The prostate cancer drug enzalutamide shortens anogenital distance in male rat offspring by blocking the androgen receptor

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Abstract

Background: Enzalutamide is a non-steroidal anti-androgen drug used to treat prostate cancer. It is a potent androgen receptor (AR) antagonist, with an in vitro Lowest Observed Effect Concentration (LOEC) of 0.05 μM . In this study, we wanted to assess its utility as a model compound for future mechanistic studies aimed at delineating mechanism-of-action of anti-androgenic effects in the developing fetus.

Methods: Enzalutamide in vitro activity was tested using an Androgen receptor reporter assay (AR-EcoScreenTM) and a steroidogenesis assay (H295R assay). For in vivo characterization, pregnant Sprague-Dawley rats were exposed to 10 mg/kg bw/day enzalutamide from gestational day 7-21. At gestational day 21, enzalutamide exposure concentrations were measured both in amniotic fluids and fetal plasma, alongside Anogenital distance (AGD). Fetal testes were collected and for testosterone measurements and gene expression profiling.

Results: Enzalutamide was a strong AR antagonist in vitro and we also observed disrupted androgen synthesis in the H295R steroidogenic assay with a LOEC of 3.1 μM . In utero exposure resulted in about 20% shorter anogenital distance (AGD) in male fetuses., as well as signs of dysregulated expression of the steroidogenic genes *Star*, *Cyp11a1* and *Cyp17a1* in the fetal testes at gestational day 21. Intra-testicular testosterone levels were unaffected.

Conclusions: Based on these observations, together with in vitro LOECs and the fetal plasma levels of enzalutamide, we propose that the effect on male AGD was caused by AR antagonism rather than suppressed androgen synthesis. Due to the characteristic mechanism of action of enzalutamide, we suggest to use it as a new model compound in research on anti-androgenic environmental chemicals.

Background

Androgen signaling is critical for male reproductive tract development and general masculinization of the fetus (1). Exposure to anti-androgens during fetal life can therefore lead to a broad range of male reproductive disorders such as hypospadias and cryptorchidism at birth (2, 3) or fertility issues in adulthood (4, 5). Anti-androgens can also cause general feminization of the male fetus, which can lead to a short male anogenital distance (AGD); a marker for disrupted fetal androgen action associated with male reproductive disorders (6–8).

Because of the strong link between fetal androgen insufficiency, male AGD, and male reproductive health disorders, both in humans and animals, AGD measurements are routinely performed in tests for developmental and reproductive toxicity in chemical risk assessment (9–12). However, it remains unclear to what extent AGD can be used as a reliable biomarker for chemicals with different anti-androgenic modes of action (8). To gain the necessary knowledge to characterize cause-effect relationships and thus better define the utility of a biomarker such as AGD, performing studies on various model compounds with known mechanism of action is of particular interest. With respect to androgen-sensitive endpoints,

selective drugs such as enzalutamide or the 5 α -reductase inhibitor finasteride are attractive model compounds due to their known, yet different, modes of action.

Enzalutamide is a second generation prostate cancer drug used to treat metastatic Castration-Resistant Prostate Cancer (mCRPC) for which the first generation drugs are not efficient (13–15). It is a non-steroidal anti-androgen that acts as an antagonist of the androgen receptor (AR) – the biological target of androgens such as testosterone and dihydrotestosterone – thereby preventing the effects of these hormones in the prostate gland (14). While first generation drugs such as bicalutamide target AR transcriptional activity by interfering with recruitment of co-activators to the transcriptional complex (16, 17), enzalutamide targets three key stages of AR signaling: blocking androgen binding, inhibiting translocation of activated AR and inhibiting binding of activated AR to the DNA (14). Thus, enzalutamide is a potent, specific inhibitor of androgen signaling.

We recently completed a study using the 5 α -reductase inhibitor finasteride as a model compound in an effort to tease out some of the underlying molecular mechanisms driving effects on male AGD (18). Based on in vitro data we found that enzalutamide apart from blocking the AR also inhibited androgen synthesis in vitro. To follow up on this, we tested enzalutamide in an in utero exposure study based on its known antiandrogenic effect in order to investigate, if the compound affected AGD and, if so, which mechanism was underlying the effect. We found that the AGD of the late gestation male fetuses were significantly shorter and that gene expression levels of steroidogenic enzymes in the fetal testis at GD21 were affected, albeit without significantly affecting the intra-testicular testosterone levels. Thus, we conclude that enzalutamide causes its AGD effect by antagonizing the AR.

Methods

Test compound

The test compound used in this study was Enzalutamide (purity 98%, CAS No.: 915087-33-1) purchased from Advanced Chemblocks Inc. (G-7963). Corn oil was purchased from Sigma-Aldrich (Copenhagen, Denmark) and used as control compound and vehicle (product number: C8267-2.5L).

AR-EcoScreen™ assay

The antagonistic effects of enzalutamide on AR were investigated using the Androgen Receptor Stably Transfected Transcriptional Activation Assay (AR STTA) described in OECD test guideline no. 458 (19). Experiments were run with three technical replicates and repeated thrice, with the mean value of technical replicates representing one biological replicate. AR-EcoScreen™ cells (JCRB1328, Japanese Collection of Research Bioresources) were grown in CellBIND® Surface cell culture flasks (Corning® Inc., Corning, New York, USA) in growth medium consisting of Gibco® DMEM/F-12 Nutrient Mixture with L-glutamine and HEPES and without phenol red supplemented with 5% FBS, 1% Penicillin-Streptomycin, 200 μ g/ml Zeocin™ Selection Reagent, and 100 μ g/ml Hygromycin B (all reagents from Invitrogen™, Life Technologies™, Carlsbad, California, USA). The cells were incubated at 37 °C with a humidified

atmosphere of 5% CO₂ and 95% air. Only cells of passage 3–15 were used for experiments. One day prior to the experiment cells were plated in white 96-well plates (Corning® Inc., Corning, New York, USA) at a density of 9,000 cells/well in assay medium consisting of Gibco® DMEM/F-12 Nutrient Mixture medium, 1% Penicillin-Streptomycin, with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) (all reagents from Invitrogen™, Life Technologies™, Carlsbad, California, USA). The following day the medium was changed to assay medium containing various concentrations of enzalutamide or the control compounds R1881 (known AR agonist, Perkin Elmer) or hydroxyflutamide (known AR antagonist, CAS No. 52806-53-8, Toronto Research Chemicals, Toronto, Ontario, Canada). Enzalutamide was added to the cells in 2-fold serial dilutions ranging from 0.05–12.5 µM. The DMSO vehicle concentrations were constant in all wells. R1881 was added at a concentration of 0.1 nM to all wells when testing for AR antagonism. After cells had been exposed to test compounds for ~ 20 hours firefly luminescence was measured in a luminometer (LUMIstar® Galaxy, BMG LABTECH, Offenburg, Germany) using Dual-Glo® Luciferase Reagent from the Dual-Glo® Luciferase Assay System from Promega (Madison, Wisconsin, USA). In order to distinguish a decrease in luciferase activity caused by pure antagonism from that caused by cytotoxicity, we measured Renilla luminescence using Dual-Glo® Stop & Glo® Reagent from the Dual-Glo® Luciferase Assay System (Promega, Madison, Wisconsin, USA).

H295R steroidogenesis assay

The ability of enzalutamide to affect steroidogenesis was investigated using the H295R Steroidogenesis Assay described in OECD test guideline no.456 (20). The H295R human adrenocortical carcinoma cell line (CRL-2128™, ATCC®, Manassas, Virginia, USA) was cultured in CellBIND® Surface cell culture flasks (Corning® Inc., Corning, New York, USA) in DMEM/F-12 growth medium with L-glutamine and HEPES and without phenol red (Invitrogen™, Life Technologies™, Carlsbad, California, USA) supplemented with 1% ITS + premix media additive (Corning® Inc., Corning, New York, USA) and 2.5% Nu-Serum™ (Corning® Inc., Corning, New York, USA). The cells were incubated at 37 °C with a humidified atmosphere of 5% CO₂ and 95% air. Only cells of passage 4–9 were used for experiments. One day prior to the experiment cells were plated in clear 24-well plates (Corning® Inc., Corning, New York, USA) at a final density of 300,000 cells/well. Cells were incubated with enzalutamide (2-fold serial dilutions ranging from 0.05–12.5 µM) for 48 hrs after which the supernatant was repositioned to new 24-well plates stored at -80 °C until quantification of hormone levels by HPLC-MS/MS. A quality control plate with the control compounds Forskolin (1 and 10 µM) (Sigma-Aldrich, Copenhagen, Denmark) and prochloraz (0.3 and 3 µM) (Ehrenstorfer, VWR & Bie & Berntsen, Denmark) were included in all experiments to monitor assay performance. Three independent experiments were performed each with technical triplicates.

After removal of the supernatant, cytotoxicity was evaluated using fluorescamine (CAS no. 38183-12-9, Sigma-Aldrich®, St. Louis, Missouri, USA). Cells were washed with 500 µl/well pre-warmed (37 °C) PBS. 150 µl/well lysis buffer were added and plates were shaken for 15 min. Hereafter, 400 µl of pre-warmed (37 °C) fluorescamine diluted in acetonitrile to a final concentration of 500 µg/ml was added to each well and placed dark in an incubator with a humidified atmosphere of 5% CO₂ and 95% air for 20 min. 300 µl of the supernatant was transferred to white opaque 24-well plates (PerkinElmer, Waltham,

Massachusetts, USA) and cell viability was evaluated by measuring fluorescence using an EnSpire® Multilabel Reader (PerkinElmer, Massachusetts, USA) with excitation and emission wavelengths of 390 nm and 460 nm, respectively.

Hormone analysis by HPLC–MS/MS

Steroid hormones were extracted and quantified as previously described (21, 22), with minor modifications. Before LC-MS/MS analysis, deuterated internal standards were added to the cell extracts and samples were centrifuged (15,000 g, 10 min). Steroid hormones were separated, detected, and quantified using on-line-SPE LC-MS/MS. For on-line SPE an Oasis HLB column (2.1 × 20 mm, 15 µm) was used. For 17β-estradiol and estrone analysis, a Kinetex C₁₈ column (2.1 × 100 mm, 2.6 µm) was used with an injection volume of 100 µl, measuring in ESI- mode using methanol, and 1 mM ammonia in water as the mobile phases (gradient flow rate was 0.4 ml/min). For the other hormones an Ascentis Express C₈ column (2.1 × 100 mm, 2.7 µm) was used with an injection volume of 100 µl, measuring in ESI-/ESI + mode with acetonitrile and 0.1% formic acid in water as the mobile phases (gradient flow rate was 0.25 ml/min). Ten hormones: testosterone, androstenedione, dehydroepiandrosterone (DHEA), corticosterone, cortisol, pregnenolone, progesterone, 17α-OH-progesterone, estradiol and estrone were quantified. The limit of quantification (LOQ) was 0.1 ng/ml for corticosterone, 1.0 ng/ml for DHEA and pregnenolone, 0.02 ng/ml for testosterone and androstenedione, 0.05 ng/ml for 17α-OH-progesterone, and 0.01 ng/ml for all the other hormones. For quantification, external calibration standards were run before and after the samples at levels of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 20 ng/ml, with 5.0 ng/ml internal standards: (testosterone-d₂, methyltestosterone-d₃, progesterone-c₂, and estradiol-d₃). The mass spectrometer was an EVOQ Elite Triple Quadrupole Instrument from Bruker (Bremen, Germany) and the UPLC system was an Ultimate 3000 system with a DGP-3600RS dual-gradient pump.

Animal study

Twelve time-mated nulliparous, young adult Sprague Dawley rats with a body weight of approximately 255 ± 25 g were supplied at gestational day (GD)3 (NTac:SD, SPF, Taconic Europe, Ejby, Denmark). The day of vaginal plug detection was designated GD1. On GD4, dams were randomized and distributed into 2 groups of 6 animals with similar body weight distributions. Animals were housed in pairs until GD17 and thereafter individually. The animals were housed under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15 × 27 × 43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden, Lynge, Denmark). They were placed in an animal room with controlled environmental conditions: 12 hr light-dark cycles with light starting at 9 pm, temperature 22 ± 1 °C, humidity 55 ± 5%, 10 air changes per hr.

All animals were fed a standard diet with Altromin 1314 (soy- and alfalfa-free, Altromin GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBT0702SU Tecniplast) were provided ad libitum. The PSU bottles and cages as well as the aspenwood shelters (instead of plastic) were used to eliminate any risk of migration of bisphenol A that could potentially confound the

study results. From GD7-21, dams were weighed daily and dosed by oral gavage by qualified animal technicians with a stainless steel probe 1.2 × 80 mm (Scanbur, Karlslunde, Denmark) with either vehicle control (corn oil) or enzalutamide (10 mg/kg bw/day) at a constant volume of 2 ml/kg bw per day. All animals were decapitated (guillotined) under CO₂/O₂-anesthesia at GD21.

Caesarean sections GD 21

Dams were decapitated (guillotined) under CO₂/O₂-anesthesia at GD21 and fetuses were collected by caesarean section. The dams were exposed 1h ± 15 min before decapitation in the same order as Caesarean sections were performed to adjust for the chemical analysis of maternal blood, fetal blood and amniotic fluid. Uteri were taken out and weighed, and the number of live fetuses, resorptions, and implantations were registered. Body weights of the fetuses were recorded prior to decapitation (by a scissor). Maternal trunk blood was collected and transferred to heparin-coated vials. Trunk blood from all fetuses was collected and transferred to heparin-coated vials and pooled for each gender within each litter. Blood samples were kept on ice and centrifuged at 4000 rpm, 4 °C for 10 min. Plasma was transferred to new tubes and stored at -80 °C. Amniotic fluid was collected from all fetuses, pooled within each litter, snap frozen in liquid nitrogen and subsequently stored at -80 °C.

AGD was measured as the distance between the genital papilla and the anus by the same, blinded technician using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) was calculated by dividing AGD by the cube root of the body weight. Fetal testes were isolated by dissection under a stereomicroscope and placed in liquid nitrogen (for steroid hormone analysis) or RNAlater (for RT-qPCR analysis) (Qiagen, Hilden, Germany) and stored at -80°C. The animal experiments were carried out at DTU Food (Mørkhøj, Denmark) with ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) and by the in-house Animal Welfare committee. All methods in this study were performed in accordance with relevant guidelines and regulations.

Enzalutamide concentrations in vivo

Acetonitrile, formic acid and 25% ammonium hydroxide, all of LC-MS grade, were obtained from Sigma Aldrich, Schneldorf, Germany. Evolute ABN express columns, 30 mg, were purchased from Biotage, Sweden. Water was purified on a Milli Q system, Millipore Corporation, US. First, 50 µl of plasma or amniotic fluid was transferred to an Eppendorf tube and 150 µl ice cold acetonitrile was added. The samples were centrifuged at 10000 x g, 4 °C for 10 minutes (Ole Dich Instrument makers, Denmark) and the supernatants were passed through an Evolute express ABN column. 20 µl eluate was transferred to an HPLC vial and 80 µl 50% acetonitrile was added prior to analysis by Liquid Chromatography – Mass Spectrometry (LC-MS).

LC was performed on a Dionex Ultimate 3000 RS (Thermo Scientific, CA) with a Poroshell SB C-18 (100 × 2.1 mm, 2.7 µm particle size) column held at 30 °C (Agilent technologies, Walbron, Germany). The solvent system consisted of A: 2.5 mM ammonium hydroxide + 0.1% formic acid in water and B: acetonitrile. Solvent programming were: 2% B from 0 to 1 min followed by a linear gradient to 95% B to 14 min, isocratic 95% B from 14 to 16 min followed by reversal to initial conditions to 16.1 min and re-

equilibration of the column to 20 min. The flow rate was 0.3 ml/min from 0 to 1 min followed by a linear gradient to 0.4 ml/min to 14 min, which was held to 16 min followed by reversal to initial conditions.

The LC system was connected to a Bruker Daltonics, maXis qTOF mass spectrometer equipped with an electrospray ion source operated in positive ion mode (Bruker Daltonics, Bremen, Germany). The ion source settings were: nebulizer pressure 2 bars, drying gas flow 8 l/min, dry gas temperature 200 °C, capillary voltage 2500 V. The scan range was from 80 to 1000 m/z with an acquisition rate of 2 Hz. Sodium formate dissolved in 50% 2-propanol was introduced in the ion source in a 0.2–0.4 min time segment and used for internal calibration of the data files. Hexakisperfluoroethoxyphosphazene was used as lock mass calibrant. Matrix matched standard samples were prepared in blank plasma at 6 different levels in the concentration range of: 3 to 10,000 nM. Standards and blanks were analyzed in the beginning of a sequence and after each set of 20 samples. Data files were processed using QuantAnalysis (Bruker Daltonics, Bremen, Germany). Extracted ion chromatograms of m/z 465.1003 $\pm$ 0.002 Da were constructed and integrated. Plasma concentrations were calculated based on linear calibrations curves constructed using 1/x weighing.

Testosterone measurements

One testicle was transferred to a beetBeater tube (Fischer Scientific, Waltham, MA, USA) and 1 mL of 80% acetonitrile (with internal standards, 1 ng/ml testosterone-d₂ (Rikilt, Wageningen, Netherlands) was added. The sample was homogenised for 1 min and 50 mg of Que Z-Sep (Sigma-Aldrich, St. Louis, Missouri, USA) was added followed by whirlmix for 1 min. The sample was centrifuged at 3000 x g, 4 °C for 10 min and placed at -18 °C for 1 hr. The sample was then centrifuged at 10000 x g 4 °C for 10 min., followed by filtration through a SPE column (Waters Corporation, Milford, MA, USA) (HLB, 30 mg), conditioned with 2 mL 80% acetonitrile. The sample was evaporated to dryness (50 °C, N₂) and dissolved in 500 µl 10% acetonitrile. Testosterone was detected and quantified using on-line-SPE LC-MS/MS. For on-line SPE, an Oasis HLB column (2.1 × 20 mm, 15 µm) was used and as analytical column an Ascentis Express C8 column (2.1 × 100 mm, 2.7 µm) was used with an injection volume of 100 µl in ES + mode with acetonitrile and 0.1% formic acid in water as the mobile phases (flow rate 0.25 ml/min, gradient method).

The MS was an EVOQ Elite Triple Quadrupole Instrument from Bruker (Bremen, Germany) and the UPLC system was an Ultimate 3000 system with a DGP-3600RS dual-gradient pump. For quantification, external calibration standards were run before and after the samples at levels of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 20 ng/ml (with 5.0 ng/ml internal standards). The limit of quantification (LOQ) for testosterone was estimated as the concentrations corresponding to six times signal-to-noise, and was < 20 pg/testicle.

Synthesis of cDNA and RT-qPCR analysis

Protocols were essentially as previously described (23). Briefly, total RNA was extracted from GD21 testis (n = 12/group) using RNeasy mini kit and on-column DNaseI treatment, according to manufacturer's instructions (Qiagen, Hilden, Germany). RNA was quantified by Nanodrop spectrophotometry (Nanodrop

Technologies, Wilmington, DE) and 500 ng total RNA used to synthesize cDNA using a random primer mix (New England Biolabs, Ipswich, MA, USA) and the Omniscript kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantitative RT-PCR (RT-qPCR) reactions were run in technical duplicates on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Thermo Fischer Scientific) in 11 µl reactions containing: 3 µl diluted (1:20) cDNA, TaqMan Fast Universal Mastermix (2X) (Life Technologies, Carlsbad, CA, USA) and TaqMan Gene Expressions Assays (Life Technologies). TaqMan assays were: Hsd3b1 Rn01774741_m1, Hsd17b1 Rn00563388_g1, Nr5a1 Rn00584298_m1, Ddx4 Rn01489814_m1, Sox9 Rn01751069_mH, Rps18 Rn01428913_gH, and Sdha Rn00590475_m1. In addition, primers and probes Cyp17a1, Cyp11a1 and Star were designed in our lab (24). The following cycling conditions were used: an initial step of 95 °C for 20 sec followed by 45 two-step thermal cycles of 95 °C for 1 sec and 60 °C for 20 sec. The relative transcript abundance was calculated using the $2^{-\Delta CT}$ method using Rps18 and Sdha as normalizing genes.

Statistics

Data from the AR-Eco Screen and H295R assay were analyzed by one-way ANOVA followed by Dunnett's post hoc test in GraphPad Prism 5 (GraphPad Software, San Diego California, USA). Results are presented as mean $\pm$ SEM for the three independent experiments. One measurement of DHEA in the H295R assay was lost so the DHEA data presented are from two independent experiments.

In vivo data on maternal parameters, fetal body weight, AGD and AGDi, were analyzed by one-way ANOVA followed by Dunnett's post hoc test, using SAS® (SAS Enterprise Guide 6.1, SAS Institute, Inc., Cary, NC, USA). AGD was analyzed using fetal weight as a covariate and fetal body weights were analyzed using the number of offspring per litter as covariate. For all analyses, the litter was the statistical unit. Statistical analyses were adjusted using litter as an independent, random and nested factor. For data presentation, group mean $\pm$ SEM was calculated from 6 litters/group based on litter means.

Analysis of enzalutamide concentrations in plasma and amniotic fluid as well as RT-qPCR data and intra-testicular testosterone levels was performed with student's t-test in GraphPad Prism 8 (GraphPad Software, San Diego California, USA). In cases of non-normal distribution or non-equal variance between groups, data was log-transformed prior to analysis, while the graphs still represent the untransformed data. For data presentation, mean $\pm$ SEM was calculated from 6 litters/group (maternal plasma, amniotic fluid and fetal plasma), 1 testicle from 6 fetuses /group (fetal intra-testicular testosterone) and 1 testicle from 12 fetuses/group (RT-qPCR).

Results

Enzalutamide acts as an AR antagonist in vitro

The AR agonistic and antagonistic potential of enzalutamide were investigated using the AR-EcoScreen™ assay (19). Enzalutamide did not show any agonistic activity (Fig. 1A) but showed AR antagonistic activity at all concentrations ($p < 0.001$) between 0.05–12.5 µM (Fig. 1B). The lowest observed effect

concentration (LOEC) was 0.05 μM while the IC_{50} value was 0.1 μM . We confirmed that the reduction in luciferase activity was not due to cytotoxicity following enzalutamide exposure (Fig. 1C).

Enzalutamide affects steroidogenesis in vitro

The H295R Steroidogenesis Assay (20) was used to test whether enzalutamide affects the synthesis of ten sex steroid hormones (Fig. 2). All steroid hormones, except for the two estrogens estrone and estradiol, were affected by enzalutamide. Pregnenolone levels were slightly increased with a LOEC of 3.1 μM . However, no increase was seen at concentrations of 25 μM and above. The progestagens, progesterone and 17 α -OH-progesterone were decreased with LOECs of 1.8 and 6.3 μM , respectively. The levels of the two androgens, androstenedione and testosterone were decreased, both with LOECs of 3.1 μM . By contrast, the adrenal androgen and precursor of the other androgens, dehydroepiandrosterone (DHEA), was increased with a LOEC of 0.8 μM . The corticosteroids, corticosterone and cortisol were generally decreased with LOECs of 6.3 and 25 μM , respectively, although cortisol was increased at 0.8 μM .

Enzalutamide was present in different biological compartments and male AGD was shorter in exposed animals at GD21

Pregnant Sprague Dawley rats were exposed to 10 mg/kg bw/day enzalutamide from GD7 to GD21 and maternal as well as fetal parameters were investigated at GD21. The distribution of enzalutamide in the different biological compartments (i.e. maternal plasma, amniotic fluid and fetal plasma) was determined at GD21 by HPLC-MS/MS. In maternal plasma the concentration was 1002 ± 145 nM, while it was 285 ± 46 nM in amniotic fluid, and 282 ± 53 nM and 122 ± 21 nM in the plasma of the female and male fetuses, respectively (Fig. 3). We measured maternal body weight, weight gain (GD 7–21), and uterus weight to determine if enzalutamide exposure resulted in maternal toxicity, but observed no treatment-related significant differences between the groups or any signs of maternal toxicity (Table 1). In addition, the number of fetuses as well as fetal weights of both males and females were similar between the two groups (Table 1). Attesting to the anti-androgenic potential of enzalutamide, we found that both AGD and AGDi was 19% shorter ($p < 0.001$) in exposed males compared to control males (Fig. 4, Table 1). There were no significant differences in either AGD or AGDi between exposed females and control females (Table 1).

Enzalutamide affects gene expression of steroidogenic enzymes in male fetal testis, without affecting intra-testicular testosterone levels

Based on our observations that enzalutamide affects steroidogenesis in vitro, we assessed expression levels of key genes encoding steroidogenic enzymes in the male fetal testis at GD21. There was an upregulation of Star ($p < 0.001$), Cyp11a1 ($p < 0.05$), Cyp17a1 ($p < 0.05$) and a downregulation of Nr5a1 ($p < 0.05$) following in utero exposure to enzalutamide (Fig. 5). The expression levels of the two hydroxysteroid dehydrogenase genes, Hsd3b1 and Hsd17b1, were unchanged, as were the germ cell marker gene Ddx4 and Sertoli cell marker Sox9 (Fig. 5). Next, we measured the intra-testicular

testosterone levels, but found no significant difference between control males and exposed males (Fig. 6). Finally, histopathological assessment of H&E stained fetal testis sections showed no adverse alterations to testis histology (Fig. 7).

Discussion

Enzalutamide is a second generation prostate cancer drug used to treat men with mCRPC (13). In this study, we explored the use of enzalutamide as a potential model compound or for investigations of anti-androgenic actions of environmental chemicals. Our particular interest in this regard is the use of target-specific compounds to delineate the molecular mechanisms driving the development of anogenital tissues. The purpose is to obtain better knowledge on the utility of AGD as a general biomarker for fetal anti-androgenic effects.

Enzalutamide is designed to specifically target the AR (14). In our AR reporter gene assay the IC_{50} for AR antagonism was calculated to be 0.05 μ M, which is close to the IC_{50} of 0.03–0.05 μ M previously reported (14, 25). Our results thereby confirmed that enzalutamide acts as an AR antagonist, without having any AR agonistic activity. Because of these specific antagonistic properties, we performed an in utero exposure study in rats, with focus on effects on the male AGD.

We measured the concentration of enzalutamide in maternal plasma, amniotic fluid and fetal plasma at GD 21 in Sprague Dawley rats and found it present in all three compartments. These data clearly show that enzalutamide can transfer across the placenta, albeit the maternal concentration was about four times higher than in the fetal compartments. Notably, we also measured almost twice the concentration of enzalutamide in male plasma compared to female. It remains unclear why this difference was observed, but sex differences in pharmacokinetics is a well-documented phenomenon, both in humans and animals (26). Furthermore, the fetal plasma concentration of 0.1–0.3 μ M is twice or more than the LOEC of 0.05 μ M for AR antagonism observed in vitro. Taken together, these data show that an exposure level of 10 mg/kg bw/day during gestation is appropriate to reach biologically active enzalutamide levels in the fetus and reduce fetal androgen signaling. As a reduced AGD caused by fetal androgen insufficiency is a well-known marker in both humans and rodents, we believe that the observed effects are translatable to humans.

Fetal androgen insufficiency, caused by low androgen levels or blockage of AR signaling, can prevent the male perineum to develop properly and result in a short AGD (8). One previous report shows that enzalutamide induces shortening of male AGD in mice, although they do not state how much shorter AGD is compared to controls (27). We found the average AGD to be 19% shorter in enzalutamide exposed males than in control males. This effect is less pronounced than previously observed with the prostate cancer drug and AR antagonist flutamide, which shortened male AGD by 19% at a dose of only 2 mg/kg (28), and between 35–43% in the 6–8 mg/kg dose range (28, 29). Conversely, enzalutamide has a greater effect on male AGD than some of the pesticides known to interfere with AR, such as vinclozolin and procymidone, where doses of 50–100 mg/kg are required to induce a similar shortening of AGD (28, 30–

32). Because of the greater potency and the known mode of action of selective pharmaceuticals like flutamide, finasteride or enzalutamide, they are often better suited as model compounds than less specific environmental chemicals when performing studies aimed at characterizing mechanisms of effects.

Apart from blocking AR action, enzalutamide was also found to disrupt hormone synthesis in the H295R in vitro steroidogenesis assay. Progestagen, androgen and corticosteroid synthesis was downregulated, whereas estrogen synthesis was unaffected. Dehydroepiandrosterone (DHEA), on the other hand, was markedly elevated. It remains unclear exactly how enzalutamide cause these effects in the steroidogenic pathway, but judging from the hormone profile, we speculate that 3 β -hydroxysteroid dehydrogenase is inhibited. With the decrease in androstenedione and testosterone, a decrease in estrogens could have been expected. It is, however, possible that sulfotransferase activity, which is responsible for metabolism of estrogens (33), was reduced. Reduced sulfotransferase activity could equalize the estrogen levels and result in unchanged levels. Another possible explanation could be induction of CYP19 as enzalutamide has previously been shown to induce other CYPs, specifically CYP2C9, CYP2C19 and CYP3A4 (34). These in vitro assays together with physiologically-based kinetic modeling have the potential to be able to predict AGD effects in the future, thereby minimizing animal testing.

Since steroidogenesis was affected in vitro, we also investigated the fetal testis for signs of steroidogenic disruption. We saw increased expression of the key steroidogenic genes Star, Cyp11a1 and Cyp17a1. We initially speculated that this could be a compensatory response to reduced testosterone levels. However, we did not detect any significant reduction in intra-testicular testosterone levels. Thus, at the functional level the effects of enzalutamide on the fetal testis may be diminishable compared to its effect on AR activation in peripheral tissues. In support of this, the in vitro LOEC on testosterone synthesis was 3.1 μ M, a concentration that is much higher than the actual measured fetal levels of 0.1 μ M. Nevertheless, our results point to some degree of disturbance to fetal testis function, but more studies are needed to elucidate the exact mechanism of action of enzalutamide on the steroidogenesis pathway.

Conclusion

In summary, enzalutamide has strong AR antagonistic effects both in vitro and in vivo. While we observed weak inhibition of steroid synthesis in the rat, this is likely of minor importance in the mode of action, as intra-testicular testosterone levels were not affected. Since enzalutamide is used to treat prostate cancer patients, fetuses will most likely not be exposed to the compound under normal circumstances; albeit, environmental exposure cannot be ruled out. The effects of enzalutamide on the developing male fetus are therefore not of immediate concern, but confirms that enzalutamide is a valuable model compound for future studies on effects of AR antagonists.

Abbreviations

AGD Anogenital distance

AR	Androgen receptor
GD	Gestational day
DHEA	Dehydroepiandrosterone
LOEC	Lowest Observed Effect Concentration
mCRPC	Metastatic Castration-Resistant Prostate Cancer

Declarations

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AUTHORS CONTRIBUTIONS

CLS, TS and AMV planned the study, all authors contributed some to the design of experiments. CLS, TS, CT, MP, HLF, KAF and HKLJ, together with support staff, performed the experiments. All authors were involved in the analysis and interpretation of data. CLS drafted the manuscript. All authors helped revise the manuscript and approved the final version.

ADDITIONAL DECLARATIONS

Ethics Statement: Animal experimentation had ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) and was overseen by DTU Food's in-house Animal Welfare committee.

Consent for Publication: Not applicable.

Disclosure Statement: All authors declare that they have no competing interests, neither financial nor non-financial, with work presented in this study.

Data Availability: All data are included in the manuscript. Additional information is available upon request to corresponding author.

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Tables

Table 1 - Pregnancy and litter data

Data represent group means, based on litter means $\pm$ SD after oral exposure GD7-21.

ENZ= Enzalutamide, 10 mg/kg bw/day.

	Control	ENZ
GD21 Caesarean section		
No. of litters	N=6	N=6
Maternal body weight (g), GD 7	262.2 $\pm$ 12.2	269.2 $\pm$ 11.4
No. of fetuses	14.0 $\pm$ 2.4	13.2 $\pm$ 2.3
Maternal bw gain GD7- 21 (g)	115.3 $\pm$ 18.8	124.5 $\pm$ 17.0
Adjusted maternal bw (g) ^	289.7 $\pm$ 20.0	307.8 $\pm$ 18.7
Uterus weight (g)	87.8 $\pm$ 10.8	85.9 $\pm$ 13.8
Fetal weight male (g) (bw)	4.3 $\pm$ 0.4	4.3 $\pm$ 0.2
Fetal weight female (g) (bw)	4.2 $\pm$ 0.3	4.0 $\pm$ 0.3
Male AGD (mm)	3.64 $\pm$ 0.2	2.96 $\pm$ 0.1**
Male AGD index	2.24 $\pm$ 0.03	1.82 $\pm$ 0.07**
Female AGD (mm)	1.81 $\pm$ 0.09	1.67 $\pm$ 0.08
Female AGD index	1.13 $\pm$ 0.04	1.06 $\pm$ 0.07

AGD index = AGD divided by cube root of the bodyweight

Figures

Figure 1

Enzalutamide acts as an AR antagonist: Dose-response curves for enzalutamide in the AR-EcoScreen™. A: Cells were incubated with increasing concentrations of enzalutamide (μ M). The values are mean $\pm$ SEM (n=3) and are presented as fold change in firefly luminescence. B: Cells were incubated with increasing concentrations of enzalutamide (μ M) in the presence of 0.1nM R1881. The values are mean $\pm$ SEM (n=3) and are presented as fold change in firefly luminescence relative to the response achieved with 0.1nM R1881, which is set to 1. ***: Significantly different from control, p<0.001. C: Induction of cytotoxicity following enzalutamide exposure. The values are mean $\pm$ SEM (n=3) and are presented as fold change in Renilla luminescence.

Figure 2

Enzalutamide affects steroidogenesis: Relative levels of produced steroid hormones by H295R (CRL-2128TM) cells exposed to increasing concentrations of enzalutamide (μM) for 48hours. Results shown are mean±SEM (n=3 and n=2 for DHEA). *: Significantly different from control, p<0.05. **: Significantly different from control, p<0.01. ***: Significantly different from control, p<0.001.

Enzalutamide at GD21

Figure 3

Enzalutamide levels in plasma and amniotic fluid: Concentration (nM) of enzalutamide in maternal plasma, amniotic fluid or plasma of the male and female fetuses at GD21 following exposure of pregnant rats from GD7 to GD21 to 10 mg/kg bw/day of enzalutamide. Results shown are mean $\pm$ SEM of pooled samples from 6 litters.

Male Anogenital Distance index

Figure 4

Enzalutamide reduces the male anogenital distance index: Anogenital distance index (AGD/cube root of bw) of control males (Control) and enzalutamide exposed males (enzalutamide, 10 mg/kg bw/day GD7-GD21). Results are shown as mean $\pm$ SEM, n=6 litters/group, with each dot representing one male fetus. **: Significantly different from control, $p < 0.01$ (with bw as covariate).

Figure 5

Enzalutamide affects gene expression of steroidogenic enzymes: RT-qPCR of steroidogenic enzymes. Results are $2^{-\Delta CT}$ values shown as mean $\pm$ SEM (n=12/group). Enz: Exposed to enzalutamide, 10mg/kg bw/day GD7-GD21. *: Significantly different from control, $p < 0.05$. **: Significantly different from control males, $p < 0.01$.

Intratesticular testosterone

Figure 6

Enzalutamide does not affect intra-testicular testosterone at GD 21: Intra-testicular testosterone concentration at GD21 (ng testosterone/testicle) following exposure to 10mg/kg bw/day of enzalutamide (Enz) from GD7 to GD21. Results shown are mean $\pm$ SEM of 1 testicle from 6 fetuses/group.

Ctrl

Enz

Figure 7

Enzalutamide does not affect fetal testis histology: Cross-sections of formalin-fixed GD21 testes from controls or enzalutamide exposed males (10mg/kg bw/day, GD7-21) were stained with hematoxylin & eosin (H&E). (n = 3); scale bar upper panel = 100 μ m, lower panel = 20 μ m.