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Evaluation of A-ring fused pyridine D-modified androstane derivatives for antiproliferative and aldo-keto reductase 1C3 inhibitory activity†

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New A-ring pyridine fused androstanes in 17 α -homo-17-oxa (D-homo lactone), 17 α -picolyl or 17(E)-picolinylidene series were synthesized and validated by X-ray crystallography, HRMS, IR and NMR spectroscopy. Novel compounds **3**, **5**, **8** and **12** were prepared by treatment of 4-en-3-one or 4-ene-3,6-dione D-modified androstane derivatives with propargylamine catalyzed by Cu(II), and evaluated for potential anti-cancer activity *in vitro* using human cancer cell lines and recombinant targets of steroidal anti-cancer drugs. Pyridine fusion to position 3,4 of the A-ring may dramatically enhance affinity of 17 α -picolyl compounds for CYP17 while conferring selective antiproliferative activity against PC-3 cells. Similarly, pyridine fusion to the A-ring of steroidal D-homo lactones led to identification of new inhibitors of aldo-keto reductase 1C3, an enzyme targeted in acute myeloid leukemia, breast and prostate cancers. One A-pyridine D-lactone steroid **5** also has selective submicromolar antiproliferative activity against HT-29 colon cancer cells. None of the new derivatives have affinity for estrogen or androgen receptors in a yeast screen, suggesting negligible estrogenicity and androgenicity. Combined, our results suggest that A-ring pyridine fusions have potential in modulating the anticancer activity of steroidal compounds.

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Introduction

Androgenic steroidal hormones are involved in the growth and progression of prostate cancers,¹ benign prostatic hyperplasia,² ovarian cancers³ and breast cancers,⁴ and impact a wide-range of normal physiological processes. Modified androstane steroids have been successfully developed into clinical drugs for the treatment of several of these diseases and are the subject of clinical and preclinical studies.⁵ Steroidal compounds such as abiraterone target androgen biosynthesis through inhibition of 17 α -hydroxylase/C_{17,20}-lyase (CYP17A1),⁶ while anti-androgens such as cyproterone acetate⁷ or the non-steroid enzalutamide⁸ target androgen recep-

tor (AR) signaling.⁹ CYP17A1 is a dual-function cytochrome P450 enzyme that is essential for the first steps of androgen biosynthesis; it catalyzes 17 α -hydroxylation of pregnenolone and progesterone and *via* 17,20-lyase activity conversion to dehydroepiandrosterone (DHEA), a precursor of testosterone and estrogen.⁶ Based on X-ray crystal structures, abiraterone binds with high affinity to the active site of CYP17A1 by coordinating the P450 heme iron through its pyridine nitrogen.¹⁰ Unfortunately prostate cancers often develop resistance to current treatments, and new targets, adjuvant therapies and more effective compounds are necessary.¹¹ Aldo-keto reductase 1C3 (AKR1C3), also known as type 5 17 β -hydroxysteroid dehydrogenase (17 β -HSD5), is a steroidogenic enzyme that, in addition to other reactions, catalyzes conversion of the weak androgen precursor androstene-3,17-dione to the potent androgen receptor ligand, testosterone.¹² AKR1C3 expression is significantly upregulated in castration resistant prostate cancer (CRPC) and has been suggested as an adjuvant drug target in both androgen-dependent prostate cancer and CRPC.^{13,14}

AKR1C3 has also been reported to play a role in the development of resistance by certain prostate cancers to enzalutamide and/or abiraterone.¹⁵ In cell models of CRPC *in vitro*, AKR1C3 inhibitors had strong antiproliferative activity and sensitized CRPC cell lines to the activity of enzalutamide¹⁶ and abiraterone.^{16–19} Less specific steroidal

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compounds that inhibit all four AKR1C isoforms (AKR1C1, -C2, -C3, -C4) may also have promise for the treatment of other cancers; for example pan-AKR1C steroidal inhibitor medroxyprogesterone acetate (MPA) stimulates the differentiation and apoptosis of AML leukemia cell lines, while specific AKR1C3 inhibitors do not show such effects.^{19,20}

Rational modification of steroid hormones is a proven strategy for the development of improved treatments for hormone-dependent and other cancers.^{5,21} For example, addition of a lactone ring to the steroid nucleus is associated with dramatic changes in bioactivity vs. parent compounds, and has led to identification of potential cytotoxic agents²² and compounds that modulate steroid signaling pathways including inhibitors of 5 α -reductase,²³ aromatase²⁴ and AKR1C3.²⁵ Testolactone, a steroidal Δ -lactone, was one of the first steroids used in clinical treatment and prevention of hormone-dependent tumors such as breast cancer,²⁶ while the steroidal Δ -lactone EM1401 is a selective inhibitor of human AKR1C3.²⁵ Similarly, C17-*exo*-heterocyclic steroids are in clinical use (e.g. abiraterone) or preclinical development (e.g. galeterone) for the treatment of prostate cancers.^{27,28} Previously^{29–31} we reported a series of C17-*exo*-heterocyclic androstanes as potential inhibitors of aromatase (CYP19) and/or CYP17.^{32,33} Based on these and other studies, the geometry of the C17 heterocyclic ring and the location of heteroatoms such as N, S or O appear critical for inhibition, likely due to coordinate interactions between inhibitor and the heme iron of CYP17.^{27,34}

Our group has synthesized steroidal derivatives in Δ -homo lactone, 17 α -picolyl and 17(*E*)-picolinylidene series with marked antiproliferative activity against human cancer cell lines.^{35–40} In the present study, we introduced a pyridine ring fused to the 3,4-position of Δ -modified androstane derivatives, in order to obtain new hybrids with modified biological activity. Pyridine is among the most frequently occurring heterocyclic structural units in pharmaceutical and agrochemical sciences, as well as in materials science, and is very important in the chemistry of natural products with biological activity.^{41,42} Modified steroids bearing heterocyclic systems as a part of their A- or D-rings display a range of biological properties such as anti-inflammatory, antimicrobial, anticancer, hypotensive, hypocholesterolemic and diuretic activities.^{43,44}

Because steroids bearing heterocycles fused to the A-ring of the steroid nucleus are of pharmaceutical interest, here we report the efficient synthesis of pyridino[2',3':3,4]-17 α -homo-17-oxa-androstan-16-one (3), pyridino[2',3':3,4]-17 α -homo-17-oxa-androstane-6,16-dione (5), 17 α -picolyl-pyridino[2',3':3,4]-androst-5-en-17 β -ol (8) and 17(*E*)-picolinyliden-pyridino[2',3':3,4]-androst-5-ene (12). Compounds were validated by X-ray crystallography (3, 8 and 12), HRMS, IR and NMR spectroscopy and evaluated for anticancer activity *in vitro* using human cancer cell lines and recombinant targets of steroidal anti-cancer drugs. Because steroidal drugs may have undesirable estrogenic or androgenic side-effects, new compounds were also tested for relative affinity to the ligand

binding domain (LBD) of estrogen receptor α (ER α), estrogen receptor β (ER β) or androgen receptor (AR) using a fluorescent cell assay in yeast.⁴⁵ To test for potential to modulate enzymes involved in androgen biosynthesis, compounds were screened for possible binding affinity to CYP17 using molecular docking, and ability to inhibit recombinant human AKR1C3 *in vitro*.

Experimental

Synthetic procedures

Infrared spectra (wave numbers in cm⁻¹) were recorded in KBr pellets (for crystals) on a NEXUS 670 SP-IR spectrometer. NMR spectra were recorded on a Bruker AC 250E spectrometer operating at 250 MHz (1H) and 62.5 MHz (13C), and are reported in ppm downfield from the tetramethylsilane internal standard. Chemical shifts are given in ppm (δ -scale). High resolution mass spectrometry (HRMS) measurements were recorded on a 6210 time-of-flight LC/MS Agilent Technologies (ESI+) instrument. Melting points were determined using an Electrothermal 9100 apparatus and are uncorrected. Chromatographic purifications were performed on silica gel columns (Kieselgel 60, 0.063–0.20 mm, Merck). All reagents used were of analytical grade.

General procedures for preparation of compounds 3 and 5. Starting compound 2 (0.161 g, 0.53 mmol) or 4³⁴ (0.145 g, 0.40 mmol) was dissolved in 95% ethanol (4 ml) and propargylamine (1.56 or 1.41 mmol) and copper(II)-nitrate-trihydrate (0.06 or 0.05 mmol) were added. The reaction mixture was stirred vigorously under reflux in an argon atmosphere for 6 or 4 h, respectively. Resulting reaction mixture was poured into water (5 ml) and extracted with dichloromethane (6 $\times$ 5 ml). Combined organic extracts were dried, solvent removed and the resulting crude product was purified by column chromatography (7 or 6 g silica gel, hexane/ethyl acetate 1 : 1 and 1 : 2), affording pure compound 3, or pure compound 5.

Pyridino[2',3':3,4]-17 α -homo-17-oxa-androstan-16-one (3). Yield 30% (0.054 g), white crystals, mp 209–211 °C, after recrystallization from hexane/ethyl acetate. IR (KBr, $\nu_{\max}$, cm⁻¹): 2932, 1731, 1574, 1441, 1382, 1241, 1188, 1041, 754. ¹H NMR (CDCl₃, δ , ppm): 0.72 (s, 3H, H-18); 1.04 (s, 3H, H-19); 2.80 (dd, 1H, J_1 = 5.9 Hz, J_2 = 18.7 Hz, H-15); 3.92 (d, 1H, J = 10.8 Hz, H-17a); 4.00 (d, 1H, J = 10.7 Hz, H-17b); 7.10 (d, 1H, J = 7.7 Hz, -N=CH-CH=CH-); 7.52 (d, 1H, J = 8.1 Hz, -N=CH-CH=CH-); 8.38 (bs, 1H, -N=CH-CH=CH-). ¹³C NMR (CDCl₃, δ , ppm): 12.21 (C-18); 15.17 (C-19); 19.65; 23.44; 29.25; 29.54; 31.90; 32.36; 34.27; 34.58; 35.23; 35.56; 44.49; 46.19; 52.16; 81.18 (C-17); 121.01 (-N=CH-CH=CH-); 132.95 (-N=CH-CH=CH-); 146.54 (-N=CH-CH=CH-); 156.51 (C-3); 170.83 (C₁₆=O). HRMS TOF (m/z): C₂₂H₂₉NO₂ [M + H]⁺ calcd. 340.22711, found 340.22652.

Pyridino[2',3':3,4]-17 α -homo-17-oxa-androstane-6,16-dione (5). Yield 16% (0.025 g), white solid, mp 193–195 °C, after recrystallization from hexane/acetone. IR (KBr, $\nu_{\max}$, cm⁻¹): 2941, 1729, 1709, 1716, 1578, 1427, 1381, 1242, 1053, 734. ¹H NMR (CDCl₃, δ , ppm): 0.75 (s, 3H, H-19); 1.07 (s, 3H, H-18);

2.72 (m, 1H, H-15a); 2.80 (m, 1H, H-15b); 3.96 (d, 1H, $J = 10.8$ Hz, H-17a); 4.05 (d, 1H, $J = 10.8$ Hz, H-17b); 7.10 (m, 1H, $-\text{N}=\text{CH}-\text{CH}=\text{CH}-$); 7.38 (d, 1H, $J = 7.6$ Hz, $-\text{N}=\text{CH}-\text{CH}=\text{CH}-$); 8.42 (d, 1H, $J = 4.3$ Hz, $-\text{N}=\text{CH}-\text{CH}=\text{CH}-$). ^{13}C NMR (CDCl_3 , δ , ppm): 13.34 (C-18); 15.00 (C-19); 19.81; 28.15; 31.47; 32.56; 34.15; 38.00; 40.02; 42.38; 44.72; 44.96; 51.86; 53.81; 80.64 (C-17); 121.33 ($-\text{N}=\text{CH}-\text{CH}=\text{CH}-$); 128.99 (C-4); 137.20 ($-\text{N}=\text{CH}-\text{CH}=\text{CH}-$); 147.56 ($-\text{N}=\text{CH}-\text{CH}=\text{CH}-$); 154.77 (C-3); 169.67 ($\text{C}_{16}=\text{O}$); 208.22 ($\text{C}_6=\text{O}$).

General procedure for preparation of compounds 8 and 12. Starting compound 7 (0.205 g, 0.54 mmol) or 11³⁵ (0.201 g, 0.56 mmol) was dissolved in 95% ethanol (4 ml), and propargylamine (2.34 mmol) and copper(II)-nitrate-trihydrate (0.09 or 0.08 mmol) were added. Reaction was stirred under reflux in an argon atmosphere for 16 h. After that, reaction mixture was precipitated to remove the catalyst, and the filtrate was evaporated to yield crude product, which was purified by column chromatography (5 g silica gel, hexane/ethyl acetate 1:1 or 4:1), yielding pure compound 8 or 12.

17 α -Picolyl-pyridino[2',3':3,4]-androst-5-en-17 β -ol (8). Yield 63% (0.142 g), white needles, mp 140–141 °C, after recrystallization from hexane/ethyl acetate. IR (KBr, ν , cm^{-1}): 3330, 2927, 2857, 1644, 1597, 1570, 1473, 1439, 1423, 1385, 1250, 1216, 1025, 755. ^1H NMR (CDCl_3 , δ , ppm): 0.72 (s, 3H, H-18); 0.98 (s, 3H, H-19); 2.83 (d, 1H, $J_{\text{gem}} = 14.5$ Hz, CH_2Py); 2.98 (m, 2H, H-2); 3.09 (d, 1H, $J_{\text{gem}} = 14.5$ Hz, CH_2Py); 6.15 (t, 1H, $J_{6,7} = 2.4$ Hz, H-6); 6.62 (bs, 1H, 17 β -OH); 7.03–7.18 (m, 3H, H-3', H-5' and H-5'', Py); 7.50–7.79 (m, 2H, H-4' and H-4'', Py); 8.36 (m, 1H, H-6'', Py); 8.46 (d, 1H, $J_{6',5'} = 4.8$ Hz, H-6', Py). ^{13}C NMR (CDCl_3 , δ , ppm): 12.24 (C-18); 14.28 (C-19); 21.05; 23.78; 29.38; 31.42; 32.21; 34.68; 35.65; 36.09; 43.21; 46.58; 46.76; 50.62; 53.54; 83.43 (C-17); 120.82 (C-5', Py); 121.30 (C-5'', Py); 121.90 (C-3', Py); 124.70 (C-6); 132.88 (C-4'', Py); 136.71 (C-4', Py); 139.67 (C-5); 146.28 (C-6', Py); 147.99 (C-6'', Py); 154.80 (C-4); 156.75 (C-2', Py); 160.84 (C-3). HRMS TOF (m/z): $\text{C}_{28}\text{H}_{35}\text{N}_2\text{O} [\text{M} + \text{H}]^+$ calcd. 415.27439, found 415.27316.

17(E)-Picolinylidene-pyridino[2',3':3,4]- androst-5-ene (12). Yield 50% (0.111 g), white solid, mp 169–170 °C, after recrystallization from hexane/ethyl acetate. IR (KBr, ν , cm^{-1}): 2934, 1652, 1584, 1471, 1439, 1427, 1372, 1215, 1149, 754. ^1H NMR (CDCl_3 , δ , ppm): 0.74 (s, 3H, H-18); 0.92 (s, 3H, H-19); 3.00 (m, 2H, H-2); 6.17 (m, 1H, H-6); 6.25 (m, 1H, H-20); 7.01–7.09 (m, 2H, H-5' and H-5'', Py); 7.30 (m, 1H, H-4', Py); 7.51–7.80 (m, 2H, H-3' and H-4'', Py); 8.37 (m, 1H, H-6'', Py); 8.57 (d, 1H, $J_{6',5'} = 4.2$ Hz, H-6', Py). ^{13}C NMR (CDCl_3 , δ , ppm): 12.27 (C-18); 18.96 (C-19); 21.42; 23.78; 25.04; 29.38; 29.89; 31.46; 34.62; 34.80; 36.00; 45.96; 46.66; 53.57; 117.89 (C-20); 120.15 (C-5', Py); 120.85 (C-5'', Py); 121.00 (C-3', Py); 122.79 (C-6); 132.92 (C-4'', Py); 134.44 (C-5); 135.79 (C-4', Py); 139.69 (C-4); 146.32 (C-6', Py); 149.16 (C-6'', Py); 156.77 (C-17); 157.65 (C-2', Py); 160.43 (C-3). HRMS TOF (m/z): $\text{C}_{28}\text{H}_{33}\text{N}_2 [\text{M} + \text{H}]^+$ calcd. 397.26383, found 397.26258.

X-ray crystal structure determination

X-ray diffraction data for compounds 3, 8 and 12 were collected at room temperature on an Agilent Technologies – Ox-

ford Diffraction Gemini S diffractometer with graphite-monochromated $\text{CuK}\alpha$ radiation ($k = 1.5418$ Å). Data reduction was performed in CrysAlis RED.⁴⁶ Space group determinations were based on analysis of the Laue class and systematically absent reflections. Collected data were corrected for absorption effects using a Multi-scan absorption correction.⁴⁷ Structures were solved by direct methods using SHELXT⁴⁸ and refined by full-matrix least-squares procedures on F^2 using SHELXL-2014/6 program.⁴⁸ Non-hydrogen atoms were refined anisotropically. The positions of C–H and O–H hydrogen atoms were found from inspection of difference Fourier maps, but other H atoms were included on calculated positions riding on their attached atoms with fixed distances 0.97 Å (CH_2) and 0.96 Å (CH_3). All calculations were performed using ORTEP-III,⁴⁹ as well as PARST⁵⁰ and PLATON⁵¹ as implemented in the WINGX⁵² system of programs. X-ray crystallography data and refinement parameters are summarized in Table 1. CCDC 1823137 (for 3), CCDC 1823138 (for 8) and CCDC 1823139 (for 12), contain supplementary crystallographic data for this work.

Materials and methods

Antiproliferative activity

Cell lines and cell culture. Six human tumor cell lines were used in the present study: estrogen receptor negative (ER–) breast adenocarcinoma cell line MDA-MB-231 (American Type Culture Collection – ATCC HTB26); ER+ breast adenocarcinoma MCF-7 (ATCC HTB22); prostate cancer PC-3 (ATCC CRL 1435); cervical carcinoma HeLa (ATCC CCL2); colon adenocarcinoma HT-29 (ATCC HTB38) and lung adenocarcinoma A549 (ATCC CCL 185), as well as one human noncancerous cell line MRC-5 (normal fetal lung fibroblasts, ATCC CCL 171). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose, supplemented with 10% fetal calf serum (FCS, Sigma) and antibiotics/antimycotic solution (Sigma). Cells were cultured in flasks (Costar, 25 cm^2) at 37 °C in 100% humidity with 5% CO_2 . Only viable cells, as determined by trypan blue dye exclusion, between the 3rd and 10th passages, were used.

Antiproliferative assay. Compounds were evaluated for anti-proliferative activity using the tetrazolium colorimetric MTT assay,⁵³ after exposure of cells to test compounds in five concentrations ranging from 10^{-8} to 10^{-4} M for 48 h. Doxorubicin (DOX), a non-selective anti-proliferative agent, and formestane, a steroidal aromatase inhibitor used as a control for general steroid toxicity, were used as reference compounds. Exponentially growing cells were harvested, counted using the trypan blue exclusion test, seeded onto 96-well plates at a density of 5000 cells per well and allowed to stand overnight. Medium containing test compounds at five different concentrations ranging from 10^{-8} to 10^{-4} M were then added to each well. After 48 h incubation viability was determined by addition of 10 μL of sterile MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) solution (5 mg mL^{-1}). The precipitated formazan crystals were

Table 1 Crystallographic data and refinement parameters for compounds **3**, **8** and **12**

	3	8	12
Chemical formula	C ₂₂ H ₂₉ NO ₂	C ₂₈ H ₃₆ N ₂ O ₂	C ₂₈ H ₃₂ N ₂
<i>M_r</i>	339.46	432.59	396.55
Crystal system, space group	Orthorhombic, <i>P</i> ₂ ₁ ₂ ₁ ₂	Monoclinic, <i>P</i> ₂ ₁	Orthorhombic, <i>P</i> ₂ ₁ ₂ ₁ ₂
<i>a</i> (Å)	6.8428(4)	11.4355(16)	7.8675(2)
<i>b</i> (Å)	12.3190(8)	7.2184(7)	12.2525(4)
<i>c</i> (Å)	21.3880(12)	14.3262(12)	23.0556(8)
β (°)	—	92.092(10)	—
<i>V</i> (Å ³)	1802.92(18)	1181.8(2)	2222.47(12)
<i>Z</i>	4	2	4
μ (mm ⁻¹)	0.62	0.59	0.52
Crystal size (mm)	0.53 × 0.43 × 0.37	0.39 × 0.26 × 0.21	0.27 × 0.22 × 0.17
Absorption correction	Multi-scan <i>CrysAlis PRO</i> , Agilent Technologies, version 1.171.36.28. Empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm		
<i>T_{min}</i> , <i>T_{max}</i>	0.950, 1.000	0.903, 1.000	0.747, 1.000
No. of measured, independent and observed [<i>I</i> > 2σ(<i>I</i>)] reflections	4335, 2874, 2521	4375, 3096, 2615	5374, 3316, 2995
<i>R_{int}</i>	0.021	0.022	0.020
θ values (°)	$\theta_{\max}$ = 70.2, $\theta_{\min}$ = 4.1	$\theta_{\max}$ = 72.5, $\theta_{\min}$ = 3.9	$\theta_{\max}$ = 72.3, $\theta_{\min}$ = 4.1
<i>R</i> [<i>F</i> ² > 2σ(<i>F</i> ²)], <i>wR</i> (<i>F</i> ²), <i>S</i>	0.045, 0.126, 1.02	0.047, 0.139, 1.07	0.054, 0.159, 1.05
No. of parameters	238	319	293
No. of restraints	0	1	0
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement		
$\Delta\rho_{\max}$, $\Delta\rho_{\min}$ (e Å ⁻³)	0.14, -0.20	0.32, -0.17	0.32, -0.32

solubilized with acidified 2-propanol (100 μL of 0.04 M HCl in 2-propanol) and absorbance was read (Multiscan Ascent, Thermo LabSystems) at 540/690 nm after several minutes incubation at room temperature. Wells containing cells without test compound were used as a control. Wells without cells containing only complete medium and MTT were used as blank.

Two independent experiments were conducted in quadruplicate for each concentration of tested compound. Mean values and standard deviations (SD) were calculated for each concentration. Anti-proliferative activity was expressed as IC₅₀ value, which is defined as the dose of compound that inhibits cell growth by 50% of untreated control cells. IC₅₀ values were calculated by median effect analysis [see Chou and Talalay 1984] in Microsoft Excel.

Relative binding affinities of test compounds for the ligand binding domain of ERα, ERβ and AR

Yeast strains, plasmids and growth conditions. *Saccharomyces cerevisiae* FY250 strain (MATα, *ura3-52*, *his3Δ00*, *leu2Δ1*, *trp1Δ6*) and plasmid constructs pRF4-6-hERα LBD-EYFP, pRF4-6-hERβ LBD-EYFP and pRF4-6-hAR LBD-EYFP (in further text LBD-YFP) for the fluorescent cellular assay were provided by Dr. Blake Peterson (University of Kansas).⁴⁵ Yeast strains were transformed using a lithium acetate/polyethylene glycol method.⁵⁴ Transformants were selected on tryptophan dropout agar (SD-Trp) for 3 days at 30 °C.

Fluorescent cellular assay in yeast. Pre-cultures were grown at 28 °C in selection media supplemented with 2% raffinose. Cell density was measured spectroscopically at 600 nm (OD₆₀₀). Saturated yeast cells were diluted in fresh selection media supplemented with 2% raffinose at OD₆₀₀ of 0.1.

Fresh culture was incubated under identical conditions until cells reached log phase, OD₆₀₀ of 0.4–0.6 and 2–3 doublings occurred (approximately 12 h). In mid-log phase expression of LBD-YFP proteins was induced with galactose (2% final concentration) and test compounds **3**, **5**, **7**, **8**, **11** or **12** or controls were added (final concentrations 10 μM). For LBD-ERα-YFP or LBD-ERβ-YFP cells estradiol was the positive control ligand and androstenedione was the negative control ligand. For LBD-AR-YFP cells, testosterone was the positive control and estradiol was the negative control. Background cell fluorescence was measured by adding DMSO without ligand at a final concentration of 1% (DMSO control). For fluorescence measurements, induced cells with or without ligand were incubated at 25 °C for 14 h in the dark. For fluorescence readings 100 μL intact cell suspension were added to a 96-well microplate (Carl Roth). Wells containing growth medium were used as a blank. Fluorescence intensity was measured at 25 °C using excitation and emission wavelengths of 485 nm and 538 nm on a fluorimeter (Fluoroskan Ascent FL). Fluorescence intensity values represent the mean of three probes. Fluorescence intensity was normalized by cell density, by measuring absorbance at 600 nm. Ligand binding affinity was expressed as fold fluorescence vs. DMSO control. Histograms were plotted in Origin Pro 8. Error bars represent propagated standard errors of the mean (SE).

AKR1C3 inhibition assay

Expression and purification of human AKR1C3. Plasmid DNA encoding N-terminal 6×-His tagged human AKR1C3 in a pET28b(+) expression vector (Novagen) was generously provided by Prof. dr Chris Bunce (University of Birmingham). Expression and purification of recombinant AKR1C3 was

conducted as described.^{55,56} Briefly, BL21(DE3) *E. coli* were transformed with AKR1C3-pET28 plasmid for bacterial expression. Cells were grown in LB medium with 50 $\mu\text{g mL}^{-1}$ kanamycin to an $\text{OD}_{595\text{nm}}$ of ~ 0.6 at 37 °C, induced with isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and grown at 20 °C for approximately 18 h. Cells were harvested and resuspended in Buffer A (25 mM sodium phosphate, 150 mM NaCl, pH 8.0), lysed by freeze thaw/sonication with lysozyme (1 mg mL^{-1}) (Sigma), and clarified by centrifugation at $10\,000 \times g$ for 45 min at 4 °C. Supernatant was loaded onto a 1 mL HisTrap nickel column (GE Biosciences). The column was washed with 20 column volumes (CV) of Buffer A and then 10 CV of Buffer A with 40 mM imidazole. Purified AKR1C3 was eluted in 5 CV Buffer A with 500 mM imidazole and applied to a Bio-Rad P10 desalting column for buffer exchange into 25 mM sodium phosphate, 150 mM NaCl, 10% glycerol, pH 8.0. Protein purity was monitored by SDS-PAGE with Coomassie staining and protein concentration was quantified using the Bradford colorimetric assay. Purified AKR1C3 was supplemented with 1.2 mM NADP⁺ (Sigma) and 50% glycerol before freezing at -80 °C.

AKR1C3 enzymatic assay. A spectrophotometric assay was used to measure reduction of the pan-AKR1C3 substrate 9,10-phenanthroquinone (PQ) by recombinant AKR1C3 as previously described.⁵⁷ Reaction mixtures containing 200 μM NADPH and 40 $\mu\text{g mL}^{-1}$ recombinant AKR1C3 were incubated with or without test compounds (6.25 μM final concentration) or AKR1C3 inhibitor ibuprofen (6.25 μM as a positive control) in 75 μL (final volume) of 50 mM sodium phosphate buffer (pH 6.5) and incubated for 15 minutes at 37 °C. AKR1C3 activity was first measured against increasing concentrations of PQ from 0–25 μM (to give 0, 0.39, 1.56, 3.12, 6.25, 12.5, 25 μM PQ). Reactions were initiated by addition of PQ and the reaction followed at 30 °C for 35 min by monitoring the decrease in absorbance at 340 nm of the NADPH co-factor ($\epsilon = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) over time. A K_m of 0.30 μM was obtained from fitting AKR1C3 activity data using the Michaelis–Menten equation implemented in GraphPad Prism 6.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) (ESI[†]). Inhibitory activity was then screened by setting the concentration of PQ near the measured K_m value of the substrate (0.39 μM). Absorbance was recorded every 30 seconds. Percent inhibition of AKR1C3 activity was calculated by comparing mean slopes from $A_{340\text{nm}}$ vs. time data for test compounds with that for ibuprofen and PQ only. Data were analyzed by fitting the first 10 minutes by linear regression in GraphPad Prism version 6.01 (GraphPad Software, San Diego California USA, www.graphpad.com). The slope obtained for PQ only was defined as 100% activity. Percent inhibition for each test compound and ibuprofen were calculated as follows: Percent inhibition = $100\% - [\text{slope}(\text{test compound})/\text{slope}(\text{PQ}) \times 100]$. All measurements were performed in triplicate, and coefficients of variability were calculated. For determination of IC_{50} , the effect of compound 3 on AKR1C3 activity was also tested at multiple con-

centrations between 1 μM and 500 μM (1.5, 3.12, 6.25, 12.5, 25, 50, 100, 200 and 500 μM) in 2% DMSO, while holding the concentration of PQ constant at 0.39 μM . AKR1C3 was incubated for 15 minutes on 37 °C with each concentration of compound 3. Prior to measurement substrate PQ (0.39 μM) was added. An IC_{50} value for compound 3 was calculated by fitting the first 12 minutes of inhibition data ($A_{340\text{nm}}$ vs. time) to a four-parameter logistic sigmoidal dose–response curve using Prism 6.01 (GraphPad, La Jolla, CA, USA).

Molecular docking

Protein (receptor) structural coordinate preparation.

Three-dimensional structural coordinates for 17,20-lyase/17 α -hydroxylase (CYP17) (PDB: 3RUK) and AKR1C3 (PDB 1ZQ5) were obtained from the protein data bank (<http://www.rcsb.org>). For CYP17 coordinates for abiraterone and water molecules were removed using a text editor. For AKR1C3, coordinates for ligand EM1404 were removed. Hydrogen atoms and Gasteiger partial charges were added using the script 'receptor.c' in VEGA ZZ 3.1.0.⁵⁸ Non-polar hydrogen atoms were merged in VEGA ZZ and receptor coordinate files saved in PDBQT format for docking.

Ligand structural coordinate preparation. Beginning with X-ray structures of 3, 7, 8, 11 and 12, 3D structural models for docking were created in AVOGADRO 1.0.3 (<http://avogadro.cc/>).⁵⁹ Hydrogen atoms were added and ligand geometries optimized (MMFF94: 500 steps conjugate gradient minimization followed by 500 steps steepest descent minimization with a convergence setting of 10×10^{-7}) in AVOGADRO. Non-polar hydrogen atoms were merged and Gasteiger partial charges were calculated using the script 'ligand.c' in VEGA ZZ and resulting ligand coordinate files were converted to PDBQT format.

Grid map calculations. Autodock grid maps were calculated for CYP17 (3RUK) or AKR1C3 (1ZQ5) using AutoGrid 4 (ref. 60) based on the coordinates of the protein crystal structure. Grid maps for CYP17 were centered on the protein molecule and a maximum grid box size was chosen (covering the entire protein), to enable unbiased docking. For AKR1C3 a grid box of $55 \times 60 \times 50$ was centered on the active site ($x, y, z = 32.15, 27.33, 15.86$). Default grid spacing of 0.375 Å was used. Maps were calculated for all atom types along with electrostatic and desolvation maps using a dielectric value of -0.1465 .

Molecular docking simulations. Initial ligand position, orientation and dihedral offset were set as random. The number of torsional degrees of freedom for each ligand was determined in AutoDockTools.⁶⁰ Docking simulations were conducted using the Lamarckian genetic algorithm. The maximum number of energy evaluations was 2 500 000. For CYP17 the GA population was 150 and a total of 10 hybrid GA-LS runs were performed. For AKR1C3, the GA population size was 200 and a total of 50 hybrid GA-LS runs were performed to enable estimation of average binding energies. Results were visualized in the program PyMol (<http://www>).

pymol.org/) and compared with X-ray structures: a) abiraterone bound to CYP17 (3RUK), b) AKR1C3 bound to flufenamic acid (1S2C) or c) AKR1C3 bound to EM1404 (1ZQ5). N-Fe ligand–receptor distances for CYP17 bound ligands were measured in PyMol. Autodock and AutoGrid calculations were also conducted using the National Biomedical Computational Resource (<http://nbc-222.ucsd.edu/opal2>) and the PyRx virtual screening tool.⁶¹ Control docking simulations using ligands present in X-ray crystal structures of the receptors were able to reproduce the ligand-protein interaction geometries present in the respective crystal structures. Based on these control docking simulations, predicted binding energies ≤ -10.00 kcal mol⁻¹ were considered to be indicative of strong binding.³⁰

Results

Synthesis and characterization of new A-ring fused pyridine D-modified androstanes

We report efficient synthesis of 17a-homo-17-oxa (Scheme 1), 17 α -picolyl and 17(*E*)-picolinylidene (Scheme 2) androstane derivatives with A-ring fused pyridine, based on convenient parent compounds, which were prepared by *Oppenauer* or *Jones* oxidation as described.^{34,35,38} Target compounds 3, 5, 8 and 12 were synthesized by convenient *one-pot* reaction⁶² from compounds 2, 4, 7 and 11, respectively, by modification of a known procedure.⁶³ The reaction was carried out with propargylamine in 95% ethanol, catalyzed by Cu(NO₃)₂, under inert atmosphere and reflux.

Structures of all synthesized compounds were confirmed by ¹H and ¹³C NMR spectra (ESI[†]). The ¹H NMR spectra of 17a-homo-17-oxa A-ring fused pyridine derivatives 3 and 5 contain new signals attributed to the introduced pyridine moiety at 7.10–8.42 ppm, compared to spectra of starting compounds 2 and 4.^{35,39} Also, new signals at 121.01–147.92

Scheme 1 a. Cyclohexanone, Al-(*O-i*-Pr)₃, toluene, co-distillation, 75 min, then HCl (1:1) or cyclohexanone, Al-(*t*-BuO)₃, reflux, 12 h, then HCl (1:1); b. propargylamine, Cu(NO₃)₂·3H₂O, 95% ethanol, argon atmosphere, 6 or 4 h, reflux; c. Jones reagent, 0 °C, 2.5 h; d. *t*-BuOK, *t*-BuOH, rt, 104 h, then HCl (1:1).

Scheme 2 a. Cyclohexanone, Al-(*O-i*-Pr)₃, toluene, co-distillation, 4 h; b. propargylamine, Cu(NO₃)₂·3H₂O, 95% ethanol, argon atmosphere, reflux, 16 h; c. Ac₂O, reflux, 6 h; d. KOH, MeOH, reflux, 1 h; e. cyclohexanone, Al-(*O-i*-Pr)₃, toluene, co-distillation, 2 h.

ppm in ¹³C NMR correspond to pyridine carbon atoms in compounds 3 and 5. In 17 α -picolyl and 17(*E*)-picolinylidene series, the presence of a pyridine fused to the A-ring in compounds 8 and 12 was confirmed according to spectroscopic data and compared to starting compounds 7 and 11.³⁶ In ¹H NMR spectra of compounds 8 and 12, multiplets originating from vinylic protons H-6 were registered at 6.15 and 6.17 ppm, respectively. Signals characteristic for seven aromatic protons from the two pyridine rings in compounds 8 and 12 appear in the range from 7.01 to 8.57 ppm. In the ¹³C NMR spectrum of compound 8, C-4'', C-5'' and C-6'' atoms from the condensed pyridine ring were observed at 132.88, 121.30 and 147.99 ppm, while signals at 120.85, 132.92 and 149.16 ppm correspond to the C-5'', C-4'' and C-6'' atoms from the condensed pyridine ring in compound 12.

Structures of A-ring fused pyridine androstane derivatives 3, 8 and 12 were unambiguously confirmed by high-resolution single crystal X-ray diffraction (Fig. 1).

In vitro antiproliferative activity of A-ring fused pyridine androstane derivatives

To test for anticancer potential, antiproliferative activity against a panel of human tumor cell lines originating from 5 different solid tumor types was evaluated (Table 2). Previously, we reported the antiproliferative activities of parent compounds in D-homo lactone (2 and 4),³⁵ 17 α -picolyl (7) or 17(*E*)-picolinylidene series (11).^{36,40} In the present study, the effect of modification of these compounds by introducing an

Fig. 1 ORTEP drawings of the X-ray structure of compounds **3** (A), **8** (B) and **12** (C) with labeled non-H atoms. Displacement ellipsoids are shown at 50% probability, and H atoms are drawn as spheres of arbitrary radii. A hydrogen bond between **8** and an ordered water is shown as a dashed line.

A-ring fused pyridine (compounds **3**, **5**, **8** and **12**) on antiproliferative activity was investigated (see Table 2). As a control, compounds were also tested against non-cancerous MRC-5 fetal lung fibroblasts. The non-selective cytotoxic drug doxorubicin and steroidal formestane were used as controls for cytotoxicity.

D-Homo lactone compounds. Parent compound **2** and new A-ring fused pyridine derivative **3** had similar strong antiproliferative activity (IC_{50} = 9.30 μ M and 9.13 μ M, respectively) against ER α -MDA-MB-231 cells, suggesting that the pyridine ring does not affect this activity, while **3** also showed strong antiproliferative activity (IC_{50} = 11.77 μ M) against HeLa cells. In contrast, the A-pyridine in compound **5** appears to interfere with the PC-3 antiproliferative activity observed for parent compound **4**. Strikingly, compound **5**

showed highly selective submicromolar activity against only HT-29 cells (IC_{50} = 0.06 μ M), ~5-fold higher than doxorubicin, and no cytotoxic effects against any other cell line tested.

17 α -Picolyl and 17(*E*)-picolinylidene compounds. Introduction of an A-fused pyridine in compound **8** resulted in strong antiproliferative activity against PC-3 cells (IC_{50} = 7.93 μ M) not observed in parent compound **7**. In contrast, no change in PC-3 antiproliferative activity was observed for compound **12** vs. parent compound **11**, although compound **12** showed notable antiproliferative activity against A549 cells (IC_{50} = 11.89 μ M) and low-to-moderate activity against MCF-7 cells (IC_{50} = 37.42 μ M). All compounds were non-toxic to normal MRC-5 cells, in contrast to doxorubicin.

Addition of an A-ring fused pyridine reverses the predicted affinities of 17 α -picolyl and 17(*E*)-picolinylidenes for CYP17 by molecular docking

Previously we predicted by docking that 17(*E*)-picolinylidene, but not 17 α -picolyl androstanes should bind CYP17 with similar geometry and affinity as abiraterone, a 17-pyridinyl androstane drug used in the treatment of prostate cancer.²⁹ Similar binding modes were reported for other C17-*exo*-heterocyclic steroidal CYP17 inhibitors, VN/124-1 (galeterone) and VN/85-1,⁶ suggesting that inhibition involves coordination between a heterocycle nitrogen and the heme iron of CYP17. Independent of their CYP17 inhibitory activity, abiraterone, galeterone and VN/85-1 also inhibit the growth of PC-3 cells *via* induction of an endoplasmic reticulum stress response.^{64,65} Similarly, 17(*E*)-picolinylidene androstanes displayed selective antiproliferative activity against PC-3 cells at the same levels as abiraterone and galeterone. In support of our docking studies, 17(*E*)-picolinylidene androstanes were reported to inhibit CYP17 *in vitro*, while 17 α -picolyl androstanes had no activity.³¹

In the present study, the same docking protocol³⁰ was used to predict the potential affinity of A-ring fused pyridine 17 α -picolyl and 17(*E*)-picolinylidene androstanes for CYP17. Docking was performed in Autodock using a maximum search space and the CYP17 receptor was held rigid with no side-chain flexibility. Control docking experiments were conducted by 're-docking' the abiraterone ligand in the X-ray

Table 2 Comparison of the antiproliferative activity of steroidal pyridine derivatives (**3**, **5**, **8** and **12**) with their parent compounds (**2**, **4**, **7** and **11**)

Compound	IC_{50} (μ M), 48 h						
	MCF-7	MDA-MB-231	PC-3	HeLa	HT-29	A549	MRC-5
2 ³⁴	>100	9.30	>100	—	—	—	>100
3	>100	9.13	>100	11.77	>100	>100	>100
4 ³⁴	>100	>100	13.00	—	—	—	>100
5	>100	>100	>100	>100	0.06	>100	>100
7 ³⁵	>100	>100	>100	57.80	57.52	>100	>100
8	>100	44.52	7.93	>100	46.08	>100	>100
11 ³⁵	>100	>100	12.9	>100	>100	>100	>100
12	37.42	87.37	11.47	>100	>100	11.89	>100
Doxorubicin	0.75	0.12	95.61	1.17	0.32	7.86	0.12
Formestane	>100	55.5	48.36	5.55	>100	>100	>100

crystal structure of human CYP17 (PDB 3RUK). Control redocking simulations reproduced all abiraterone–CYP17 interactions present in the X-ray structure with an RMSD <0.6 Å. As previously reported, the parent 17(*E*)-picolinylidene androstane (11) coordinates the heme-Fe of CYP17 (N–Fe distance 2.66 Å) with similar geometry and affinity (–12.19 kcal mol^{–1}) as abiraterone (–11.59 kcal mol^{–1}), while the 17 α -picolyl parent compound (7) could clash with the heme (see Fig. 2).

In contrast, the presence of a fused A-ring pyridine appears to reverse these results. Based on docking simulations, A-pyridine 17(*E*)-picolinylidene androstanes would have no affinity for CYP17, while A-pyridine 17 α -picolyl androstanes are predicted to bind CYP17 with high affinity (–11.84 kcal mol^{–1}) similar to that predicted for abiraterone (–11.59 kcal mol^{–1}). Structurally, the A-ring pyridine 17(*E*)-picolinylidene androstane (12) is now too long (15.84 Å length) to fit in the CYP17 binding pocket, while the A-pyridinyl nitrogen of the shorter 17 α -picolyl (compound 8, 13.81 Å length) is capable of coordination with the heme Fe of CYP17 (Fig. 3). Interestingly, the A-pyridine in 17 α -picolyl compound 8 also appears to have conferred PC-3 antiproliferative activity not observed in the parent compound 7, while compound 12 retained PC-3 antiproliferative activity despite apparent loss of CYP17 affinity by docking.

A-ring fused pyridine 17 α -homo-17-oxa androstane 3 strongly inhibits AKR1C3 activity *in vitro*

AKR1C3 reduces androstene-3,17-dione and estrone to yield the more potent hormones testosterone and 17 β -estradiol,

Fig. 2 A) Molecular docking of parent 17(*E*)-picolinylidene (11, green) CYP17. Abiraterone is shown in magenta. 17(*E*)-Picolinylidene (11) is predicted to bind to CYP17 in the same manner as abiraterone via N coordination with the heme-Fe. B) Manual docking suggests that 17 α -picolyl (7, blue) may clash with the heme group without extensive conformational change in the CYP17 binding pocket. Molecular docking results for compound 11 (green) are shown for comparison.

Fig. 3 Molecular docking of A-ring fused pyridine 17 α -picolyl A) (8) and 17(*E*)-picolinylidene B) (12) androstanes with CYP17. Abiraterone is shown in magenta. A-pyridine 17 α -picolyl (green) (8) is predicted to bind to CYP17 through hydrogen bond interactions with R239 and N202 as well as pyridine-N mediated coordination with the heme-Fe. A-pyridine 17(*E*)-picolinylidene (blue) (12) would likely clash with R239 without extensive conformational change in the CYP17 binding pocket.

and is a potential drug target in the treatment of breast, prostate and other cancers.⁶⁶ Steroidal inhibitors of AKR1C3 have been reported with Δ -lactone modifications, and the X-ray structure of a Δ -lactone steroidal inhibitor (EM1404) has been solved in complex with AKR1C3.²⁵ Moreover, in preliminary studies we observed that Δ -lactone modified estranes displayed moderate AKR1C3 inhibitory activity (not shown). Because of the above we wanted to determine if A-ring fused pyridine Δ -lactone androstanes are potential inhibitors of AKR1C3. Recombinant AKR1C3 catalyzes the reduction of 9,10-phenanthrenequinone (PQ) in the presence of cofactor NADPH, and AKR1C3 activity can be determined by monitoring the decrease in absorbance at 340 nm over time, corresponding to loss of NADPH. Reduction of the AKR1C3 substrate PQ by recombinant AKR1C3 was monitored spectrophotometrically as described.⁵⁷ AKR1C3 activity was first measured against increasing concentrations of PQ from 0–25 μ M, and a K_m of 0.30 μ M was obtained from fitting the data using the Michaelis–Menten equation implemented in GraphPad Prism (ESI†). The effect of incubation with test compounds (3, 5, 8 or 12) or ibuprofen (positive control AKR1C3 inhibitor,⁶⁶) on AKR1C3 activity was then measured (Fig. 4) by setting the concentration of PQ near the measured K_m value of the substrate (0.39 μ M). Data for A_{340nm} vs. time were then analyzed by linear regression, where the slope obtained for the first 10 minutes of the PQ only control was considered to represent 100% AKR1C3 activity. The relative percent inhibition for each test compound and ibuprofen were then calculated. The A-pyridine Δ -lactone compound (3)

strongly inhibited PQ reduction ($64.35 \pm 1.02\%$) at a level similar to the known AKR1C3 inhibitor ibuprofen ($59.67 \pm 1.52\%$), while addition of compounds 5, 8 and 12 actually increased the activity of AKR1C3 vs. PQ only controls ($-67.42 \pm 2.66\%$, $-43.37 \pm 0.42\%$ and $-64.69 \pm 0.53\%$ for 5, 8 and 12 respectively).

The effect of increasing concentrations of compound 3 on AKR1C3 activity was then tested at 1.5, 3.12, 6.25, 12.5, 25, 50, 100, 200 and 500 μM while holding the concentration of substrate PQ constant at 0.39 μM . An IC_{50} value for compound 3 was estimated by fitting the inhibition data (A_{340} vs. time) to a four-parameter logistic sigmoidal dose-response curve (Fig. 4B). The IC_{50} for inhibition of AKR1C3 activity by compound 3 was $\sim 15 \mu\text{M}$, in the range of reported values for ibuprofen ($\text{IC}_{50} \sim 10 \mu\text{M}$,⁶⁶).

Molecular docking suggests that A-ring pyridine D-lactone androstanes are potential ligands for human AKR1C3

To rationalize the AKR1C3 inhibitory activity of compound 3, docking simulations were conducted in AutoDock. Compounds 3, 5, 8 and 12 were docked using the X-ray structure of AKR1C3 in complex with steroidal lactone inhibitor EM1404

as the receptor (PDB 1ZQ5).²⁵ This structure was chosen for docking because the bound ligand is a steroid derivative similar to our test compounds. For docking, the receptor was kept rigid with no side-chain flexibility. Control redocking simulations reproduced EM1404-AKR1C3 interactions present in the X-ray structure with an RMSD of 0.812 Å and a high binding energy of $-12.9 \text{ kcal mol}^{-1}$. The AKR1C3 substrate androstenedione was then cross-docked onto this structure, giving a predicted binding energy of $-10.1 \text{ kcal mol}^{-1}$ that we used as a reference value indicative of strong binding. In agreement with our *in vitro* results, compounds 8 and 12 were predicted to have low affinity for AKR1C3, with binding energies of -8.4 and $-7.6 \text{ kcal mol}^{-1}$, while compound 3 was predicted to bind with higher affinity similar to androstenedione ($-9.8 \text{ kcal mol}^{-1}$). Molecular docking also suggests that compound 5 could bind to AKR1C3 with a similar binding energy of $-9.6 \text{ kcal mol}^{-1}$. This is not unexpected given that 3 and 5 are identical with the exception of a single carbonyl group on the B-ring of compound 5, and no attempts were made to model potential 'induced-fit' effects upon ligand binding. However, in crystal structures of human AKR1C3, ligand binding is associated with varying degrees of conformational changes in the active site.^{25,55,66} The presence of multiple binding sites and the potential for "induced-fit" effects in the case of AKR1C3 mean that *in silico* simulation results should be interpreted in conjunction with available *in vitro* and structural data for identification of AKR1C3 inhibitors.⁶⁶ With this in mind, docking does suggest that A-ring pyridine D-lactone androstanes as a class may be useful scaffolds for design of new AKR1C3 inhibitors. Specifically, molecular docking predicts that the D-lactone modification may interact with the oxyanion site of AKR1C3 near the NADP cofactor, forming hydrogen bonds with Y55 and possibly H117, while the A-ring pyridine interacts with residues in the proposed SP1 site (Fig. 5B, for a detailed description of AKR1C3 binding sites see ref. 66). A similar mechanism of binding was observed in crystal structures of AKR1C3 in complex with non-steroidal anti-inflammatory drugs (e.g. ibuprofen/PDB:3R8G and flufenamic acid/PDB 1S2C) which form hydrogen bonds with oxyanion site residues Y55 and H117, while the other end of both compounds form van Der Waals contacts with SP1 (Fig. 5A).^{55,66} In contrast, in the X-ray structure of EM1404 + AKR1C3, the steroidal lactone interacts in a different location with S118 of the SP2 site, while an acetate ion present during crystallization was located in the oxyanion site (Fig. 5A).²⁵

Fig. 4 Inhibition of recombinant human AKR1C3 by A-pyridine D-lactone androstane derivative 3. A) Reactions containing 200 μM NADPH and 40 $\mu\text{g mL}^{-1}$ recombinant AKR1C3 were incubated with or without test compounds 3, 5, 8, or 12 (6.25 μM), or AKR1C3 inhibitor ibuprofen (IBU, 6.25 μM , positive control). A) Reactions were initiated by addition of PQ (0.39 μM) and the decrease in NADPH absorbance at 340 nm was monitored. B) Effect of increasing concentrations of compound 3 (1.5, 3.12, 6.25, 12.5, 25, 50, 100, 200 and 500 μM in 2% DMSO) on AKR1C3 activity, reflected by a decrease in $A_{340\text{nm}}$ over time, at constant substrate concentration (PQ = 0.39 μM). $\epsilon_{\text{NADPH}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and path length (l = 0.562 cm).

New A-ring fused pyridine D-modified androstane derivatives have no apparent affinity for the ligand binding domain of ER α , ER β or androgen receptors

Because they are based on natural estrogens or androgens, steroidal compounds designed as inhibitors of steroidogenic enzymes may have off-target interactions with androgen or estrogen receptors. In the case of compounds intended for the treatment of hormone-sensitive cancers, potential estrogenic or androgenic effects may be undesirable. Because of this, compounds (3, 5, 7, 8, 11 and 12) were tested for affinity

Fig. 5 A) Structure of human AKR1C3 in complex with non-steroidal inhibitor flufenamic acid (PDB 1S2C) shows hydrogen bonding between the O1 carboxylic acid of flufenamic acid (blue) and oxyanion site residues Y55 and H117, while the CF₃ group is located in the SP1 site in contact with Y216. To illustrate the properties of the oxyanion site, an acetate ion present at the same location in the structure of AKR1C3 + EM1404 (1ZQ5) is superimposed (B) molecular docking of compound **3** (green) with AKR1C3 (PDB 1ZQ5) predicts that the α -lactone group is also capable of hydrogen bonding with Y55 in the oxyanion site. The NADP cofactor is shown in yellow CPK.

to the ligand binding domains (LBD) of estrogen receptor α (ER α), estrogen receptor β (ER β) or androgen receptor (AR) using a fluorescent screen in yeast adapted from Muddana and Peterson⁴⁵ (Fig. 6). LBDs of steroid receptors were expressed in-frame with yellow fluorescent protein (YFP) in *Saccharomyces cerevisiae*. Addition of positive control steroidal ligands to yeast expressing the relevant cognate receptor results in increased YFP fluorescence intensity, enabling estimation of relative receptor binding affinities. Relative binding affinities of A-pyridine α -modified steroidal derivatives **3**, **5**, **7**, **8**, **11**, and **12** were evaluated by fluorimetry. Estradiol and androstenedione were used as positive and negative controls, respectively, in cells expressing LBD-ER α -YFP and -ER β -YFP. For cells expressing LBD-AR-YFP, testosterone was used as a positive control and estradiol was used as negative control. As can be seen in Fig. 6, no test compounds displayed affinity for any of the steroid hormone receptors suggesting they lack estrogenic or androgenic properties.

Conclusions

We report a convenient and efficient synthesis of A-ring fused pyridine α -homo lactone, 17 α -picolyl and 17(*E*)-picolinylidene

Fig. 6 New A-ring fused pyridine α -modified steroids **3**, **5**, **7**, **8**, **11** and **12** do not have affinity for the ligand binding domains of estrogen receptor α , estrogen receptor β or the androgen receptor based on a fluorescent screen in yeast.

androstane derivatives. Addition of an A-ring fused pyridine resulted in significant changes in anticancer properties against human tumor cell lines and selected protein targets of breast and prostate cancer. A-ring fused pyridine 17 α -picolyl androstane was predicted to have increased affinity for CYP17 by molecular docking, while conferring selective antiproliferative activity against PC-3 prostate cancer cells. Similarly, A-ring fused pyridine α -homo lactone androstanes may represent potential starting compounds for the development of new AKR1C3 inhibitors. Strikingly, one A-ring fused pyridine α -lactone steroid **5** was also characterized with highly selective submicromolar antiproliferative activity against only HT-29 colon cancer cells. None of the new A-fused pyridine androstane derivatives had affinity for estrogen or androgen receptor in a yeast screen, suggesting negligible estrogenicity and androgenicity. Combined, our results suggest that A-ring fused pyridine modifications have potential in modulating the anticancer activity of steroidal anticancer compounds. Several compounds deserve further studies.

Conflicts of interest

The authors declare no competing interest.

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