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Expression of human aldo-keto reductase 1C2 in cell lines of peritoneal endometriosis: Potential implications in metabolism of progesterone and dydrogesterone and inhibition by progestins

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ABSTRACT

The human aldo-keto reductase AKR1C2 converts 5α-dihydrotestosterone to the less active 3α-androstanediol and has a minor 20-ketosteroid reductase activity that metabolises progesterone to 20α-hydroxyprogesterone. AKR1C2 is expressed in different peripheral tissues, but its role in uterine diseases like endometriosis has not been studied in detail. Some progestins used for treatment of endometriosis inhibit AKR1C1 and AKR1C3, with unknown effects on AKR1C2. In this study we investigated expression of *AKR1C2* in the model cell lines of peritoneal endometriosis, and examined the ability of recombinant AKR1C2 to metabolise progesterone and progestin dydrogesterone, as well as its potential inhibition by progestins. *AKR1C2* is expressed in epithelial and stromal endometriotic cell lines at the mRNA level. The recombinant enzyme catalyses reduction of progesterone to 20α-hydroxyprogesterone with a 10-fold lower catalytic efficiency than the major 20-ketosteroid reductase, AKR1C1. AKR1C2 also metabolises progestin dydrogesterone to its 20α-dihydrodydrogesterone, with 8.6-fold higher catalytic efficiency than 5α-dihydrotestosterone. Among the progestins that are currently used for treatment of endometriosis, dydrogesterone, medroxyprogesterone acetate and 20α-dihydrodydrogesterone act as AKR1C2 inhibitors with low μM K_i values *in vitro*. Their potential *in vivo* effects should be further studied.

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1. Introduction

Endometriosis is a complex disease that is defined as the presence of endometrial glands and stroma outside the uterine cavity [1]. Most commonly, it is diagnosed in women in reproductive age, and it affects up to 15% of all premenopausal women [1]. Ectopic endometrium can be found on the ovaries, the pelvic peritoneum, or different parts of the rectovaginal tract, thus forming three different entities with different pathogenesis: ovarian endometriosis, peritoneal endometriosis and deep infiltrating endometriosis, respectively [2–4]. Endometriosis is usually treated surgically, although up to 40% of patients face recurrence within 9 years of surgery [5]. The combination of surgical and medical treatment with gonadotropin-releasing hormone (GnRH) agonists or aromatase inhibitors can provide improved outcomes. These treatments are not suitable for long-term application; therefore, other therapeutic strategies are needed. One possibility is the application of progestins and/or combined oral contraceptives, which can be effective, well tolerated and safer than GnRH agonist use [6].

Progestins are defined as synthetic progestational agents, which act as agonists of the PRs and have progestogenic effects on the estrogen-primed endometrium. There are different classes of progestins: progesterone derivatives (retroprogesterone dydrogesterone, 17-hydroxy progesterone, and 19-norprogesterone derivatives); testosterone derivatives (19-nortestosterone derivatives); and spironolactone derivatives (e.g. drospirenone) [7,8]. Their detailed mechanism of action is not understood in detail, but the studies suggest that progestins generally affect the hypothalamic–pituitary–ovary axis and in this manner suppress ovarian steroidogenesis and also cause decidual transformation of the eutopic endometrium, and to some degree of ectopic lesions as well, with different effects seen with the different progestins [9]. Several progestins have been used for treatment of endometriosis,

Abbreviations: 5α-DHT, 5α-dihydrotestosterone; 5α-DHP, 5α-dihydroprogesterone.

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although with different success rates [10]. The retroprogesterone dydrogesterone has a better bioavailability than progesterone, and its main metabolite, 20α-dihydrodydrogesterone, is also active [11].

We recently showed that the recombinant human aldo-keto reductase 1C1 (AKR1C1) converts dydrogesterone to its active metabolite 20α-dihydrodydrogesterone [12]. We also showed that progestins dydrogesterone, 20α-dihydrodydrogesterone, medroxyprogesterone acetate and norethinodrone, inhibit AKR1C1 and aldo-keto reductase 1C3 (AKR1C3) with μM K_i values [12]. Our experimental data demonstrate that *AKR1C1* and *AKR1C3* are overexpressed in ovarian endometriosis at the mRNA level [13]. This elevated mRNA levels of *AKR1C1* and *AKR1C3* may contribute to the persistence of endometriosis through the metabolism of progesterone, thus treatment with progestins may reduce the activity of AKR1C1/AKR1C3. Also the homologous AKR1C2, which differs from AKR1C1 only in 7 amino acid residues, is overexpressed in ovarian endometriosis [13], suggesting that this enzyme also contribute to progesterone and dydrogesterone metabolism and may be inhibited by progestins as well.

The human aldo-keto reductase AKR1C2 *in vivo* catalyzes reduction of 5α-dihydrotestosterone (5α-DHT), the most potent androgen, to its less active counterpart 3α-androstanediol [14] (Fig. 1, Table 1). This enzyme has minor 20-keto- and 17-ketosteroid reductase activities, and thus converts progesterone to the less active 20α-hydroxyprogesterone, and estrone to the potent estradiol [14] (Fig. 1). AKR1C2 also converts 5α-dihydroprogesterone (5α-DHP) mainly to 5α-pregnane-3α-ol-20-one, the most potent positive allosteric modulator of the γ-aminobutyric acid type A ($GABA_A$) receptor [14], as well as to 5α-pregnane-20α-ol-3-one, and is thus involved in the production of active neurosteroids [15,16] (Fig. 1, Table 1). The expression of AKR1C2 has been observed in different tissues including uterus, where AKR1C2 probably forms 3α-androstanediol and 20α-hydroxyprogesterone, and it might thus be involved in termination of pregnancy [14]. However, the role of AKR1C2 in progesterone and 5α-DHP metabolism in uterine diseases, such as endometrial cancer and endometriosis, has not been examined. We hypothesized that the observed increased levels of AKR1C2 in ovarian

Table 1
Kinetic parameters of AKR1C2-catalyzed reactions.

Substrate	K_M(μM)	k_{cat}(min^{-1})	k_{cat}/K_M(min^{-1} μM^{-1})
5α-DHT[a]	5.7	3.0	0.52
5α-DHP[b]	0.6	0.48	0.80
Progesterone[a]	7.7	0.21	0.030
Dydrogesterone[a]	1.6	7.0	4.480

5α-DHT, 5α-dihydrotestosterone; 5α-DHP, 5α-dihydroprogesterone.
[a] Present study.
[b] Usami et al. [16].

OH
O
H
NADPH + H⁺
NADP⁺
OH
HO
H
5α-Dihydrotestosterone
3α-Androstanediol

O
O
H
NADPH + H⁺
NADP⁺
O
HO
H
5α-Dihydroprogesterone
5α-Pregnane-3α-ol, 20-one

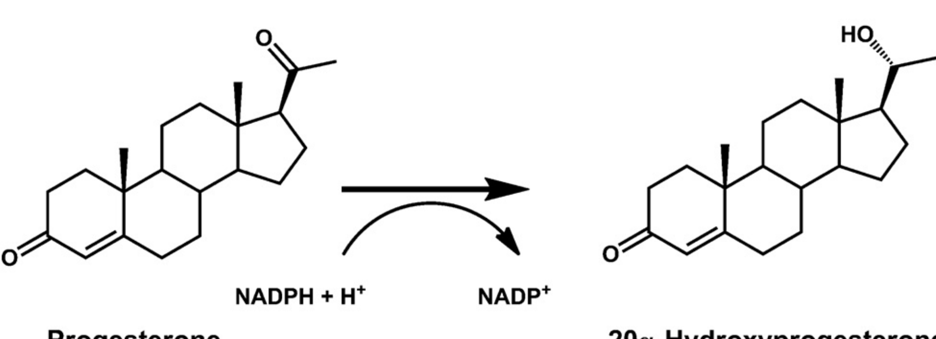

Fig. 1. Different enzymatic activities of AKR1C2. AKR1C2 can act as a 3-ketosteroid reductase, converting 5α-dihydrotestosterone to 3α-androstanediol and 5α-dihydroprogesterone to 5α-pregnane-3α-ol-20-one, and also as a 20-ketosteroid reductase, converting progesterone to 20α-hydroxyprogesterone.

endometriosis enhance metabolism of progesterone to the less active 20α-hydroxyprogesterone, increase the production of 20α-dihydrodydrogesterone from dydrogesterone, and that similarly as AKR1C1 and AKR1C3 also AKR1C2 may be inhibited by progestins.

The aim of this study was thus to evaluate the role of AKR1C2 in endometriosis, especially in progesterone and dydrogesterone metabolism. We investigated expression of *AKR1C2* in the model cell lines of peritoneal endometriosis, examined the ability of AKR1C2 to metabolise progesterone and progestin dydrogesterone, and evaluated the inhibitory effects of different progestins that are currently used for the treatment of endometriosis.

2. Materials and methods

2.1. Materials

Medroxyprogesterone acetate, desogestrel, norethindrone, levonorgestrel, danazol, progesterone, 5α-dihydrotestosterone, 1-acenaphthenol, ursodeoxycholic acid, litocholic acid, Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM)/F-12, Dulbecco's modified Eagle's medium (DMEM), Minimum essential Eagle's medium (MEM), foetal bovine serum, penicillin, streptomycin, trypsin–EDTA solution, and DNase I (AMP-D1) were obtained from Sigma-Aldrich Chemie GmbH (Diesenhofen, Germany). Progesterone (1,2,6,7-^{3}H) and dihydrotestosterone (1,2,4,5,6,7^{-3}H)-(5α androstan-17β-ol-3-one) were purchased from Perkin Elmer (Boston, MA USA). TaqMan® Universal PCR Master Mix was purchased from Applied Biosystems (Foster City, California, USA), and SuperScript Vilo cDNA Synthesis Kit was obtained from Invitrogen (Carlsbad, California, USA). Dydrogesterone and 20α-dihydrodydrogesterone were kindly provided by Abbott Products GmbH Hannover, Germany.

2.2. Cell culture

We used immortalised human endometriotic epithelial cells (12-Z) and stromal cells (22-B) kindly provided by Dr. Starzinski-Powitz [16]. The endometriotic cell lines were established from light red peritoneal endometriotic lesions by transfection with SV40T antigen and have been well characterized showing that they represent good models of peritoneal endometriosis [17–19]. The endometriotic cells were cultured in DMEM/F-12 containing 5% foetal bovine serum and penicillin (100 U/mL), streptomycin (100 μg/mL) in humidified 5% CO_2 and 95% air at 37 °C. Ishikawa cells were obtained from European Collection of Cell Cultures (ECACC, 99040201) and were maintained at 37 °C and 5% CO_2 in MEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 5% foetal bovine serum. HepG2 cells were obtained from American Type Culture Collection (ATCC, HB-8065) and were cultured in DMEM containing 10% foetal bovine serum in humified 5% CO_2 and 95% air at 37 °C.

2.3. Real-time PCR analysis

The 12-Z, 22-B, Ishikawa and HepG2 cells were cultured in T75 flasks. At 80–90% confluency the cells were harvested, washed with phosphate-buffered saline and used for total RNA isolation, using QuickGene-810 (FujiFilm) according to the manufacturer instructions. RNA quantity and quality were assessed with NanoDrop and Agilent 2100 Bioanalyser instruments, respectively. The average RNA integrity number was 9.9. DNAse treatment was performed on all samples, and cDNA synthesis was carried out with SuperScript Vilo cDNA Synthesis Kit. Two μg of total RNA was converted into cDNA (40 μl), according to the manufacturer instructions, and then stored at −20 °C.

The expression of *AKR1C2* and three previously selected reference genes, *PPIA*, *GAPDH* and *RPLP0* [20], was examined by real-time TaqMan® PCR assay. *PPIA*, *GAPDH* and *RPLP0* expression levels were determined with the exon-spanning hydrolysis probes (FAM or VIC dye labelled) that are commercially available as 'Assay on Demand' (Applied Biosystems, Foster City, CA, USA), with optimised primer and probe concentrations [13,20]. The primers and fluorescent TaqMan® MGB probes for the specific amplification of *AKR1C2* were designed in our laboratory [21]. Quantification was accomplished with the LightCycler® 480 Real-Time PCR System (Roche) using TaqMan® Universal PCR Master Mix and the universal thermocycling parameters recommended by Applied Biosystems. RT-PCR samples were run in triplicates using 0.25 μL cDNA. The reactions were performed in 384-well plates (Roche) with a reaction volume of 5 μL. The gene expression normalisation factor for each sample was calculated based on the geometric mean of all three of the selected reference genes [22]. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines were considered in the performance and interpretations of the qPCR reactions [23].

2.4. Construction of pGex-AKR1C2

The pET41a(+)-AKR1C2 was constructed from a pcDNA3-AKR1C2 vector (kindly provided by Dr. Trevor M. Penning). The forward oligonucleotide primer with a restriction site for *EcoRV* (5′-CCTTGAT↓ATCTGATGGATTCGAAATACCAG-3′), and reverse oligonucleotide primer with a restriction site for *Xho*I (5′-TTTAC↓TCGAGCATTAATATTCATCAGAAAATGG-3′), were used for amplification of the AKR1C2 coding sequence by PCR. The PCR product and the pET41a(+) vector (Novagen, Merck KGaA, Darmstadt, Germany) were digested with *EcoRV* and *Xho*I, gel purified, and ligated.

2.5. Expression and purification of recombinant AKR1C2

The pET41a(+)-AKR1C2 construct was verified by sequencing (Sequiserve, Vaterstetten, Germany) and transferred into the *Escherichia coli* BL21-CodonPlus(DE3)-RIL strain. The cells were grown in Luria-Bertani medium containing 50 μg/mL kanamycin at 37 °C in a rotary shaker, until an OD_{600} of 1.0 had been reached. Expression was induced by isopropyl beta-D-thiogalactoside at a final concentration of 1 mM, and the incubation was continued for 16 h at 24 °C, as described for AKR1C1 [24]. The preparation of cell extracts, purification of the glutathione-S-transferase (GST)-fusion protein by affinity binding to glutathione–sepharose, and the cleavage with thrombin were performed as described previously [25]. Protein concentrations of samples were determined using the Bradford method, with bovine serum albumin as the standard. The purity of AKR1C2 was examined by SDS PAGE followed by Coomasie blue staining, where only a single band with a molecular mass of 37 kDa was seen (Supplementary Fig. S1).

2.6. AKR1C2 enzymatic assays

The 20-ketosteroid reductase activity of AKR1C2 was measured following reduction of different concentrations of [^{3}H]-labelled progesterone or 5α-DHT (final total concentrations: 1–40 μM or 0.5–30 μM, respectively) in 100 mM phosphate buffer (pH 6.5) in the presence of 100 μM NADPH, and 4% acetonitrile as a co-solvent, 0.3 μM or 50 nM AKR1C2 with progesterone or 5α-DHT as substrates, respectively. The reactions were performed at 37 °C and stopped by the addition of 500 μL ethyl acetate, after different times (2–90 min or 3–20 min with progesterone or 5α-DHT, respectively). The substrate progesterone or 5α-DHT and the product 20α-hydroxyprogesterone or 5α-androstane-3α-ol-17β-ol were

extracted from the organic, ethyl acetate phase of the reaction mixture. The organic phase was evaporated under reduced pressure, and the residue was dissolved in 100 μL acetonitrile and separated using an HPLC system (Knauer, Berlin, Germany) equipped with an ODS Hypersil reverse phase column (5 μm, 250 mm × 4 mm; Thermo Fisher Scientific, Waltham, MA, USA) and detector of radioactivity. The mobile phase consisted of acetonitrile and water (ratio 55:45 [v/v] and 40/60 [v/v] with progesterone and 5α-DHT as substrates, respectively), at a flow rate of 1 mL/min. The linear range of the enzymatic reaction was determined, and the K_M and V_{max} values were estimated from the plots of initial velocity *versus* concentration of substrate, using GraphPad Prism Version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). The reduction of different concentrations of unlabelled dydrogesterone (final concentrations: 1–20 μM) in the presence of 90 nM AKR1C2 were also measured, as described above. These reactions were performed at 37 °C and stopped with ethyl acetate after different times (1–8 min). The substrate dydrogesterone and the product 20α-dihydrodydrogesterone were extracted from the reaction mixture in ethyl acetate, analysed as described above and detected by measuring absorbance at 300 nm.

2.7. NMR analysis of 20α-dihydrodydrogesterone

The stereoselectivity of dydrogesterone metabolism by AKR1C2 was also evaluated. The metabolite was prepared in 100 mM phosphate buffer (pH 6.5) in the presence of 300 μM NADPH, 4% acetonitrile as a co-solvent, 0.3 μM AKR1C2 and 50 μM dydrogesterone. Total reaction volume was 10 mL. Parallel reactions were performed at 37 °C and stopped by the addition of 10 mL ethyl acetate after 24 h. The substrate dydrogesterone and the metabolite were extracted from the reaction mixture in ethyl acetate. The metabolite was further purified with column chromatography using Merck silica gel 60 (mesh 70–230) as the stationary phase and a mixture of chloroform, ethyl acetate and methanol in a 20:4:1 ratio as the mobile phase. The ^1H NMR spectrum was recorded on a Bruker Advance 400 DPX spectrometer at 302 K using solvent as an internal standard ($CDCl_3$ at 7.26 ppm). In the spectrum the signals that unambiguously prove the identity of the produced 20α-dihydrodydrogesterone (the chemical shifts are identical to those published previously [26]) are clearly visible and are broadened in Fig. 4. The signals that are easily distinguished are the singlet at 0.76 ppm and a symmetrical multiplet in the 3.70–3.80 ppm range that are representing the H-18 proton and the H-20 proton, respectively. The third characteristic signal at 1.26 ppm (the doublet) is unfortunately overlapped with residual water in the deuterated chloroform.

2.8. Inhibition assay

Human recombinant AKR1C2 catalyses oxidation of the artificial substrate 1-acenaphthenol in the presence of the coenzyme NAD^+. The reaction was followed spectrophotometrically by measuring the increase in NADH absorbance ($\varepsilon_{\lambda 340}$ = 6600 M^{-1} cm^{-1}) in the absence and presence of each of the compounds. The assays for AKR1C2 were carried out in 96-well microtitre plates in a 0.3-mL volume that included 100 mM phosphate buffer (pH 9.0), 0.005% Triton X-114 and 6.5% DMSO as co-solvent. A substrate concentration close to the determined K_M (60 μM) was used, with 2.3 mM coenzyme NAD^+ and 181 nM human recombinant AKR1C2. The concentrations of the compounds tested ranged from 50 nM to 50 μM. The measurements were performed on a Biotek PowerWave XS2 microplate spectrophotometer at 37 °C. Initial reaction velocities were calculated, and the IC_{50} values were determined graphically from the plots of residual activity *versus* $\log_{10}$ [inhibitor], using GraphPad Prism Version 5.00 (GraphPad Software, Inc.). Using the Cheng–Prusoff relationship for competitive inhibition, the K_i values were calculated for the best inhibitors. Inhibition assays with progesterone as a substrate were carried out in 100 mM phosphate buffer (pH 6.5) in the presence of 100 μM NADPH, 0.6 μM AKR1C2, and 4% acetonitrile as a co-solvent, with the final total concentrations of 7.7 μM progesterone (unlabeled plus [^{3}H]-labelled progesterone). Dydrogesterone and 20α-dihydrodydrogesterone were dissolved in acetonitrile, and the reactions were stopped with 500 μL of ethyl acetate after 35 minutes. The substrate and product were extracted from the reaction mixture and analysed by HPLC, as described in Section 2.6.

2.9. Docking simulations

Automated docking was used to locate the appropriate binding orientations of progesterone, dydrogesterone, 20α-dihydrodydrogesterone and medroxyprogesterone acetate within the active sites of human AKR1C2. The crystal structures of AKR1C2 were retrieved from the Protein Data Bank. Computational procedures were carried out on a workstation with two quad-core Intel Xeon 2.2 GHz processors, 8 GB of RAM, 320 GB and 1000 GB hard drives, and a Nvidia Quadro FX 4800 graphic card, running the current version of 64-bit Arch Linux. For active site detection, the crystal structure of AKR1C2 co-crystallized with $NADP^+$ and ursodeoxycholate (PDB entry 1IHI) was used and the active site was defined as the area of AKR1C2 within 6.5 Å of the co-crystallized ursodeoxycholate (25 amino acid residues). The crystal structure of AKR1C2 co-crystallized with NADPH (PDB entry 2HDJ) was then used for docking experiment. The compounds were docked to the active site involving the same 25 amino acid residues as detected in 1IHI crystal structure, using eHiTS 2009 from SimBioSys Inc (SimBioSys Inc., Toronto, Canada). The cofactor NADPH was left in the model. The scoring was done with eHiTS_Score, which is a statistically derived empirical scoring function that is included in the eHiTS software package [27,28]. PyMol (PyMOL Molecular Graphics System, Version 1.3; Schrodinger, LLC, USA) was used for visual inspection of the results and the graphical representations.

3. Results and discussion

3.1. AKR1C2 is expressed in endometriotic cell lines

Progestins, including dydrogesterone, are often used for the treatment of endometriosis. Therefore, it is important to understand the metabolism of progestins as well as the effects of progestins on progesterone-metabolising enzymes in endometriotic tissue. Recently, we found increased mRNA levels of *AKR1C1*, *AKR1C2* and *AKR1C3* in ovarian endometriosis, compared to normal endometrium [13,29], and higher protein levels of AKR1C2 in stromal cells of ovarian endometriosis [13]. However, expression of AKR1C isozymes in other types of endometriosis has not yet been examined. Here, we studied expression of *AKR1C2* in the model cell lines of peritoneal endometriosis, epithelial cells 12-Z, and stromal cells 22-B, by real-time PCR analysis. *AKR1C2* was expressed in both cell lines (Fig. 2). Higher mRNA levels were seen in the 22-B stromal cell line, which is in agreement with the published study [30]. The mRNA levels in control cell lines, endometrial cancer cell line Ishikawa and liver cancer cell line HepG2, were about 8-fold and 50-fold higher as compared to endometriosis cell line 22-B, respectively (Fig. 2). *AKR1C2* expression in specimens of normal endometrium was comparable to expression in the peritoneal endometriosis cell line 22-B, while we saw about 7-fold higher levels in specimens of ovarian endometriosis. This diverse AKR1C2 levels in ovarian and peritoneal endometriosis may be explained by different pathogenesis of different types of endometriosis. Our

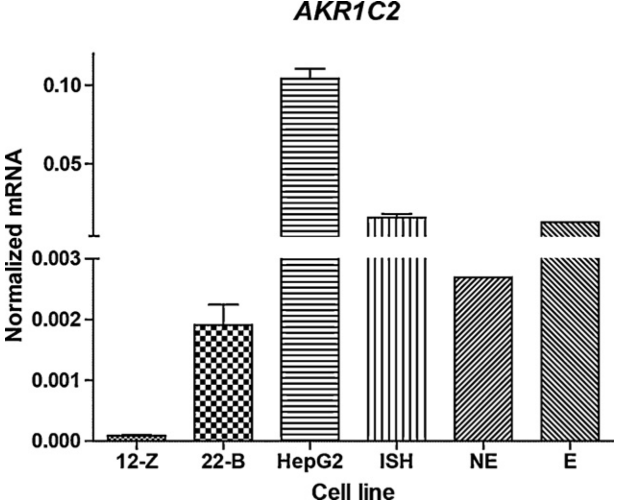

Fig. 2. *AKR1C2* expression in the endometriotic and control cell lines and diseased tissue. mRNA expression levels of AKR1C2 in epithelial (12-Z) and stromal (22-B) endometriotic cell lines; control cell lines: liver cancer cell line HepG2, endometrial cancer cell line Ishikawa; and tissue of ovarian endometriosis and normal endometrium. Expression levels were normalised to the expression of the housekeeping genes (*PPIA*, *GAPDH* and *RPLP0*). Mean ± SEM values are shown.

data on *AKR1C2* expression in endometriotic cell lines derived from peritoneal lesions and the reported higher expression in ovarian endometriosis thus suggest that together with AKR1C1 and AKR1C3, AKR1C2 may also be involved in pathogenesis of this disease. To further characterize AKR1C2 we next proceeded with its over-expression in *E. coli*.

3.2. AKR1C2 catalyses the metabolism of progesterone and progestin dydrogesterone

The enzymatic activity of the recombinant AKR1C2 was first determined spectrophotometrically by measuring NAD^+-dependent oxidation of the artificial substrate 1-acenaphthenol. The specific activity was 2.1 μmol/min/mg, which is comparable to the specific activity reported by others (2.5 μmol/min/mg) [31]. Similarly as reported previously the homogenous recombinant AKR1C2 catalyzed reduction of 5α-DHT to 5α-androstane-3α-ol-17β-ol and converted progesterone to 20α-hydroxyprogesterone (14), as expected no formation of 3α-hydroxyprogesterone was observed (Fig. 3). This confirmed that with progesterone as a substrate, AKR1C2 acts only as a 20-ketosteroid reductase. By following reduction of progesterone to 20α-hydroxyprogesterone we determined 17-fold lower catalytic efficiency for reduction of progesterone comparing to efficiency for 5α-DHT reduction (Table 1). AKR1C2 was reported to have only a minor 20-ketosteroid reductase activity [14], and we show here that its catalytic efficiency for progesterone reduction is comparable to the efficiency of AKR1C3 [12,32]; and about 10-fold lower than the catalytic efficiency of AKR1C1 [12,33].

As we observed a similar efficiency of AKR1C2 for progesterone reduction as compared to AKR1C3 and 10-fold lower efficiency as compared to AKR1C1, we also examined the ability of this enzyme to metabolise progestin dydrogesterone. This progestin is converted into its 20α-dihydro-metabolite mostly by AKR1C1 and very poorly by AKR1C3 [12]. AKR1C2 also catalyzed the reduction of this progestin at the 20-keto position to form 20α-dihydro-metabolite as confirmed by NMR analysis (Table 1, Figs. 3 and 4). Surprisingly a 3.7-fold higher catalytic efficiency was observed compared with AKR1C1 [12]. Dydrogesterone, which is widely used for menstrual disorders, endometriosis, threatened and habitual abortion, and postmenopausal hormone replacement therapy, can thus be

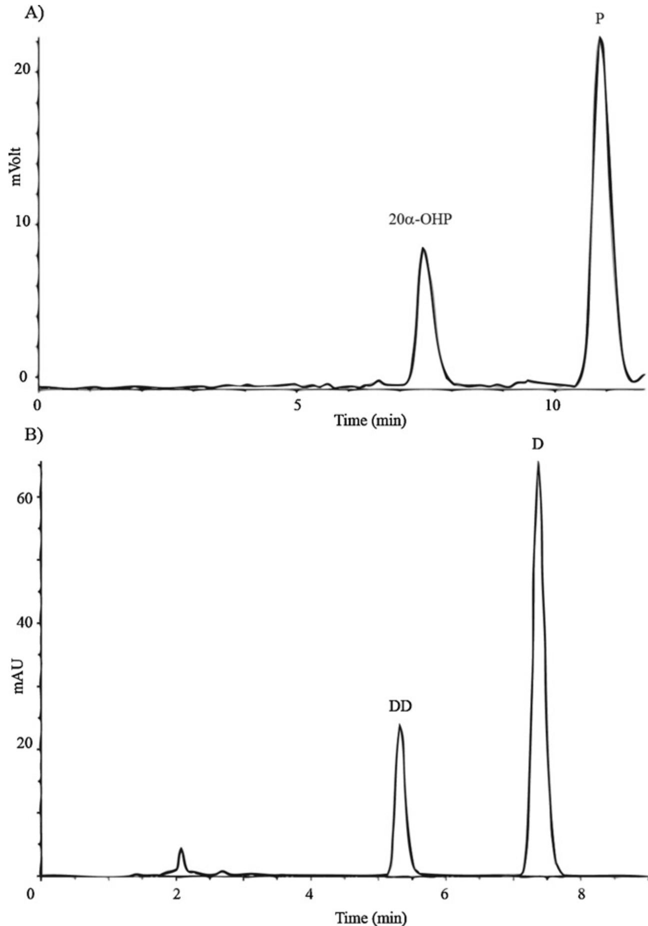

Fig. 3. AKR1C2 converts progesterone to 20α-hydroxyprogesterone and dydrogesterone to 20α-dihydrodydrogesterone. HPLC elution profile shows separation of: (A) [^{3}H]-labelled progesterone (P) and its metabolite 20α-hydroxyprogesteone (20α-OHP), both detected by radioactive detection and (B) unlabelled dydrogesterone (D) and its metabolite 20α-dihydrodydrogesterone (DD), both detected by measuring absorbance at 300 nm. The minor peak corresponds to acetonitrile, which absorbs at 300 nm and was used as a solvent.

metabolised to its major metabolite 20α-dihydrodydrogesterone by two human AKR1C isoforms, AKR1C1 and AKR1C2. The local metabolism of dydrogesterone by these two enzymes might thus affect its concentrations and actions in particular tissues.

3.3. Progestins inhibit AKR1C2 in vitro

As progestins act as inhibitors of AKR1C1 and AKR1C3 [12], we examined here whether progestins can interfere with AKR1C2-catalyzed reactions as well. We examined seven compounds that are currently used for treatment of endometriosis for their inhibition of 1-acenaphthenol oxidation by the recombinant AKR1C2: three progesterone derivatives (medroxyprogesterone acetate, dydrogesterone, and its metabolite 20α-dihydrodydrogesterone); three 19-nortestosterone derivatives (desogestrel, noretindrone and levonorgestrel); and the androgen danazol. Dydrogesterone, medroxyprogesterone acetate and 20α-dihydrodydrogesterone were tested also for their inhibition of progesterone reduction. Additionally, we tested litocholic and ursodeoxycholic acid, known potent inhibitors of AKR1C2 [15,34], as controls. First, we determined the percentages of inhibition at 60 μM substrate 1-acenaphthenol in the presence of 10 μM and 50 μM progestins, and 1 μM bile acids (Table 2). In further studies, the IC_{50} values were determined for oxidation of 1-acenaphthenol and reduction

Table 2
Progestins evaluated for inhibition of AKR1C2.

Compound	Structure	Inhibition (%)	
Dydrogesterone		75.7[a]	94.2[b]
Medroxyprogesterone acetate		66.2[a]	93.4[b]
20α-Dihydrodydrogesterone		55.1[a]	87.9[b]
Levonorgestrel		NI[a]	45.2[b]
Norethindrone		NI[a]	40.6[b]
Danazol		33.2[a]	35.7[b]
Desogestrel		NI[a]	18.0[b]
Litocholic acid[A]		81.0[c]	

Table 2 (*Continued*)

Compound	Structure	Inhibition (%)
Usodeoxycholic acid[A]		74.2[c]

Percentages of enzyme inhibition at 60 μM 1-acenaphthenol and (a) 10 μM, (b) 50 μM, and (c) 1 μM of each compound shown. Data are means of at least three measurements determined in two independent experiments.

NI, no inhibition observed.

[A] Litocholic and ursodeoxycholic acid are potent inhibitors of AKR1C2 and served as controls.

of progesterone for all of the progestins that showed more than 50% inhibition initially; with the K_i values calculated using the Cheng–Prusoff equation for competitive inhibition (Table 3). As progestins resemble the steroidal substrates, and AKR1C2 converts dydrogesterone to 20α-dihydrodydrogesterone, progestins were expected to bind to the active site and act as competitive inhibitors.

The retroprogestrone dydrogesterone, with an inverted configuration at C9 and C10 and an additional double bond at C6, was the most potent inhibitor of 1-acenaphthenol oxidation by AKR1C2 (1.3 μM K_i) and similarly potent inhibitor of progesterone reduction as medroxyprogesterone acetate (4.4 μM and 3.8 μM K_i, respectively). The inhibitory effect was decreased if the 20-keto group of dydrogesterone was reduced, as with 20α-dihydrodydrogesterone (2.3 μM K_i). Medroxyprogesterone acetate has an additional methyl group at C6 and an acetate group at C17, and it showed similar inhibitory potential to 20α-dihydrodydrogesterone (2.1 μM K_i). When compared to the known inhibitors, the ursodeoxycholic and litocholic acid, progesterone derivatives were only 9–16-fold less active when 1-acenaphtenol was used as a substrate and 25–47-fold less

Table 3
IC_{50} and K_i values of progestin inhibitors of AKR1C2.

Progestin	IC_{50} (μM)	K_i (μM)
Dydrogesterone	2.6/8.7[a]	1.3/4.4[a]
Medroxyprogesterone acetate	4.1/7.6[a]	2.1/3.8[a]
20α-Dihydrodydrogesterone	4.4/13.2[a]	2.3/6.6[a]
Litocholic acid[b]	0.28	0.14
Ursodeoxycholic acid[b]	0.29	0.15

IC_{50} and K_i values for oxidation of 1-acenaphthenol are shown.

[a] IC_{50} and K_i values for progesterone reduction by AKR1C2.

[b] Litocholic and ursodeoxycholic acid are known inhibitors of AKR1C2 and served as controls.

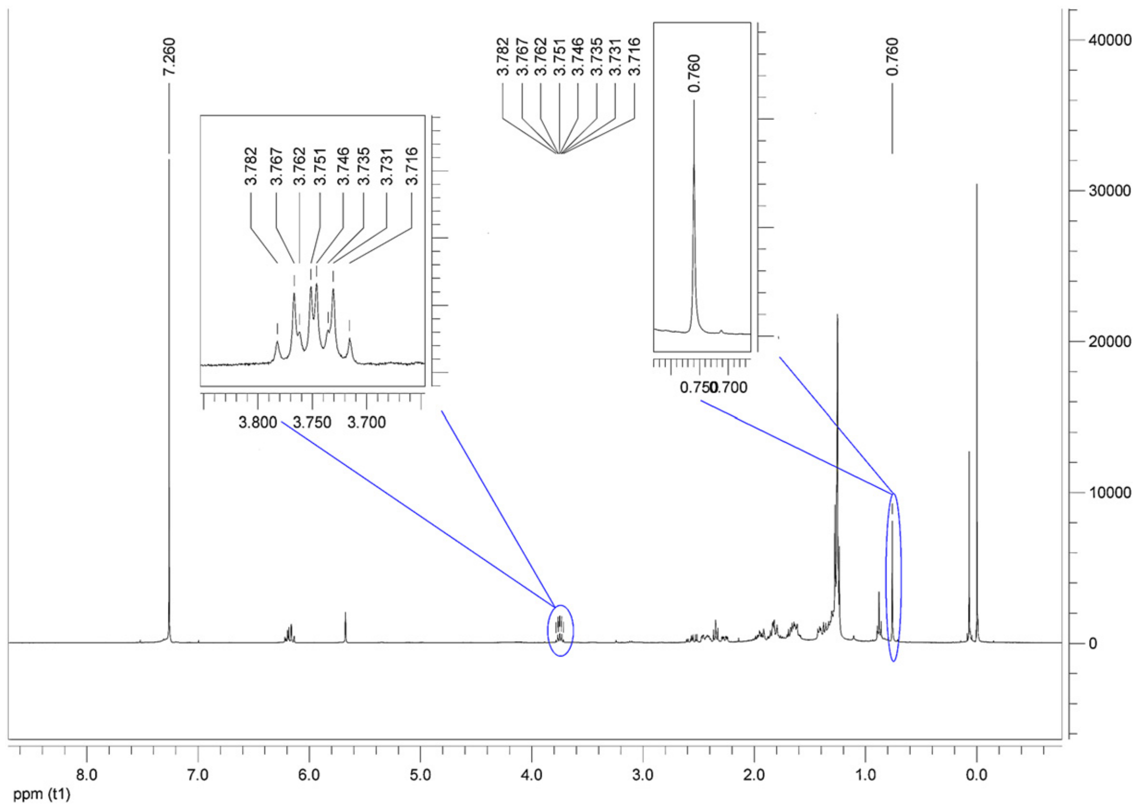

Fig. 4. NMR analysis of 20α-dihydrodydrogesterone. Proton NMR spectra of the purified 20α-dihydrodydrogesterone with broadened two portions of the spectra annotating the most significant signals of the compound are shown.

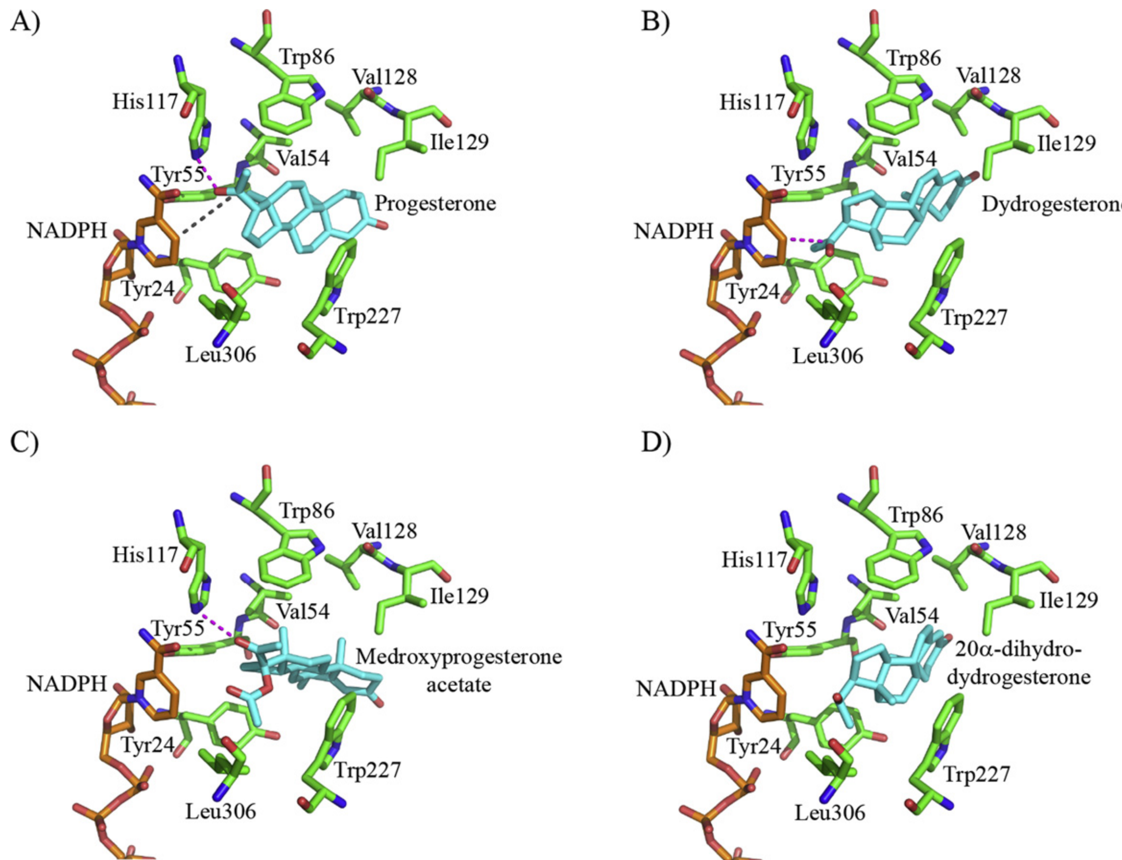

Fig. 5. Docking of progesterone, dydrogesterone, medroxyprogesterone acetate and 20α-dihydrodydrogesterone into the active site of AKR1C2. The docking positions of progesterone and the progestins within the AKR1C2 active site (PDB code 2HDJ): (A) Progesterone, coenzyme NADPH, catalytic amino acids Tyr55 and His117, and amino acids Tyr24, Trp86, Val128, Ile129, Val154, Trp227, and Leu306. (B) Dydrogesterone, coenzyme NADPH, and catalytic amino acids Tyr55, His117, and amino acids Tyr24, Val54, Trp86, Val128, Ile129, Trp227, and Leu306. (C) Medroxyprogesterone acetate, coenzyme NADPH, and catalytic amino acids Tyr55 and His117, and amino acids Tyr24, Val54, Trp86, Val128, Ile129, Trp227 and Leu306. (D) 20α-dihydrodydrogesterone, coenzyme NADPH, and catalytic amino acids Tyr55, His117, and amino acids Tyr24, Val54, Trp86, Val128, Ile129, Trp227, and Leu306.

active with progesterone (Table 3). A similar inhibition profile, with low K_i values for dydrogesterone and medroxyprogesterone acetate, was recently observed for AKR1C3 catalyzed oxidation of 1-acenaphthenol (unpublished data) as well as for AKR1C1 and AKR1C3 catalyzed reductions of progesterone [12]. However, compared to AKR1C2, the K_i values for 20α-dihydrodydrogesterone inhibition of progesterone reduction by AKR1C1 and AKR1C3 were about 3-fold higher. The plasma concentrations of 20α-dihydrodydrogesterone after oral intake of 10 mg dydrogesterone can reach 0.2 μM [35], and also plasma concentrations of medroxyprogesterone acetate after 100–250 mg dose can be as high as 136 nM [36], which both may be sufficient to elicit a weak physiological inhibitory effect on AKR1C2.

The 19-nortestosterone derivatives were less potent inhibitors than the progesterone derivatives. Levonorgestrel and norethindrone, with a keto group at position 3, showed better inhibition, 45.2% and 40.6%, respectively. These two compounds differed only in the substituent at position 13, where an ethyl group (levonorgestrel) showed slightly higher inhibitory effects than a methyl group (norethindrone). The 19-nortestosterone derivative desogestrel lacks the 3-keto group but has an additional methylene group at position 11 and ethyl group at C13; this was the weakest inhibitor (18.0% inhibition). Androgen danazol has an additional heterocyclic ring and a methyl at C19; this was less potent (35.7% inhibition) than levonorgestrel and norethindrone, but showed better inhibition than desogestrel. Interestingly, when tested against AKR1C1 and AKR1C3, danazol was a less potent inhibitor than desogestrel [12].

Similarly, as reported recently for AKR1C1 and AKR1C3 [12], we showed here that 3-keto, 17-acetyl and 19-methyl substituents of progestins are important functional groups for inhibition of AKR1C2. By inhibiting AKR1C2, the progestins at high therapeutic doses, especially 20α-dihydrodydrogesterone and medroxyprogesterone acetate, might affect the local metabolism of 5α-DHP, 5α-DHT and progesterone, but the *in vivo* effects should be further studied.

3.4. Docking simulations of progesterone and progestins into the AKR1C2 active site support the experimental data

To better understand the 20α-HSD activity of AKR1C2, and the inhibitory action of progestins we preformed docking simulations of the most potent progestin inhibitors dydrogesterone, medroxyprogesterone acetate and 20α-dihydrodydrogesterone, and of the substrate progesterone into the crystal structure of AKR1C2 co-crystallized with NADPH (PDB code 2HDJ) (Fig. 5). Docking simulations by eHits revealed that the progestins can enter the substrate-binding region of the active site. Within the active site, all three of these steroids occupy the position where the C20 atoms of the steroids were correctly oriented towards C4 of the pyridine head of the cofactor and the catalytic amino-acid residues Tyr55 and His117. Although only dydrogesterone was at an appropriate distance to the nicotinamide moiety for hydride transfer (3.2 Å and 4.4 Å, for dydrogesterone and progesterone, respectively) both, progesterone and dydrogesterone were correctly oriented for the formation of the 20α-dihydro-metabolite. None of the steroids

were close enough to interact with the catalytic Tyr55 and only progesterone and MPA were at an appropriate distance from His117 for H-bond formation. Although site-directed mutagenesis studies suggest that Tyr55 is a proton donor in the AKR1C enzymes [37], it was also suggested that a water molecule occupying the oxyanion hole would bridge the hydrogen bond network between the steroid ketone and the catalytic tetrad [38], and this may explain reduction of dydrogesterone and progesterone by AKR1C2.

Progesterone and all progestins were additionally stabilised by hydrophobic interactions (medroxyprogesterone acetate with Tyr24, Val54, Tyr55, Trp86, Val128, Ile129, Trp227, Leu306 and Leu308; progesterone with Tyr24, Val54, Tyr55, Trp86, Ile129, Trp227 and Leu308; dydrogesterone with Tyr24, Val54, Tyr55, Trp86, Val128, Ile129, Trp227, Leu306 and Leu308; and 20α-dihydrodydrogesterone with Tyr24, Val54, Trp86, Val128, Ile129, Trp227, Leu306 and Leu308). The positions with low docking energies support our experimental data, showing high inhibitory potential of medroxyprogesterone acetate, dydrogesterone, and 20α-dihydrodydrogesterone and a higher catalytic efficiency for reduction of dydrogesterone than progesterone.

4. Conclusions

The recombinant AKR1C2 catalyses the reduction of progesterone to 20α-hydroxyprogesterone with a similar catalytic efficiency to that of AKR1C3. Additionally, AKR1C2 metabolises progestin dydrogesterone to its 20α-dihydro-metabolite with about a 150-fold higher efficiency than progesterone and a 8.6-fold higher efficiency than 5α-DHT. AKR1C2 is thus the most efficient dydrogesterone-metabolising enzyme among the AKR1C1-AKR1C3 isozymes. *AKR1C2* is over-expressed in ovarian endometriosis and was here detected in the model cell lines of peritoneal endometriosis. Among the progestins that are currently used for the treatment of endometriosis, the progestin derivatives medroxyprogesterone acetate, dydrogesterone, and its metabolite 20α-dihydrodydrogesterone act *in vitro* as AKR1C2 inhibitors with low μM K_i values. The relatively high plasma concentrations of 20α-dihydrodydrogesterone and medroxyprogesterone acetate suggest that these two progestins may affect AKR1C2 also *in vivo*, which should be evaluated in further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.12.011.

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