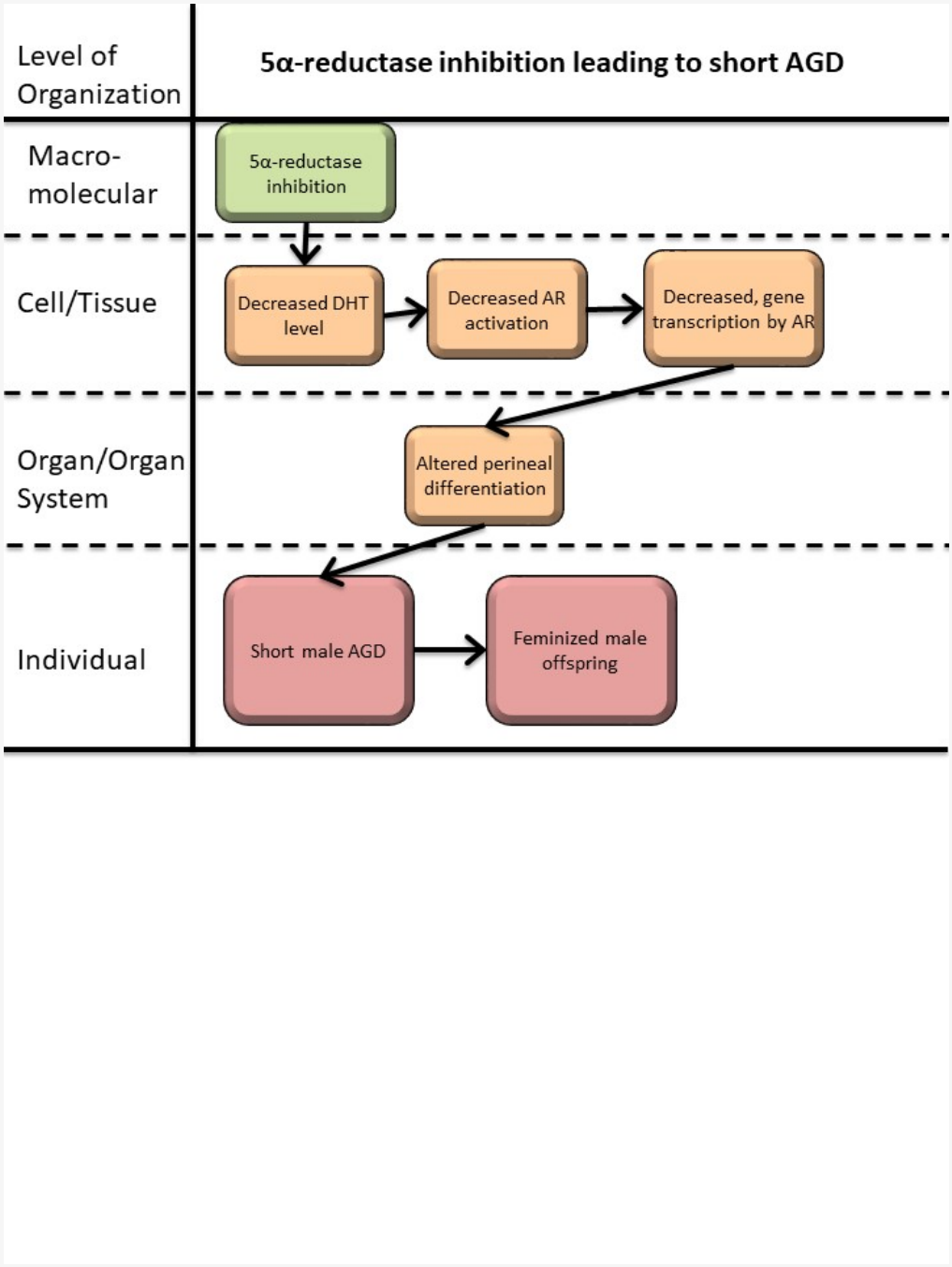
AOP ID and Title:

AOP 305: 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring
Short Title: 5α-reductase inhibition leading to short AGD

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.90	Included in OECD Work Plan

Abstract

Abstract

This AOP links 5α-reductase inhibition 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).

5α-reductase is an enzyme responsible for the conversion of testosterone to DHT in target tissues. DHT is more potent agonist of the Androgen receptor (AR) than testosterone, so that DHT is necessary for proper masculinization of e.g. male external genitalia. 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 masculinization of the developing fetus, including differentiation of the levator ani/bulbocavernosus (LABC) muscle complex in males. 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 inhibition of 5α-reductase that converts testosterone into the more potent DHT in androgen sensitive target tissues. This includes developing perineal region, which, when DHT levels are low or absent, leads to inactivation of the AR and failure to properly masculinize the perineum/LABC complex.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1617	Inhibition, 5α-reductase	Inhibition, 5α-reductase
2	KE	1613	Decrease, dihydrotestosterone (DHT) level	Decrease, DHT level
3	KE	1614	Decrease, androgen receptor activation	Decrease, AR activation
	KE	286	Altered, Transcription of genes by the androgen receptor	Altered, Transcription of genes by the AR
5	AO	1688	anogenital distance (AGD), decreased	AGD, decreased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, 5α-reductase	adjacent	Decrease, dihydrotestosterone (DHT) level	High	High
Decrease, dihydrotestosterone (DHT) level	adjacent	Decrease, androgen receptor activation		
Decrease, androgen receptor activation	non-adjacent	anogenital distance (AGD), decreased		

Stressors

Name	Evidence
Finasteride	High

Finasteride

Finasteride is a type II 5alpha-reductase inhibitor that blocks conversion of testosterone to dihydrotestosterone (Clark et al 1990; Imperato-McGinley et al 1992). Intrauterine exposure in rats can result in shorter male AGD in male offspring (Bowman et al 2003; Christiansen et al 2009; Schwartz et al 2019)

References:

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

Clark et al (1990), Teratology 42:91-100; doi: 10.1002/tera.1420420111

Imperato-McGinley (1992), J Clin Endocrinol Metab 75:1022-1026; doi: 10.1210/jcem.75.4.1400866

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

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: 1617: Inhibition, 5α-reductase

Short Name: Inhibition, 5α-reductase

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	MolecularInitiatingEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

eukaryotic cell

Cell term

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

This KE is applicable to both sexes, across developmental stages into adulthood, in many different tissues and across taxa.

Essentially the reaction performed by the isozymes is the same, but the enzyme is differentially expressed in the body. 5α-reductase type 1 is mainly linked to the production of neurosteroids, 5α-reductase type 2 is mainly involved in production of 5α-DHT, whereas 5α-reductase type 3 is involved in N-glycosylation (Robitaille & Langlois, 2020).

The expression profile of the three 5α-reductase isoforms depends on the developmental stage, the tissue of interest, and the disease state of the tissue. The enzymes have been identified in, for instance, non-genital and genital skin, scalp, prostate, liver, seminal vesicle, epididymis, testis, ovary, kidney, exocrine pancreas, and brain (Azzouni, 2012, Uhlen 2015).

5α-reductase is well-conserved, all primary species in Eukaryota contain all three isoforms (from plant, amoeba, yeast to vertebrates) (Azzouni, 2012) and the enzymes are expressed in both males and females (Langlois, 2010, Uhlen 2015).

Key Event Description

This KE describes the inhibition of 5α-reductases (3-oxo-5α-steroid 4-dehydrogenases). These enzymes are widely expressed in tissues of both sexes and responsible for conversion of steroid hormones.

There are three isozymes: 5α-reductase type 1, 2, and 3. The substrates for 5α-reductases are 3-oxo (3-keto), $\Delta^{4,5}$ C19/C21 steroids such as testosterone, progesterone, androstenedione, epi-testosterone, cortisol, aldosterone, and deoxycorticosterone. The enzymatic reaction leads to an irreversible breakage of the double bond between carbon 4 and 5 and subsequent insertion of a hydride anion at carbon 5 and insertion of a proton at carbon 4. The reaction is aided by the cofactor NADPH. The substrate affinity and reaction velocity differ depending on the combination of substrate and enzyme isoform, for instance 5α-reductase type 2 has a higher substrate affinity for testosterone than the type 1 isoform of the enzyme, and the enzymatic reaction occurs at a higher velocity under optimal conditions. Likewise, inhibitors of 5α-reductase may exhibit differential effects depending on isoforms (Azzouni et al., 2012).

How it is Measured or Detected

There is currently (as of 2023) no OECD test guideline for the measurement of 5α-reductase inhibition.

Inhibition of 5α-reductase can be assessed using transfected cell lines. This has been demonstrated in HEK-293 cells stably transfected with human 5α-reductase type 1, 2, and 3 (Yamana et al., 2010), in CHO cells stably transfected with human 5α-reductase type 1 and 2 (Thigpens et al., 1993), and COS cells transfected with human and rat 5α-reductase with unspecified isoforms (Andersson & Russell, 1990). The transfected cells are typically used as intact cells or cell homogenates. Further, 5α-reductase 1 and 2 has been successfully expressed and isolated from *Escherichia coli* with subsequent functionality allowing for examination of enzyme inhibition (Peng et al., 2020).

The output of the above methods could be decreased dihydrotestosterone (DHT) with increasing test chemical concentrations. Other substrates exist for the different isoforms that could be used to assess the enzymatic inhibition (Peng et al., 2020). The use of radiolabeled steroids has historic and continued use for 5α-reductase inhibition examination (Andersson & Russell, 1990; Peng et al., 2020; Thigpens et al., 1993; Yamana et al., 2010); however, alternative methods are available, such as conventional ELISA kits or advanced analytical methods such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

References

Andersson, S., & Russell, D. W. (1990). Structural and biochemical properties of cloned and expressed human and rat steroid 5a-reductases. *Proc. Natl. Acad. Sci. USA*, *87*, 3640–3644. https://www.pnas.org

Azzouni, F., Godoy, A., Li, Y., & Mohler, J. (2012). The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases. In *Advances in Urology*. https://doi.org/10.1155/2012/530121

Peng, H. M., Valentin-Goyco, J., Im, S. C., Han, B., Liu, J., Qiao, J., & Auchus, R. J. (2020). Expression in escherichia coli, purification, and functional reconstitution of human steroid 5α-reductases. *Endocrinology (United States)*, *161*(8), 1–11.

https://doi.org/10.1210/ENDOCR/BQAA117

Robitaille, J., & Langlois, V. S. (2020). Consequences of steroid-5α-reductase deficiency and inhibition in vertebrates. In *General and Comparative Endocrinology* (Vol. 290). Academic Press Inc. https://doi.org/10.1016/j.ygcen.2020.113400

Thigpens, A. E., Cala, K. M., & Russell, D. W. (1993). Characterization of Chinese Hamster Ovary Cell Lines Expressing Human Steroid 5a-Reductase Isozymes. *The Journal of Biological Chemistry*, *268*(23), 17404–17412.

Yamana, K., Fernand, L., Luu-The, V., & Luu-The, V. (2010). Human type 3 5α-reductase is expressed in peripheral tissues at higher levels than types 1 and 2 and its activity is potently inhibited by finasteride and dutasteride. *Hormone Molecular Biology and Clinical Investigation*, *2*(3), 293–299. https://doi.org/10.1515/HMBCI.2010.035

List of Key Events in the AOP

Event: 1613: Decrease, dihydrotestosterone (DHT) level

Short Name: Decrease, DHT level

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:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	KeyEvent
Aop:305 - 5α-reductase inhibition 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

Biological Context

Level of Biological Organization

Tissue

Cell term

Cell term

eukaryotic cell

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

This KE is applicable to both sexes, across developmental stages and adulthood, in many different tissues and across vertebrate taxa.

In both humans and rodents, DHT is important for the *in utero* differentiation and growth of the prostate and male external genitalia (Azzouni et al., 2012; Gerald & Raj, 2022). Besides its critical role in development, DHT also induces growth of facial and body hair during puberty in humans (Azzouni et al., 2012).

In mammals, the role of DHT in females is less established (Swerdloff et al., 2017), however studies suggest that androgens are important in e.g. bone metabolism and growth, as well as female reproduction from follicle development to parturition (Hammes & Levin, 2019).

Key Event Description

Dihydrotestosterone (DHT) is an endogenous steroid hormone and a potent androgen. The level of DHT in tissue or blood is dependent on several factors, such as the synthesis, uptake/release, metabolism, and elimination from the system, which again can be dependent on biological compartment and developmental stage.

DHT is primarily synthesized from testosterone (T) via the irreversible enzymatic reaction facilitated by 5α-Reductases (5α-REDs) (Swerdloff et al., 2017). Different isoforms of this enzyme are differentially expressed in specific tissues (e.g. prostate, skin, liver, and hair follicles) at different developmental stages, and depending on disease status (Azzouni et al., 2012; Uhlén et al., 2015), which ultimately affects the local production of DHT.

An alternative (“backdoor”) pathway , exists for DHT formation that is independent of T and androstenedione as precursors. This pathway relies on the conversion of progesterone (P) or 17-OH-P to androsterone and then androstanediol through several enzymatic reactions and finally, the conversion of androstanediol into DHT probably by HSD17B6 (Miller & Auchus, 2019; Naamneh Elzenaty et al., 2022). The “backdoor” synthesis pathway is a result of an interplay between placenta, adrenal gland, and liver during fetal life (Miller & Auchus, 2019).

The conversion of T to DHT by 5α-RED in peripheral tissue is mainly responsible for the circulating levels of DHT, though some tissues express enzymes needed for further metabolism of DHT consequently leading to little release and contribution to circulating levels (Swerdloff et al.).

The initial conversion of DHT into inactive steroids is primarily through 3α-hydroxysteroid dehydrogenase (3α-HSD) and 3β-HSD in liver, intestine, skin, and androgen-sensitive tissues. The subsequent conjugation is mainly mediated by uridine 5´-diphospho (UDP)-glucuronyltransferase 2 (UGT2) leading to biliary and urinary elimination from the system. Conjugation also occurs locally to control levels of highly potent androgens (Swerdloff et al., 2017).

Disruption of any of the aforementioned processes may lead to decreased DHT levels, either systemically or at tissue level.

How it is Measured or Detected

Several methods exist for DHT identification and quantification, such as conventional immunoassay methods (ELISA or RIA) and advanced analytical methods as liquid chromatography tandem mass spectrometry (LC-MS/MS). The methods can have differences in detection and quantification limits, which should be considered depending on the DHT levels in the sample of interest. Further, the origin of the sample (e.g. cell culture, tissue, or blood) will have implications for the sample preparation.

Conventional immunoassays have limitations in that they can overestimate the levels of DHT compared to levels determined by gas chromatography mass spectrometry and liquid chromatography tandem mass spectrometry (Hsing et al., 2007; Shiraishi et al., 2008). This overestimation may be explained by lack of specificity of the DHT antibody used in the RIA and cross-reactivity with T in samples (Swerdloff et al., 2017).

References

Azzouni, F., Godoy, A., Li, Y., & Mohler, J. (2012). The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases. In *Advances in Urology*. https://doi.org/10.1155/2012/530121

Gerald, T., & Raj, G. (2022). Testosterone and the Androgen Receptor. In *Urologic Clinics of North America* (Vol. 49, Issue 4, pp. 603–614). W.B. Saunders. https://doi.org/10.1016/j.ucl.2022.07.004

Hammes, S. R., & Levin, E. R. (2019). Impact of estrogens in males and androgens in females. In *Journal of Clinical Investigation* (Vol. 129, Issue 5, pp. 1818–1826). American Society for Clinical Investigation. https://doi.org/10.1172/JCI125755

Hsing, A. W., Stanczyk, F. Z., Bélanger, A., Schroeder, P., Chang, L., Falk, R. T., & Fears, T. R. (2007). Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry. *Cancer Epidemiology Biomarkers and Prevention*, *16*(5), 1004–1008. https://doi.org/10.1158/1055-9965.EPI-06-0792

Miller, W. L., & Auchus, R. J. (2019). The “backdoor pathway” of androgen synthesis in human male sexual development. *PLoS Biology*, *17*(4). https://doi.org/10.1371/journal.pbio.3000198

Naamneh Elzenaty, R., du Toit, T., & Flück, C. E. (2022). Basics of androgen synthesis and action. In *Best Practice and Research: Clinical Endocrinology and Metabolism* (Vol. 36, Issue 4). Bailliere Tindall Ltd. https://doi.org/10.1016/j.beem.2022.101665

Shiraishi, S., Lee, P. W. N., Leung, A., Goh, V. H. H., Swerdloff, R. S., & Wang, C. (2008). Simultaneous measurement of serum testosterone and dihydrotestosterone by liquid chromatography-tandem mass spectrometry. *Clinical Chemistry*, *54*(11), 1855–1863. https://doi.org/10.1373/clinchem.2008.103846

Swerdloff, R. S., Dudley, R. E., Page, S. T., Wang, C., & Salameh, W. A. (2017). Dihydrotestosterone: Biochemistry, physiology, and clinical implications of elevated blood levels. In *Endocrine Reviews* (Vol. 38, Issue 3, pp. 220–254). Endocrine Society. https://doi.org/10.1210/er.2016-1067

Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I. M., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A. K., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., … Pontén, F.

(2015). Tissue-based map of the human proteome. *Science*, *347*(6220). https://doi.org/10.1126/science.1260419

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
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).

References

Davey, R. A., & Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *The Clinical Biochemist. Reviews*, *37*(1), 3–15.

Gao, W., Bohl, C. E., & Dalton, J. T. (2005). Chemistry and structural biology of androgen receptor. *Chemical Reviews*, *105*(9), 3352–3370. https://doi.org/10.1021/cr020456u

Hutson, J. M. (1985). A biphasic model for the hormonal control of testicular descent. *The Lancet*, *24*, 419–421. https://doi.org/https://doi.org/10.1016/S0140-6736(85)92739-4

Kaftanovskaya, E. M., Huang, Z., Barbara, A. M., de Gendt, K., Verhoeven, G., Gorlov, I. P., & Agoulnik, A. I. (2012). Cryptorchidism in mice with an androgen receptor ablation in gubernaculum testis. *Molecular Endocrinology*, *26*(4), 598–607. https://doi.org/10.1210/me.2011-1283

Lee, S. H., Hong, K. Y., Seo, H., Lee, H. S., & Park, Y. (2021). Mechanistic insight into human androgen receptor-mediated endocrine-disrupting potentials by a stable bioluminescence resonance energy transfer-based dimerization assay. *Chemico-Biological Interactions*, *349*. https://doi.org/10.1016/j.cbi.2021.109655

Leung, J. K., & Sadar, M. D. (2017). Non-Genomic Actions of the Androgen Receptor in Prostate Cancer. *Frontiers in Endocrinology*, *8*. https://doi.org/10.3389/fendo.2017.00002

OECD (2022). Test No. 251: Rapid Androgen Disruption Activity Reporter (RADAR) assay. Paris: OECD Publishing doi:10.1787/da264d82-en.

Pang, T. P. S., Clarke, M. v., Ghasem-Zadeh, A., Lee, N. K. L., Davey, R. A., & MacLean, H. E. (2012). A physiological role for androgen actions in the absence of androgen receptor DNA binding activity. *Molecular and Cellular Endocrinology*, *348*(1), 189–197. https://doi.org/10.1016/j.mce.2011.08.017

Event: 286: Altered, Transcription of genes by the androgen receptor

Short Name: Altered, Transcription of genes by the AR

Key Event Component

Process	Object	Action
regulation of gene expression	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:19 - Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)	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:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent

AOP ID and Name	Event Type
Aop:495 - Androgen receptor activation leading to prostate cancer	KeyEvent

Stressors

Name
Bicalutamide
Cyproterone acetate
Epoxiconazole
Flutamide
Flusilazole
Prochloraz
Propiconazole
Stressor:286 Tebuconazole
Triticonazole
Vinclozalin

Biological Context

Level of Biological Organization
Tissue

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Bicalutamide

Using analysis of androgen-regulated gene expression in the LNCaP prostate cancer cell line (Ngan et al. 2009).

Cyproterone acetate

Using analysis of androgen-regulated gene expression in the LNCaP prostate cancer cell line (Ngan et al. 2009) and using the AR-CALUX reporter assay in antagonism mode, cyproterone acetate showed an IC50 of 7.1 nM (Sonneveld et al. 2005).

Epoxiconazole

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

Flutamide

Analysis of androgen-regulated gene expression in the LNCaP prostate cancer cell line (Ngan et al. 2009) and using the AR-CALUX reporter assay in antagonism mode, flutamide showed an IC50 of 1.3 uM (Sonneveld et al. 2005).

Flusilazole

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

Prochloraz

Using gene expression analysis of the androgen-regulated genes ornithine decarboxylase, prostatic binding protein C3 as well as insulin-like growth factor I. Gene expression levels were reduced in ventral prostates of male Wistar pups at postnatal day 16 following *in utero* and lactational exposure from maternal perinatal dosing with prochloraz (50 and 150 mg/kg/day) from gestational day 7 to postnatal day 16 (Laier et al. 2006). Also, using transiently AR-transfected CHO cells, prochloraz showed a LOEC of 6.3 mM and an IC50 of 13 mM (Kjærstad et al. 2010).

Propiconazole

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

Stressor:286 Tebuconazole

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

Triticonazole

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

Vinclozalin

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 and Grossmann 2016). Despite certain inter-species differences, AR function mediated through gene expression is highly conserved, with mutation 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 considered broadly applicable across vertebrate taxa, sex and developmental stages, as all vertebrate animals express the AR in numerous cells and tissues where it regulates gene transcription required for developmental processes and function.

Key Event Description

This KE refers to transcription of genes by the androgen receptor (AR) as occurring in complex biological systems such as tissues and organs *in vivo*.

The Androgen Receptor and its function

The AR belongs to the steroid hormone nuclear receptor family. It is a ligand-activated transcription factor with three domains: the N-terminal domain, the DNA-binding domain, and the ligand-binding domain with the latter being the most evolutionary conserved (Davey and Grossmann 2016). Androgens (such as dihydrotestosterone and testosterone) are AR ligands and act by binding to the AR in androgen-responsive tissues (Davey and Grossmann 2016). Human AR mutations and mouse knockout models have established a fundamental role for AR in masculinization and spermatogenesis (Maclean et al.; Walters et al. 2010; Rana et al. 2014). The AR is also expressed in many other tissues such as bone, muscles, ovaries and within the immune system (Rana et al.

2014).

Altered transcription of genes by the AR as a Key Event

Upon activation by ligand-binding, the AR translocates from the cytoplasm to the cell nucleus, dimerizes, binds to androgen response elements in the DNA to modulate gene transcription (Davey and Grossmann 2016). The transcriptional targets vary between cells and tissues, as well as with developmental stages and is also dependent on available co-regulators (Bevan and Parker 1999; Heemers and Tindall 2007). It should also be mentioned that the AR can work in other 'non-canonial' ways such as non-genomic signaling, and ligand-independent activation (Davey & Grossmann, 2016; Estrada et al, 2003; Jin et al, 2013).

A large number of known, and proposed, target genes of AR canonical signaling have been identified by analysis of gene expression following treatments with AR agonists (Bolton et al. 2007; Ngan et al. 2009, Jin et al. 2013).

How it is Measured or Detected

Altered transcription of genes by the AR can be measured by measuring the transcription level of known downstream target genes by RT-qPCR or other transcription analyses approaches, e.g. transcriptomics.

References

Bevan C, Parker M (1999) The role of coactivators in steroid hormone action. Exp. Cell Res. 253:349–356

Bolton EC, So AY, Chaivorapol C, et al (2007) Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev 21:2005–2017. doi: 10.1101/gad.1564207

Davey RA, Grossmann M (2016) Androgen Receptor Structure, Function and Biology: From Bench to Bedside. Clin Biochem Rev 37:3–15

Estrada M, Espinosa A, Müller M, Jaimovich E (2003) Testosterone Stimulates Intracellular Calcium Release and Mitogen-Activated Protein Kinases Via a G Protein-Coupled Receptor in Skeletal Muscle Cells. Endocrinology 144:3586–3597. doi: 10.1210/en.2002-0164

Heemers H V., Tindall DJ (2007) Androgen receptor (AR) coregulators: A diversity of functions converging on and regulating the AR transcriptional complex. Endocr. Rev. 28:778–808

Jin, Hong Jian, Jung Kim, and Jindan Yu. 2013. "Androgen Receptor Genomic Regulation." Translational Andrology and Urology 2(3):158–77. doi: 10.3978/j.issn.2223-4683.2013.09.01

Maclean HE, Chu S, Warne GL, Zajact JD Related Individuals with Different Androgen Receptor Gene Deletions

MacLeod DJ, Sharpe RM, Welsh M, et al (2010) Androgen action in the masculinization programming window and development of male reproductive organs. In: International Journal of Andrology. Blackwell Publishing Ltd, pp 279–287

Ngan S, Stronach EA, Photiou A, et al (2009) Microarray coupled to quantitative RT–PCR analysis of androgen-regulated genes in human LNCaP prostate cancer cells. Oncogene 28:2051–2063. doi: 10.1038/onc.2009.68

Ogino, Y., Ansai, S., Watanabe, E., Yasugi, M., Katayama, Y., Sakamoto, H., et al. (2023). Evolutionary differentiation of androgen receptor is responsible for sexual characteristic development in a teleost fish. *Nat. Commun. 2023 141* 14, 1–16. doi:10.1038/s41467-023-37026-6.

Ogino, Y., Hirakawa, I., Inohaya, K., Sumiya, E., Miyagawa, S., Denslow, N., et al. (2014). Bmp7 and Lef1 Are the Downstream Effectors of Androgen Signaling in Androgen-Induced Sex Characteristics Development in Medaka. *Endocrinology* 155, 449–462. doi:10.1210/EN.2013-1507.

Rana K, Davey RA, Zajac JD (2014) Human androgen deficiency: Insights gained from androgen receptor knockout mouse models. Asian J. Androl. 16:169–177

Walters KA, Simanainen U, Handelsman DJ (2010) Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models. Hum Reprod Update 16:543–558. doi: 10.1093/humupd/dmq003

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
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’.

References

Aydoğan Ahbab M, Barlas N (2015) Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats. *Toxicol Lett* **233:** 125-137

Boberg J, Axelstad M, Svingen T, Mandrup K, Christiansen S, Vinggaard AM, Hass U (2016) Multiple endocrine disrupting effects in rats perinatally exposed to butylparaben. *Toxicol Sci* **152:** 244-256

Boberg J, Metzdorff S, Wortziger R, Axelstad M, Brokken L, Vinggaard AM, Dalgaard M, Nellemann C (2008) Impact of diisobutyl phthalate and other PPAR agonists on steroidogenesis and plasma insulin and leptin levels in fetal rats. *Toxicology* **250:** 75-81

Bowman CJ, Barlow NJ, Turner KJ, Wallace DG, Foster PM (2003) Effects of in utero exposure to finasteride on androgen-dependent reproductive development in the male rat. *Toxicol Sci* **74:** 393-406

Christiansen S, Boberg J, Axelstad M, Dalgaard M, Vinggaard AM, Metzdorff SB, Hass U (2010) Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. *Reprod Toxicol* **30:** 313-321

Christiansen S, Scholze M, Dalgaard M, Vinggaard AM, Axelstad M, Kortenkamp A, Hass U (2009) Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. *Environ Health Perspect* **117:** 1839-1846

Draskau MK, Boberg J, Taxvig C, Pedersen M, Frandsen HL, Christiansen S, Svingen T (2019) In vitro and in vivo endocrine disrupting effects of the azole fungicides triticonazole and flusilazole. *Environ Pollut* **255:** 113309

Ema M, Miyawaki E (2002) Effects on development of the reproductive system in male offspring of rats given butyl benzyl phthalate during late pregnancy. *Reprod Toxicol* **16:** 71-76

Ema M, Miyawaki E, Hirose A, Kamata E (2003) Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate. *Reprod Toxicol* **17:** 407-412

Foster PM, Harris MW (2005) Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. *Toxicol Sci* **85:** 1024-1032

Gray LE, Jr., Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L (2000) Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci* **58:** 350-365

Gray LEJ, Ostby JS, Kelce WR (1994) Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol* **129:** 46-52

Hass U, Boberg J, Christiansen S, Jacobsen PR, Vinggaard AM, Taxvig C, Poulsen ME, Herrmann SS, Jensen BH, Petersen A,

Clemmensen LH, Axelstad M (2012) Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reprod Toxicol* **34:** 261-274

Hass U, Scholze M, Christiansen S, Dalgaard M, Vinggaard AM, Axelstad M, Metzdorff SB, Kortenkamp A (2007) Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. *Environ Health Perspect* **115 Suppl. 1:** 122-128

Hoshino N, Iwai M, Okazaki Y (2005) A two-generation reproductive toxicity study of dicyclohexyl phthalate in rats. *J Toxicol Sci* **30 Spec No:** 79-96

Hotchkiss AK, Parks-Saldutti LG, Ostby JS, Lambright C, Furr J, Vandenbergh JG, Gray LEJ (2004) A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biol Reprod* **71:** 1852-1861

Howdeshell KL, Furr J, Lambright CR, Rider CV, Wilson VS, Gray LE, Jr. (2007) Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. *Toxicol Sci* **99:** 190-202

Ipulan LA, Suzuki K, Sakamoto Y, Murashima A, Imai Y, Omori A, Nakagata N, Nishinakamura R, Valasek P, Yamada G (2014) Nonmyocytic androgen receptor regulates the sexually dimorphic development of the embryonic bulbocavernosus muscle. *Endocrinology* **155:** 2467-2479

Jarfelt K, Dalgaard M, Hass U, Borch J, Jacobsen H, Ladefoged O (2005) Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethylhexyl) phthalate and di(2-ethylhexyl) adipate. *Reprod Toxicol* **19:** 505-515

Jost A (1953) Problems of fetal endocrinology: The gonadal and hypophyseal hormones. *Recent Prog Horm Res* **8:** 379-418

Juul A, Almstrup K, Andersson AM, Jensen TK, Jorgensen N, Main KM, Rajpert-De Meyts E, Toppari J, Skakkebaek NE (2014) Possible fetal determinants of male infertility. *Nat Rev Endocrinol* **10:** 553-562

Kita DH, Meyer KB, Venturelli AC, Adams R, Machado DL, Morais RN, Swan SH, Gennings C, Martino-Andrade AJ (2016) Manipulation of pre and postnatal androgen environments and anogenital distance in rats. *Toxicology* **368-369:** 152-161

Laier P, Metzdorff SB, Borch J, Hagen ML, Hass U, Christiansen S, Axelstad M, Kledal T, Dalgaard M, McKinnell C, Brokken LJ, Vinggaard AM (2006) Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicol Appl Pharmacol* **213:** 2

Li M, Qiu L, Zhang Y, Hua Y, Tu S, He Y, Wen S, Wang Q, Wei G (2013) Dose-related effect by maternal exposure to di-(2-ethylhexyl) phthalate plasticizer on inducing hypospadiac male rats. *Environ Toxicol Pharmacol* **35:** 55-60

Lin H, Lian QQ, Hu GX, Jin Y, Zhang Y, Hardy DO, Chen GR, Lu ZQ, Sottas CM, Hardy MP, Ge RS (2009) In utero and lactational exposures to diethylhexyl-phthalate affect two populations of Leydig cells in male Long-Evans rats. *Biol Reprod* **80:** 882-888

Loeffler IK, Peterson RE (1999) Interactive effects of TCDD and p,p'-DDE on male reproductive tract development in in utero and lactationally exposed rats. *Toxicol Appl Pharmacol* **154:** 28-39

MacLeod DJ, Sharpe RM, Welsh M, Fisken M, Scott HM, Hutchison GR, Drake AJ, van den Driesche S (2010) Androgen action in the masculinization programming window and development of male reproductive organs. *Int J Androl* **33:** 279-287

Matsuura I, Saitoh T, Ashina M, Wako Y, Iwata H, Toyota N, Ishizuka Y, Namiki M, Hoshino N, Tsuchitani M (2005) Evaluation of a two-generation reproduction toxicity study adding endpoints to detect endocrine disrupting activity using vinclozolin. *J Toxicol Sci* **30 Spec No:** 163-168

McIntyre BS, Barlow NJ, Foster PM (2001) Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci* **62:** 236-249

McIntyre BS, Barlow NJ, Sar M, Wallace DG, Foster PM (2002) Effects of in utero linuron exposure on rat Wolffian duct development. *Reprod Toxicol* **16:** 131-139

Melching-Kollmuss S, Fussell KC, Schneider S, Buesen R, Groeters S, Strauss V, van Ravenzwaay B (2017) Comparing effect levels of regulatory studies with endpoints derived in targeted anti-androgenic studies: example prochloraz. *Arch Toxicol* **91:** 143-162

Moore RW, Rudy TA, Lin TM, Ko K, Peterson RE (2001) Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) phthalate. *Environ Health Perspect* **109:** 229-237

Mylchreest E, Sar M, Cattley RC, Foster PM (1999) Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* **156:** 81-95

Nagao T, Ohta R, Marumo H, Shindo T, Yoshimura S, Ono H (2000) Effect of butyl benzyl phthalate in Sprague-Dawley rats after gavage administration: a two-generation reproductive study. *Reprod Toxicol* **14:** 513-532

Nardelli TC, Albert O, Lalancette C, Culty M, Hales BF, Robaire B (2017) In utero and lactational exposure study in rats to identify replacements for di(2-ethylhexyl) phthalate. *Sci Rep* **7:** 3862

Noriega NC, Ostby J, Lambright C, Wilson VS, Gray LE, Jr. (2005) Late gestational exposure to the fungicide prochloraz delays the onset of parturition and causes reproductive malformations in male but not female rat offspring. *Biol Reprod* **72:** 1324-1335

OECD. (2013) Guidance document in support of the test guideline on the extended one generation reproductive toxicity study No. 151.

Ostby J, Kelce WR, Lambright C, Wolf CJ, Mann P, Gray CLJ (1999) The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. *Toxicol Ind Health* **15:** 80-93

Saillenfait AM, Gallissot F, Sabaté JP (2009a) Differential developmental toxicities of di-n-hexyl phthalate and dicyclohexyl phthalate administered orally to rats. *J Appl Toxicol* **29:** 510-521

Saillenfait AM, Roudot AC, Gallissot F, Sabaté JP (2011) Prenatal developmental toxicity studies on di-n-heptyl and di-n-octyl phthalates in Sprague-Dawley rats. *Reprod Toxicol* **32:** 268-276

Saillenfait AM, Sabaté JP, Gallissot F (2009b) Effects of in utero exposure to di-n-hexyl phthalate on the reproductive development of the male rat. *Reprod Toxicol* **28:** 468-476

Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, Longnecker MP, Hernandez-Avila M (2004) Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. *Environ Health* **3:** 8

Schneider S, Kaufmann W, Strauss V, van Ravenzwaay B (2011) Vinclozolin: a feasibility and sensitivity study of the ILSI-HESI F1-extended one-generation rat reproduction protocol. *Regulatory Toxicology and Pharmacology* **59:** 91-100

Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, 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

Scott HM, Hutchison GR, Mahood IK, Hallmark N, Welsh M, De Gendt K, Verhoeven H, O'Shaughnessy P, Sharpe RM (2007) Role of androgens in fetal testis development and dysgenesis. *Endocrinology* **148:** 2027-2036

Skakkebaek NE, Rajpert-De Meyts E, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* **16:** 972-978

Taxvig C, Vinggaard AM, Hass U, Axelstad M, Metzdorff S, Nellemann C (2008) Endocrine-disrupting properties in vivo of widely used azole fungicides. *Int J Androl* **31:** 170-177

Turner KJ, Barlow NJ, Struve MF, Wallace DG, Gaido KW, Dorman DC, Foster PM (2002) Effects of in utero exposure to the organophosphate insecticide fenitrothion on androgen-dependent reproductive development in the Crl:CD(SD)BR rat. *Toxicol Sci* **68:** 174-183

Tyl RW, Myers CB, Marr MC, Fail PA, Seely JC, Brine DR, Barter RA, Butala JH (2004) Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. *Reprod Toxicol* **18:** 241-264

Van den Driesche S, Kolovos P, Platts S, Drake AJ, Sharpe RM (2012) Inter-relationship between testicular dysgenesis and Leydig cell function in the masculinization programming window in the rat. *PloS one* **7:** e30111

Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB, Sharpe RM (2008) Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* **118:** 1479-1490

Welsh M, Saunders PT, Sharpe RM (2007) The critical time window for androgen-dependent development of the Wolffian duct in the rat. *Endocrinology* **148:** 3185-3195

Wolf CJ, LeBlanc GA, Gray LE, Jr. (2004) Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat. *Toxicol Sci* **78:** 135-143

Wolf CJJ, Lambright C, Mann P, Price M, Cooper RL, Ostby J, Gray CLJ (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* **15:** 94-118

Zhang L, Dong L, Ding S, Qiao P, Wang C, Zhang M, Zhang L, Du Q, Li Y, Tang N, Chang B (2014) Effects of n-butylparaben on steroidogenesis and spermatogenesis through changed E_2 levels in male rat offspring. *Environ Toxicol Pharmacol* **37:** 705-717

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1880: Inhibition, 5α-reductase leads to Decrease, DHT level

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of 5α-reductase leading to impaired fecundity in female fish	adjacent	High	High
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	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

Key Event Relationship Description

This key event relationship (KER) links inhibition of 5α-reductase activity to decreased dihydrotestosterone (DHT) levels.

There are three isozymes of 5α-reductase: type 1, 2, and 3. 5α-reductase type 2 is mainly involved in the synthesis of 5α-DHT from testosterone (T) (Robitaille & Langlois, 2020), although 5α-reductase type 1 can also facilitate this reaction, but with lower affinity for T (Nikolaou et al., 2021). The type 1 isoform is also involved in the alternative ('backdoor') pathway for DHT formation, facilitating the conversion of progesterone or 17OH-progesterone to dihydroprogesterone or 5α-pregnan-17α-ol-3,20-dione, respectively, whereafter several subsequent reactions will ultimately lead to the formation of DHT (Miller & Auchus, 2019). The quantitative importance of the alternative pathway remains unclear (Alemany, 2022). The type 1 and type 2 isoforms of 5α-reductase are the primary focus of this KER.

The direct conversion of T to 5α-DHT mainly takes place in the target tissue (Robitaille & Langlois, 2020). In mammals, the type 1 isoform is found in the scalp and other peripheral tissues (Miller & Auchus, 2011), such as liver, skin, prostate (Azzouni et al., 2012), bone, ovaries, and adipose tissue (Nikolaou et al., 2021). The type 2 isoform is expressed mainly in male reproductive tissues (Miller & Auchus, 2011), but also in liver, scalp and skin (Nikolaou et al., 2021). The expression level of both isoforms depend on the developmental stage and the tissue.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this KER is considered high.

5α-reductase can catalyze the conversion of T to DHT. The substrates for 5α-reductases are 3-oxo (3-keto), $\Delta^{4,5}$ C19/C21 steroids such as testosterone and progesterone. The enzymatic reaction leads to an irreversible breakage of the double bond between carbon 4 and 5 and subsequent insertion of a hydride anion at carbon 5 and insertion of a proton at carbon 4. The reaction is aided by the cofactor NADPH (Azzouni et al., 2012). By inhibiting this enzyme, the described catalyzed reaction will be inhibited leading to a decrease in DHT levels.

In both humans and rodents, DHT is important for the *in utero* differentiation and growth of the prostate and male external genitalia. Besides its critical role during fetal development, DHT also induces growth of facial and body hair during puberty in humans (Azzouni et al., 2012).

Empirical Evidence

The empirical evidence for this KER is considered high

Dose concordance

Several inhibitors of 5α-reductases have been developed for pharmacological uses. Inhibition of the enzymatic conversion of radiolabeled substrate has been illustrated (Table 1) and data display dose-concordance, with increasing concentrations of inhibitor leading to lower 5α-reductase product formation.

Table 1: Dose concordance from selected in vitro test systems

Test system	**Model description**	**Stressor**	**Effect**	**Reference**
HEK-293 cells	Cells stably transfected human 5α-reductase type 1 and 2 used to measure conversion of [^{14}C]labeled steroids	Finasteride	Type 1: IC_{50} = 106.9 µM Type 2: IC_{50} = 14.3 µM	(Yamana et al., 2010)
		Dutasteride	Type 1: IC_{50} = 8.7 µM Type 2: IC_{50} = 57 µM	
COS cells	Cell homogenates from transfected cells with human and rat 5α-reductase (unknown isoform) used to measure conversion of radiolabeled testosterone	Finasteride	Human: IC_{50} ≈ 1 µM K_i = 340-620 nM Rat: IC_{50} ≈ 0.1 µM K_i = 3-5 nM	(Andersson & Russell, 1990)
		4-MA	Human: IC_{50} ≈ 0.1 µM K_i = 7-8 nM Rat: IC_{50} ≈ 0.1 µM K_i = 5-7 nM	
CHO cells	Stably transfected with human 5α-reductase type 1 and 2	Finasteride	Type 1: K_i = 325 nM Type 2: K_i = 12 nM	(Thigpens et al., 1993)
		4-MA	Type 1: K_i = 8 nM Type 2: K_i = 4 nM	
Isolated enzyme	Human 5α-reductase type 1 and 2 used to measure conversion of radiolabeled substrate of both isoforms	Finasteride	Type 1: K_i = > 200 nM Type 2: K_i = 0.45 nM	(Peng et al., 2020)
		Dutasteride	Type 1: K_i = 39 nM Type 2: K_i = 1.1 nM	

These in vitro studies clearly show effects on the enzymatic reaction induced by 5α-reductases in a concentration dependent manner (Andersson & Russell, 1990; Thigpens et al., 1993; Yamana et al., 2010).

In the intact organism, when 5α-reductase type 2 activity is lacking through e.g. inhibitor treatment or knockout, this will results in decreased 5α-DHT locally in the tissues, but also in blood (Robitaille & Langlois, 2020). This has been demonstrated in humans, rats, monkeys, and mice (Robitaille et al. 2020).

Finasteride is a specific inhibitor of 5α-reductase type 2 (Russell & Wilson, 1994). Men with androgenic alopecia were treated with increasing concentrations of finasteride and presented with decreased DHT levels in biopsies from scalp, as well as a decrease in serum DHT levels with dose dependency being most apparent in serum, up to about 70% decrease (Drake et al., 1999). Likewise, men treated with dutasteride exhibited a clear dose dependent decrease in serum DHT after 24 weeks treatment with a maximum efficacy of about 98% (Clark et al., 2004).

Other evidence

The phenotype of males with deficiency in 5α-reductases are typically born with ambiguous external genitalia. They also present with small prostate, minimal facial hair and acne, or temporal hair loss. Comparison of affected individuals to non-affected individuals in regard to T/DHT ratio, conversion of infused radioactive T, and ratios of urinary metabolites of 5α-reductase and 5β-reductase concluded that these phenotypic characteristics were due to 5α-reductase defects that resulted in less conversion of T to DHT (Okeigwe et al. 2014). Mutations in the 5α-reductase gene can result in boys being born with moderate to severe undervirilization phenotypes (Elzenaty 2022).

Quantitative Understanding of the Linkage

Inhibitors of 5α-reductase are important for the prevention and treatment of many diseases. There are several compounds that have been developed for pharmaceutical purposes and they can target the different isoforms with different affinity. Examples of inhibitors are finasteride and dutasteride. Finasteride mainly has specificity for the type 2 isoform, whereas dutasteride inhibits both type 1 and 2 isoforms (Miller & Auchus, 2011).

These differences in isoform specificity reflects in the effects on DHT serum levels, hence the broader specificity of dutasteride leads to > 90% decrease in patients with benign prostatic hyperplasia, in comparison to 70% with finasteride administration (Nikolaou et al., 2021).

Response-response relationship

Enzyme inhibition can occur in different ways e.g. both competitive and noncompetitive. The inhibition model depends on the specific inhibitor and hence a generic quantitative response-response relationship is difficult to derive.

Time-scale

An inhibition of 5α-reductases would lead to an immediate change in DHT levels at the molecular level. However, the time-scale for systemic effects on hormone levels are challenging to estimate.

Known Feedforward/Feedback loops influencing this KER

Androgens can regulate gene expression of 5α-reductases (Andersson et al., 1989; Berman & Russell, 1993).

References

Alemany, M. (2022). The Roles of Androgens in Humans: Biology, Metabolic Regulation and Health. In *International Journal of Molecular Sciences* (Vol. 23, Issue 19). MDPI. https://doi.org/10.3390/ijms231911952

Andersson, S., Bishop, R. W., & Russell$, D. W. (1989). *THE JOURNAL OF BIOLOGICAL CHEMISTRY Expression Cloning and Regulation of Steroid 5cw-Reductase, an Enzyme Essential for Male Sexual Differentiation** (Vol. 264, Issue 27).

Andersson, S., & Russell, D. W. (1990). Structural and biochemical properties of cloned and expressed human and rat steroid 5a-reductases. *Proc. Natl. Acad. Sci. USA*, *87*, 3640–3644. https://www.pnas.org

Azzouni, F., Godoy, A., Li, Y., & Mohler, J. (2012). The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases. In *Advances in Urology*. https://doi.org/10.1155/2012/530121

Berman, D. M., & Russell, D. W. (1993). Cell-type-specific expression of rat steroid 5a-reductase isozymes (sexual development/androgens/prostate/stroma/epithelium). In *Proc. Natl. Acad. Sci. USA* (Vol. 90). https://www.pnas.org

Clark, R. V., Hermann, D. J., Cunningham, G. R., Wilson, T. H., Morrill, B. B., & Hobbs, S. (2004). Marked Suppression of Dihydrotestosterone in Men with Benign Prostatic Hyperplasia by Dutasteride, a Dual 5α-Reductase Inhibitor. *Journal of Clinical Endocrinology and Metabolism*, *89*(5), 2179–2184. https://doi.org/10.1210/jc.2003-030330

Drake, L., Hordinsky, M., Fiedler, V., Swinehart, J., Unger, W. P., Cotterill, P. C., Thiboutot, D. M., Lowe, N., Jacobson, C., Whiting, D., Stieglitz, S., Kraus, S. J., Griffin, E. I., Weiss, D., Carrington, P., Gencheff, C., Cole, G. W., Pariser, D. M., Epstein, E. S., … City, O. (1999). *The effects of finasteride on scalp skin and serum androgen levels in men with androgenetic alopecia*.

Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews*, *32*(1), 81–151. https://doi.org/10.1210/er.2010-0013

Miller, W. L., & Auchus, R. J. (2019). The “backdoor pathway” of androgen synthesis in human male sexual development. *PLoS Biology*, *17*(4). https://doi.org/10.1371/journal.pbio.3000198

Nikolaou, N., Hodson, L., & Tomlinson, J. W. (2021). The role of 5-reduction in physiology and metabolic disease: evidence from cellular, pre-

clinical and human studies. In *Journal of Steroid Biochemistry and Molecular Biology* (Vol. 207). Elsevier Ltd. https://doi.org/10.1016/j.jsbmb.2021.105808

Peng, H. M., Valentin-Goyco, J., Im, S. C., Han, B., Liu, J., Qiao, J., & Auchus, R. J. (2020). Expression in escherichia coli, purification, and functional reconstitution of human steroid 5α-reductases. *Endocrinology (United States)*, *161*(8), 1–11. https://doi.org/10.1210/ENDOCR/BQAA117

Robitaille, J., & Langlois, V. S. (2020). Consequences of steroid-5α-reductase deficiency and inhibition in vertebrates. In *General and Comparative Endocrinology* (Vol. 290). Academic Press Inc. https://doi.org/10.1016/j.ygcen.2020.113400

Russell, D. W., & Wilson, J. D. (1994). *STEROID Sa-REDUCTASE: TWO GENES/TWO ENZYMES*. www.annualreviews.org

Thigpens, A. E., Cala, K. M., & Russell, D. W. (1993). Characterization of Chinese Hamster Ovary Cell Lines Expressing Human Steroid 5a-Reductase Isozymes. *The Journal of Biological Chemistry*, *268*(23), 17404–17412.

Yamana, K., Fernand, L., Luu-The, V., & Luu-The, V. (2010). Human type 3 5α-reductase is expressed in peripheral tissues at higher levels than types 1 and 2 and its activity is potently inhibited by finasteride and dutasteride. *Hormone Molecular Biology and Clinical Investigation*, *2*(3), 293–299. https://doi.org/10.1515/HMBCI.2010.035

Relationship: 1935: Decrease, DHT level leads to Decrease, AR activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	adjacent	High	High
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	Moderate
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent		

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

Taxonomic applicability

KER1935 is assessed applicable to vertebrates, as DHT and AR activation are known to be related in these species.

Sex applicability

KER1935 is assessed applicable to both sexes, as DHT activates AR in both males and females.

Life-stage applicability

KER1935 is considered applicable to developmental and adult life stages, as DHT-mediated AR activation is relevant from the AR is expressed.

Key Event Relationship Description

Dihydrotestosterone (DHT) is a primary ligand for the Androgen receptor (AR), a nuclear receptor and transcription factor. DHT is an endogenous sex hormone that is synthesized from e.g. testosterone by the enzyme 5α-reductase in different tissues and organs (Davey & Grossmann, 2016; Marks, 2004). In the absence of ligand (e.g. DHT) the AR is localized in the cytoplasm in complex with molecular chaperones. Upon ligand binding, AR is activated, translocated into the nucleus, and dimerizes to carry out its 'genomic function' (Davey & Grossmann, 2016). Hence, AR transcriptional function is directly dependent on the presence of ligands, with DHT being a more potent AR

activator than testosterone (Grino et al, 1990). Reduced levels of DHT may thus lead to reduced AR activation. Besides its genomic actions, the AR can also mediate rapid, non-genomic second messenger signaling (Davey and Grossmann, 2016). Decreased DHT levels that lead to reduced AR activation can thus entail downstream effects on both genomic and non-genomic signaling.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of KER1935 is considered high.

The activation of AR is dependent on binding of ligands (though a few cases of ligand-independent AR activation has been shown, see *uncertainties and inconsistencies*), primarily testosterone and DHT in most vertebrates and 11-ketotestosterone in teleost fishes (Schuppe et al., 2020). Without ligand activation, the AR will remain in the cytoplasm associated with heat-shock and other chaperones and not be able to carry out its canonical ('genomic') function. Upon androgen binding, the AR undergoes a conformational change, chaperones dissociate, and a nuclear localization signal is exposed. The androgen/AR complex can now translocate to the nucleus, dimerize and bind AR response elements to regulate target gene expression (Davey and Grossmann, 2016; Eder et al., 2001).

The requirement for androgens binding to the AR for transcriptional activity has been extensively studied and proven and is generally considered textbook knowledge. The OECD test guideline no. 458 uses DHT as the reference chemical for testing androgen receptor activation *in vitro* (OECD, 2020). In the absence of DHT during development caused by 5α-reductase deficiency (i.e. still in the presence of testosterone) male fetuses fail to masculinize properly. This is evidenced by, for instance, individuals with congenital 5α-reductase deficiency conditions (Costa et al., 2012); conditions not limited to humans (Robitaille and Langlois, 2020), testifying to the importance of specifically DHT for AR activation and subsequent masculinization of certain reproductive tissues.

Binding of testosterone or DHT has differential effects in different tissues. E.g. in the developing mammalian male; testosterone is required for development of the internal sex organs (epididymis, vas deferens and the seminal vesicles), whereas DHT is crucial for development of the external sex organs (Keller et al., 1996; Robitaille and Langlois, 2020).

Empirical Evidence

The empirical support for KER1935 is considered high.

Dose concordance:

- Increasing concentrations of DHT lead to increasing AR activation *in vitro* in AR reporter gene assays (OECD, 2020; Williams et al., 2017).
- In cell lines where proliferation can be induced by androgens (such as prostate cancer cells) proliferation can be used as a readout for AR-activation. Finasteride, a 5α-reductase inhibitor, dose-dependently decreases AR-mediated prostate cancer cell line proliferation (Bologna et al., 1995). 0.001 μM finasteride decreased the growth rate with 44%, 0.1 μM decreased the growth rate with 80%.
- Specific events of masculinization during development are dependent on AR activation by DHT, including the development and length of the perineum which can be measured as the anogenital distance (AGD, (Schwartz et al., 2019)). E.g. a dose-dependent effect of rat *in utero* exposure to the 5α-reductase inhibitor finasteride was observed on the length of the AGD, where 0.01 mg/kg bw/day finasteride reduced the AGD measured at pup day 1 by 8%, whereas 1 mg/kg bw/day reduced the AGD by 23% (Bowman et al., 2003).

Other evidence:

- Male individuals with congenital 5α-reductase deficiency (absence of DHT) fail to masculinize properly (Costa et al., 2012).
- A major driver of prostate cancer growth is AR activation (Davey and Grossmann, 2016; Huggins and Hodges, 1941). Androgen deprivation is used as treatment including 5α-reductase inhibitors to reduce DHT levels (Aggarwal et al., 2010).

Uncertainties and Inconsistencies

Ligand-independent actions of the AR have been identified. To what extent and of which biological consequences is not well defined (Bennesch and Picard, 2015).

It should be noted, that in tissues, that are not DHT-dependent but rather respond to T, a decrease in DHT level may not influence AR activation significantly in that specific tissue.

Quantitative Understanding of the Linkage

Response-response relationship

There is a positive dose-response relationship between increasing concentrations of DHT and AR activation (Dalton et al., 1998; OECD, 2020). However, there is not enough data, or overview of the data, to define a quantitative linkage *in vivo*, and such a relationship will differ between biological systems (species, tissue, cell type).

Time-scale

Upon DHT binding to the AR, a conformational change that brings the amino (N) and carboxy (C) termini into close proximity occurs with a $t_{1/2}$ of approximately 3.5 minutes, around 6 minutes later the AR dimerizes as shown in transfected HeLa cells (Schaufele et al., 2005). Addition of 5 nM DHT to the culture medium of 'AR-resistant' transfected prostatic cancer cells resulted in a rapid (from 15 minutes, maximal at 30 minutes) nuclear translocation of the AR with minimal residual cytosolic expression (Nightingale et al., 2003). AR and promoter interactions

occur within 15 minutes of ligand binding, and RNA polymerase II and coactivator recruitment are then proposed to occur transiently with cycles of approximately 90 minutes (Kang et al., 2002).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Age	AR expression changes with aging	Tissue-specific alterations in AR activity with aging	(Supakar et al., 1993; Wu et al., 2009)
Genotype	Number of CAG repeats in the first exon of AR	Decreased AR activation with increased number of CAGs	(Chamberlain et al., 1994; Tut et al., 1997)
Androgen deficiency syndrome	Low circulating testosterone levels due to primary (testicular) or secondary (pituitary-hypothalamic) hypogonadism	Reduced levels of circulating testosterone, precurser of DHT	(Bhasin et al., 2010)
Castration	Removal of testicles	Reduced levels of circulating testosterone, precurser of DHT	(Krotkiewski et al., 1980)

Known Feedforward/Feedback loops influencing this KER

Androgens can upregulate and downregulate AR expression as well as 5α-reductase expression, but for 5α-reductase, each isoform in each tissue is differently regulated by androgens and can display sexual dimorphism (Lee and Chang, 2003; Robitaille and Langlois, 2020).

References

Aggarwal, S., Thareja, S., Verma, A., Bhardwaj, T.R., Kumar, M., 2010. An overview on 5α-reductase inhibitors. Steroids 75, 109–153. https://doi.org/10.1016/j.steroids.2009.10.005

Bennesch, M.A., Picard, D., 2015. Minireview: Tipping the Balance: Ligand-Independent Activation of Steroid Receptors. Mol. Endocrinol. 29, 349–363. https://doi.org/10.1210/ME.2014-1315

Bhasin, S., Cunningham, G.R., Hayes, F.J., Matsumoto, A.M., Snyder, P.J., Swerdloff, R.S., Montori, V.M., 2010. Testosterone Therapy in Men with Androgen Deficiency Syndromes: An Endocrine Society Clinical Practice Guideline. J. Clin. Endocrinol. Metab. 95, 2536–2559. https://doi.org/10.1210/JC.2009-2354

Bologna, M., Muzi, P., Biordi, L., Festuccia, C., Vicentini, C., 1995. Finasteride dose-dependently reduces the proliferation rate of the LnCap human prostatic cancer cell line in vitro. Urology 45, 282–290. https://doi.org/10.1016/0090-4295(95)80019-0

Bowman, C.J., Barlow, N.J., Turner, K.J., Wallace, D.G., Foster, P.M.D., 2003. Effects of in Utero Exposure to Finasteride on Androgen-Dependent Reproductive Development in the Male Rat. Toxicol. Sci. 74, 393–406. https://doi.org/10.1093/TOXSCI/KFG128

Chamberlain, N.L., Driver, E.D., Miesfeld, R.L., 1994. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. Nucleic Acids Res. 22, 3181. https://doi.org/10.1093/NAR/22.15.3181

Costa, E.F., Domenice, S., Sircili, M., Inacio, M., Mendonca, B., 2012. DSD due to 5α-reductase 2 deficiency - From diagnosis to long term outcome. Semin. Reprod. Med. 30, 427–431. https://doi.org/10.1055/S-0032-1324727/ID/JR00766-20/BIB

Dalton, J.T., Mukherjee, A., Zhu, Z., Kirkovsky, L., Miller, D.D., 1998. Discovery of nonsteroidal androgens. Biochem. Biophys. Res. Commun. 244, 1–4. https://doi.org/10.1006/bbrc.1998.8209

Davey, R.A., Grossmann, M., 2016. Androgen Receptor Structure, Function and Biology: From Bench to Bedside. Clin. Biochem. Rev. 37, 3–15.

Eder, I.E., Culig, Z., Putz, T., Nessler-Menardi, C., Bartsch, G., Klocker, H., 2001. Molecular Biology of the Androgen Receptor: From Molecular Understanding to the Clinic. Eur. Urol. 40, 241–251. https://doi.org/10.1159/000049782

Grino, P.B., Griffin, J.E., Wilson, J.D., 1990. Testosterone at High Concentrations Interacts with the Human Androgen Receptor Similarly to Dihydrotestosterone. Endocrinology 126, 1165–1172. https://doi.org/10.1210/endo-126-2-1165

Huggins, C., Hodges, C. V., 1941. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res. 1, 293–297.

Kang, Z., Pirskanen, A., Jänne, O.A., Palvimo, J.J., 2002. Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. J. Biol. Chem. 277, 48366–48371. https://doi.org/10.1074/jbc.M209074200

Keller, E.T., Ershler, W.B., Chang, C., 1996. The androgen receptor: a mediator of diverse responses. Front. Biosci. (Landmark Ed) 1, 59–71. https://doi.org/10.2741/A116

Krotkiewski, M., Kral, J.G., Karlsson, J., 1980. Effects of castration and testosterone substitution on body composition and muscle metabolism in rats. Acta Physiol. Scand. 109, 233–237. https://doi.org/10.1111/J.1748-1716.1980.TB06592.X

Lee, D.K., Chang, C., 2003. Expression and Degradation of Androgen Receptor: Mechanism and Clinical Implication. J. Clin. Endocrinol. Metab. 88, 4043–4054. https://doi.org/10.1210/JC.2003-030261

Marks, L.S., 2004. 5Alpha-Reductase: History and Clinical Importance. Rev. Urol. 6 Suppl 9, S11-21.

Nightingale, J., Chaudhary, K.S., Abel, P.D., Stubbs, A.P., Romanska, H.M., Mitchell, S.E., Stamp, G.W.H., Lalani, E.N., 2003. Ligand Activation of the Androgen Receptor Downregulates E-Cadherin-Mediated Cell Adhesion and Promotes Apoptosis of Prostatic Cancer Cells. Neoplasia 5, 347. https://doi.org/10.1016/S1476-5586(03)80028-3

OECD, 2020. Test No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264264366-en

Robitaille, J., Langlois, V.S., 2020. Consequences of steroid-5α-reductase deficiency and inhibition in vertebrates. Gen. Comp. Endocrinol. 290. https://doi.org/10.1016/j.ygcen.2020.113400

Schaufele, F., Carbonell, X., Guerbadot, M., Borngraeber, S., Chapman, M.S., Ma, A.A.K., Miner, J.N., Diamond, M.I., 2005. The structural basis of androgen receptor activation: Intramolecular and intermolecular amino-carboxy interactions. Proc. Natl. Acad. Sci. U. S. A. 102, 9802–9807. https://doi.org/10.1073/pnas.0408819102

Schuppe, E.R., Miles, M.C., Fuxjager, M.J., 2020. Evolution of the androgen receptor: Perspectives from human health to dancing birds. Mol. Cell. Endocrinol. 499, 110577. https://doi.org/10.1016/J.MCE.2019.110577

Schwartz, C.L., Christiansen, S., Vinggaard, A.M., Axelstad, M., Hass, U., 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. https://doi.org/10.1007/s00204-018-2350-5

Supakar, P.C., Song, C.S., Jung, M.H., Slomczynska, M.A., Kim, J.M., Vellanoweth, R.L., Chatterjee, B., Roy, A.K., 1993. A novel regulatory element associated with age-dependent expression of the rat androgen receptor gene. J. Biol. Chem. 268, 26400–26408. https://doi.org/10.1016/S0021-9258(19)74328-2

Tut, T.G., Ghadessy, F.J., Trifiro, M.A., Pinsky, L., Yong, E.L., 1997. Long Polyglutamine Tracts in the Androgen Receptor Are Associated with Reduced Trans-Activation, Impaired Sperm Production, and Male Infertility. J. Clin. Endocrinol. Metab. 82, 3777–3782. https://doi.org/10.1210/JCEM.82.11.4385

Williams, A.J., Grulke, C.M., Edwards, J., McEachran, A.D., Mansouri, K., Baker, N.C., Patlewicz, G., Shah, I., Wambaugh, J.F., Judson, R.S., Richard, A.M., 2017. The CompTox Chemistry Dashboard: a community data resource for environmental chemistry. J. Cheminform. 9, 61. https://doi.org/10.1186/s13321-017-0247-6

Wu, D., Lin, G., Gore, A.C., 2009. Age-related Changes in Hypothalamic Androgen Receptor and Estrogen Receptor α in Male Rats. J. Comp. Neurol. 512, 688. https://doi.org/10.1002/CNE.21925

List of Non Adjacent Key Event Relationships

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		