al., 2003).

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Relationship: 2128: Decrease, AR activation leads to decrease, transcription of genes by AR

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Relationship: 2129: decrease, transcription of genes by AR leads to AGD, decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	Moderate	Low

List of Non Adjacent Key Event Relationships

Relationship: 2123: Antagonism, Androgen receptor leads to AGD, decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent	Moderate	Low

Relationship: 2820: Decrease, AR activation leads to AGD, decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent		
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent		
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent		