CYP17A1 inhibitor abiraterone, an anti-prostate cancer drug, also inhibits the 21-hydroxylase activity of CYP21A2

Jana Malikova[1,2,#], Simone Brixius-Anderko[3, ¶, #], Sameer S Udhane[1,2,Ψ], Shaheena Parween[1,2], Bernhard Dick[4], Rita Bernhardt[3] and Amit V. Pandey[1,2,*]

[1]Pediatric Endocrinology, Diabetology and Metabolism, University Children's Hospital, Inselspital, Bern, Switzerland
[2]Department for BioMedical Research, University of Bern, Bern, Switzerland
[3]Department of Biochemistry, Faculty of Technical and Natural Sciences, Saarland University, 66123 Saarbrücken, Germany.
[3] Department of Nephrology, Hypertension and Clinical Pharmacology, University Hospital of Bern, Bern, Switzerland.

# These authors contributed equally to this work.

¶Current address: Medicinal Chemistry Department, College of Pharmacy, University of Michigan, Ann Arbor MI 48109, USA

Ψ Current address: Department of Pathology, Medical College of Wisconsin Cancer Center, Milwaukee, WI, 53226, USA.

Running title: Inhibition of CYP21A2 activity by abiraterone.

Keywords: Abiraterone, CYP21A2, CYP17A1, prostate cancer, cytochrome P450, steroidogenesis, androgens.

* Address for Correspondence:
PD Dr. Amit V Pandey
Pediatric Endocrinology, Diabetology & Metabolism,
KIKL C837, University Children's Hospital Bern,
Freiburgstrasse 15,
CH-3010 Bern Switzerland
Tel: +41 31 632 9637
Email: amit@pandeylab.org

Abstract

Abiraterone is an inhibitor of CYP17A1 which is used for the treatment of castration resistant prostate cancer. Abiraterone is known to inhibit several drug metabolizing cytochrome P450 enzymes including CYP1A2, CYP2D6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and CYP3A5, but its effects on steroid metabolizing P450 enzymes are not clear. In preliminary results, we had observed inhibition of CYP21A2 by 1μM abiraterone. Here we are reporting the effect of abiraterone on activities of CYP21A2 in human adrenal cells as well as with purified recombinant CYP21A2. Cells were treated with varying concentrations of abiraterone for 24 hours and CYP21A2 activity was measured using [^{3}H] 17-hydroxyprogesterone as substrate. Whole steroid profile changes were determined by gas chromatography-mass spectrometry. Binding of abiraterone to purified CYP21A2 protein was measured spectroscopically. Computational docking was used to study the binding and interaction of abiraterone with CYP21A2. Abiraterone caused significant reduction in CYP21A2 activity in assays with cells and an inhibition of CYP21A2 activity was also observed in experiments using recombinant purified proteins. Abiraterone binds to CYP21A2 with an estimated Kd of 6.3 μM. These inhibitory effects of abiraterone are at clinically used concentrations. A loss of CYP21A2 activity in combination with reduction of CYP17A1 activities by abiraterone could result in lower cortisol levels and may require monitoring for any potential adverse effects.

2

1 Introduction

Androgenic steroids are required for a wide range of functions necessary for life, from the salt balance by mineralocorticoids, sugar balance by glucocorticoids to the growth, reproductive and sexual functions by sex steroids. Biosynthesis of androgens occurs in the human adrenal cortex (zona reticularis) and gonads (ovaries / testes) [1]. Enzymes for production of androgens and genes encoding these enzymes are known but the mechanism of regulation of androgen production remains unclear [2]. Steroid hormones are synthesized from cholesterol starting from conversion of cholesterol to pregnenolone by CYP11A1, a member of cytochrome P450 gene family, which is the quantitative regulator of steroidogenesis [3]. Pregnenolone can then be directed to one of three principal pathways by CYP17A1, the qualitative regulator of steroidogenesis, which catalyzes both 17α-hydroxylase and 17,20 lyase activities [4, 5] (Fig. 1).

In the absence of CYP17A1, pregnenolone is converted to C21 steroids, including progesterone, corticosterone and aldosterone. In presence of the 17α-hydroxylase activity of CYP17A1, the adrenal zona fasciculata produces C21 17α-hydroxy steroids including cortisol [6, 7]. When both 17α-hydroxylase and 17,20 lyase activities are present, the adrenal zona reticularis and gonads produce dehydroepiandrosterone (DHEA), which is the precursor of androgens and estrogens. The CYP17A1 acts as a qualitative regulator of sex steroid biosynthesis in humans [8]. CYP17A1 catalyzes two distinct reactions in the steroid pathway [5, 9, 10]; its 17α-hydroxylase activity is essential for producing 17OH-pregnenolone and 17OH-progesterone precursors of cortisol, and its 17,20 lyase activity is needed for the production of the precursor of sex steroids, dehydroepiandrosterone (DHEA) . The two activities of CYP17A1 determine the type of steroid hormone synthesized in different cells and tissues; if CYP17A1 is absent, mineralocorticoids are produced, if only 17α-hydroxylase

3

87 activity is present, glucocorticoids are made, and if both activities are present sex steroid

88 precursors can be produced. Overproduction of androgens by specific activation of

89 CYP17A1-17,20 lyase activity has been implicated in the pathogenesis of the polycystic

90 ovary syndrome [8].

91

92 The overproduction of steroid hormones, especially hypercortisolemia during the

93 Cushing´s syndrome, is a potentially life-threatening situation [11, 12]. The

94 hyperandrogenism is not a life threatening condition itself but brings many severe

95 complications during fetal, childhood as well as adult phases of life [13]. Androgens are

96 regulators of both, the female and male sexual differentiation [13, 14]. The hyperandrogenism

97 during childhood and adulthood is mainly recognized in females due to virilisation, hirsutism,

98 oligomenorhea, infertility etc. Non-tumoral cases of hyperandrogenism are polycystic ovary

99 syndrome, Cushing syndrome or congenital adrenal hyperplasia due to 21-hydroxylase

100 deficiency [15-18]. The hyperandrogenism can also be the first sign of adrenocortical or

101 ovarian tumors [15, 19]. Overproduction of cortisol and androgens could be therapeutically

102 influenced by drugs.

103

104 The adrenal steroidogenesis inhibitors block various steps in steroid production.

105 Currently few drugs are approved as steroidogenesis inhibitors (Ketoconazole, Metyrapone,

106 Etomidate, Mitatone) in the European Union or in the United States. All these drugs inhibit

107 the CYP17A1, CYP11A1 and CYP11B1 [20]. Several other drugs like Osilodrostat (inhibitor

108 of CYP11B and other CYP enzymes) are being studied [20-22]. In addition, new drugs such

109 as orteronel and galeterone which are able to inhibit androgen productions in androgen

110 depended prostate cancers are being tested [23-28].

111

4

112 Abiraterone was designed as a CYP17A1 inhibitor and its effect on androgen production in

113 the treatment of androgen depended prostate cancer was confirmed by clinical trials [29]. The

114 U.S. Food and Drug Administration approved abiraterone acetate (Zytiga Tablets,

115 http://www.zytiga.com)) for use in combination with prednisone for the treatment of patients

116 with metastatic castration-resistant prostate cancer (mCRPC) who have received prior

117 chemotherapy containing docetaxel. Abiraterone was tested in our laboratory to elucidate its

118 effect on adrenal androgen production. The CYP17A1 inhibitors in use target both the 17α-

119 hydroxylase and 17,20 lyase activities and require steroid supplementation [26, 30-33]. In our

120 previous study abiraterone inhibited both the 17α-hydroxylase and the 17,20-lyase activities

121 of CYP17A1. Surprisingly, abiraterone also completely inhibited the 21-hydroxylase activity

122 of CYP21A2 at the concentration for the clinical uses (1 μM) [26].

123

124 Both the CYP17A1 and the CYP21A2 enzymes are localized in the endoplasmic reticulum,

125 and catalyze important steps in the biosynthesis of steroids [8]. The CYP17A1 is the source of

126 17α-hydroxylase and 17,20-lyase activities in adrenals and gonads whereas the CYP21A2 is

127 localized only in the adrenals and catalyzes the 21-hydroxylation of progesterone/17OHP to

128 DOC /11-deoxycortisol for the biosynthesis of mineralocorticoids and glucocorticoids. Both

129 of these enzymes have sequence similarities and belong to the cytochrome P450 family of

130 proteins [8] and depend on P450 oxidoreductase for redox equivalents [34]. The deficiency of

131 CYP21A2 leads to variable symptoms depending on the amount of residual activity, ranging

132 from the severe adrenal crisis with salt wasting symptoms to mild hyperandrogenism [8, 35,

133 36].

134

135 Here, we are reporting detailed studies on the effect of abiraterone on 21-hydroxylase activity.

136 The mode of action of abiraterone on CYP21A2 was tested using recombinant purified human

137 CYP21A2 protein expressed in bacteria as well as in cell based assays using a human adrenal

138 cell line. All studies show the inhibition of 21-hydroxylase activity of CYP21A2 at clinically

139 used concentrations of abiraterone, indicating that treatment with abiraterone should be used

140 with caution, especially in treatment of non-cancerous hyperandrogenic disorders like PCOS.

141

2 Materials and Methods

143

2.1 Materials

145 Abiraterone was purchased from Selleckchem (Houston, TX, USA). Radio-labeled [^{3}H]-17α-

146 hydroxyprogesterone (17OH-PROG) was from American Radiolabel Chemicals Inc. (St.

147 Louis, MO, USA). All other chemicals were purchased from Sigma Chemical company (St.

148 Louis, MO, USA)

149

2.2 Protein expression and purification

151 Human CYP21A2 was recombinantly produced in *E. coli* strain C43(DE3) (Lucigen,

152 Middleton, MI, USA) and purified via metal chelate (IMAC) and ion exchange

153 chromatography as described previously. Carbon monoxide difference spectroscopy was

154 performed to determine the enzyme quality and quantity by monitoring the absorption peaks

155 at 450 nm. An extinction coefficient of 91 mM^{-1}*cm^{-1} was used for calculation of

156 cytochrome P450 content [37]. Human NADPH- cytochrome P450 reductase (POR) was

157 produced as recombinant protein in *E. coli* C43(DE3) and purified by IMAC using established

158 protocols [38].

159

2.3 Study of Abiraterone binding to CYP21A2 by difference absorption spectroscopy

161 Difference spectroscopy was performed with tandem cuvettes for determination of the

162 dissociation constant (K_d) for abiraterone according to earlier protocols. Cuvettes contained 1

163 µM of purified CYP21A2 in phosphate buffer (50 mM potassium phosphate (pH 7.4), 0.5%

164 sodium cholate, 0.05% Tween 20 and 20% glycerol). Titration was performed by adding

165 increasing amounts of abiraterone dissolved in DMSO. Difference spectra were monitored in

166 the wavelength range of 350 to 500 nm. The binding titrations were carried out in three

167 different experiments. To determine the K_d, the average of ΔA (absorbance difference of

168 peak-to-trough) was plotted against the concentration of abiraterone. The plots were fitted for

169 hyperbolic regression using OriginPro 9 software (OriginLab Corp, MA, USA).

170

171 2.4 CYP21A2 Inhibition studies with purified recombinant protein

172 The inhibition studies were performed in reconstituted *in vitro* assays in 50 mM HEPES

173 buffer (pH 7.4) containing 20% glycerol and 100 µM 1,2-dilauroyl-sn-glycero-3-

174 phosphocholine. Prior to use, the buffer was sonicated in a water bath for 5 minutes for the

175 reconstitution of 1,2-dilauroyl-sn-glycero-3-phosphocholine vesicles. The concentration of

176 human CYP21A2 in reactions was 0.1 − 0.3 µM and equal amounts of human POR were

177 added. Additionally, the reaction contained a NADPH regeneration system consisting of 5

178 mM glucose-6-phosphate, 1 mM $MgCl_2$ and glucose-6-phosphate dehydrogenase. The 17OH-

179 PROG (substrate) was varied at a concentration of 1, 2.5 and 5 µM and the abiraterone

180 (inhibitor) was at concentrations of 0.25, 0.5, 1, 2.5 and 5 µM. The substrate concentrations

181 were kept below saturation, but in excess over K_m for CYP21A2. The final DMSO

182 concentration was kept below 2%. The reaction was initiated by addition of 5 mM NADPH

183 and incubation was performed in a water bath with shaking for 4 - 7 min at 37°C. After the

184 incubation, reactions were stopped by addition of chloroform and steroids were extracted with

7

185 chloroform. Extraction process was repeated twice, then steroids were dried by evaporation

186 and stored at -20°C for quantitative analysis by HPLC.

187

2.5 Steroid analysis via RP-HPLC

189 Steroid analysis was carried out by RP-HPLC using a Jasco reversed phase LC900 HPLC

190 system (Jasco Inc, Easton, MD, USA) and a 4.6 mm × 125 mm NucleoDur C18 Isis Reversed

191 Phase column (Macherey-Nagel, Düren, Germany). Samples were measured within 30 min at

192 240 nm and a flow rate of 0.8 mL/min with the gradient: 80% solvent A (10% acetonitrile in

193 water) for 13 min, 60% solvent A for 7 min, 80% solvent B (100% acetonitrile) for 2 min and

194 80% solvent A for 8 min.

195

2.6 Inhibition of CYP21A2 activity by abiraterone in human adrenal cells

197 Human adrenal carcinoma cell line (NCI-H295R) was purchased from American Type

198 Culture Collection (ATCC, CRL-2128). The NCI-H295R cells were cultured under standard

199 condition in DMEM/Ham's F-12 medium containing L-glutamine (GIBCO) supplemented

200 with 5% NU-I serum (BD biosciences), 0.1% insulin, transferrin, and selenium (100 U/ml;

201 GIBCO), penicillin (100 U/ml, GIBCO) and streptomycin (100 µg/ml, GIBCO). Abiraterone

202 was dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 200 µM; final

203 concentrations used for treatment were in the range of 0.001 to 1 µM.

204

205   For the experiments cells were grown in twelve-well plates. Twenty-four hours after

206 plating the cells, medium was replaced and treatment was added in normal growth medium

207 for 24 h. After 24 hour cells were treated with 1µM trilostane (a specific blocker of HSD3B)

208 for 90 min before adding [3H] 17OH-PROG. 17OH-PROG was added at two concentrations

209 (1 and 5 µM). Control cells were treated with 0.1% (v/v) DMSO. Radiolabeled [3H]- 17OH-

PROG (50 000 cpm) was added to the culture medium for the last 60 min of incubation.

Steroids were extracted from cell supernatants and separated by thin layer chromatography

(TLC) on silicagel (SIL G/UV$_{254}$) TLC plates (Macherey-Nagel, Oensingen, Switzerland) as

previously described [26, 39-42]. The steroids were visualized on a Fuji FLA-7000

PhosphoImager (Fujifilm, Dielsdorf, Switzerland) and quantified using Multi Gauge software

(Fujifilm, Dielsdorf, Switzerland). The conversion of 17OH-PROG to 11-deoxycorticosterone

(11DOC) showed 21-hydroxylase activity. Steroid conversion was assessed as a percentage of

incorporated radioactivity into a specific steroid product in relation to total radioactivity

measured for the whole sample (internal control). Data were analyzed based on Michaelis-

Menten enzyme kinetics [43] using the method of Dixon to determine the Ki values [44].

2.7 Steroid profiling from cell culture

Steroid metabolites from cell cultures were measured by gas chromatography-mass

spectrometry (GC/MS) according to established protocols [45, 46] . The cells were grown in

10 cm plates in normal growth medium for 24 h, then medium was replaced, and cells were

treated with 1 µM abiraterone in medium without NU-I serum for 24 h. After 12 h of

treatment, 1 µM pregnenolone was added. At the end of incubations supernatant was collected

and concentrated samples were used for steroid analysis by GC–MS. All measurements were

performed in the steroid laboratory of the Department of Nephrology, Hypertension and

Clinical Pharmacology at the University Hospital of Bern, Switzerland.

2.8 Protein structure analysis

The published 3D structure of CYP21A2 waw obtained from PDB database (www.rcsb.org).

We performed several sequence alignments with multiple CYP21A2 protein sequences from

different organisms and made in-silico calculations with the programs YASARA [47] and

235 WHATIF [48]. For all experiments, a crystal structure (PDB # 4Y8W) of CYP21A2 was

236 used. Missing hydrogen atoms were added with YASARA [47] that was also used for all

237 subsequent computations unless stated otherwise. Afterwards system was subjected to 500 ps

238 explicit solvent MD simulations at 310 K, preceded by 500 steps of steepest decent and

239 simulated annealing minimization with the AMBER03 force field and the TIP3P water model

240 [49, 50]. All subsequent MD simulations retained these settings. The resulting minimum

241 energy structure was used for AutoDock VINA [51] to perform docking experiments with

242 abiraterone (orthorhombic docking was grid established around the central heme). The final

243 poses were selected based on their docking scores and resemblance to the co-crystallized

244 ligand in the template structure (PDB: 4Y8W). Structure models were depicted with Pymol

245 (www.pymol.org) and rendered as ray traced images with POVRAY (www.povray.org).

246 Ligand interactions were analysed and depicted with LIGPLOT+

247 (http://www.ebi.ac.uk/thornton-srv/software/LigPlus/)

248

2.9 Statistical Analysis

250 Statistical analysis was performed with Microsoft Excel and GraphPad Prism 6 (Graph Pad

251 Software, Inc. San Diego, CA, USA). Statistical differences between values were calculated

252 using the Student's t test. Quantitative data represent the mean of three independent

253 experiments, error bars indicate the mean $\pm$ SEM. Significance was set at $*p < 0.05$ and

254 $**p < 0.01$, $***p < 0.001$.

255

3 Results

257

3.1 Effect of abiraterone on steroid production by human adrenal cells. We measured the

259 global changes in steroid production of human adrenal NCI-H295A cells upon abiraterone

260 treatment (Table 1). Abiraterone lowered the production of both testosterone, and

261 dihydrotestosterone. In addition, androsterone, etiocholanolone and their 11β-hydroxy

262 metabolites were also lowered. Abiraterone caused major changes in cortisol metabolites with

263 a decrease of tetrahydro deoxycorticosterone and cortisol and increase in tetrahydrocortison,

264 α-Cortolon, β-Cortolon, tetrahydrocortisol and 5α-tetrahydrocortisol. Overall in addition to

265 inhibition of CYP17A1 activities, abiraterone seems to affect a wide range of steroid

266 metabolizing enzymes.

267

3.2 Inhibition of CYP21A2 activity by abiraterone in human adrenal cells.

269 The 21-hydroxylase activity was monitored in H295R cells treated with control (DMSO) and

270 1 μM abiraterone for 24 h. CYP21A2 activities were measured by observing the conversion

271 of [^{3}H] 17-OH-PROG (17α-hydroxyprogesterone) to 11-deoxycortisol using two different

272 substrate concentrations (Figure 2A). Data are presented as mean ± SD of three independent

273 experiments for each set of substrate concentrations. We observed significantly decreased 21-

274 hydroxalyse activity in cells treated with abiraterone at concentrations from 0.03 μM to 1 μM.

275 The calculated IC_{50} for abiraterone inhibition of CYP21A2 activity in our experiments was 25

276 nM at 1μM substrate concentration and 54 nM at 5 μM substrate concentration. We also

277 calculated the K_i value for abiraterone inhibition of CYP21A2 activity by Dixon plot analysis

278 [44]. A plot of 1/v versus increasing abiraterone concentrations at two different substrate

279 concentrations showed a competitive inhibition / simple mixed inhibition pattern with an

280 estimated K_i value of 23 nM. The strong inhibition of CYP21A2 activity at the lower end of

281 clinically used concentrations of abiraterone indicated that abiraterone is a potent inhibitor of

282 CYP21A2 activity, in addition to its effects of CYP17A1 activities. Comparison of IC_{50}

283 values obtained at lower substrate concentration and the K_i value derived from Dixon plot

284 indicated a pattern of a simple mixed inhibition according to the Cheng-Prusoff equation [52, 53] for the mechanism of abiraterone effect of CYP21A2 activity.

286

3.3 Computational docking of abiraterone into the human CYP21A2 crystal structure.

288 Abiraterone was docked into the crystal structure of human CYP21A2 using Autodock VINA

289 (Figure 3A). Superimposition of CYP21A2 structures with either its substrate or abiraterone

290 docked into the active site revealed similar binding poses (Figure 3B). We observed a

291 nitrogen-iron binding pattern from the docking of abiraterone into the active site of CYP21A2

292 crystal structure (Figure 3C). Binding pose of abiraterone to CYP21A2 was also similar to its

293 binding into the CYP17A1. A comparison of the CYP17A1 crystal structure in complex with

294 abiraterone and docked abiraterone into the crystal structure of CYP21A2 revealed similar

295 binding conformations and the distance of the imidazole nitrogen of abiraterone to the central

296 heme iron of both, CYP21A2 and CYP17A1, was similar (3.2 Å vs 2.7Å) (Figure 3D).

297 Binding of abiraterone with CYP21A2 shares many similarities with its binding to CYP17A1

298 with many similar active site residues involved in binding for both proteins (Table 2).

299

3.4 Determination of abiraterone-CYP21A2 dissociation constant by difference spectroscopy

302 Since our cell culture experiments demonstrate a significant inhibition of CYP21A2 by

303 abiraterone, which was also supported by docking studies using the crystal structure of

304 CYP21A2, we were interested to evaluate the mechanism of this effect using in-vitro

305 investigations with purified enzymes. To confirm the computational docking of abiraterone

306 into the CYP21A2 structure binding of abiraterone to purified human CYP21A2 was studied.

307 The dissociation constant for binding of abiraterone to CYP21A2 was determined by

308 difference absorption spectroscopy (Figure 4). The formation of complex between an inhibitor

12

309 and CYP21A2 could be determined spectroscopically by observing the type II shift in spectral

310 changes caused by the displacement of a water molecule upon coordination of a nitrogen-

311 containing ligand to the P450 heme iron. Titration of recombinant bacterially expressed

312 CYP21A2 with increasing concentrations of abiraterone shows a typical type II shift with an

313 absorption decrease at 410 nm and an increase at 424 nm (Figure 4 inset), confirming the

314 computational docking experiments which showed nitrogen-iron co-ordination for binding of

315 abiraterone to CYP21A2 active site. Plotting of the absorbance differences produced a

316 hyperbolic curve which gave a K_d of 6.3 ± 0.2 µM (Figure 4). The micro molar range of the

317 dissociation constant indicates a strong binding of abiraterone to human CYP21A2, which is,

318 however, weaker than that for 17OH-PROG, which has a K_d value of 0.03 µM as reported by

319 Pallan et al. [54].

320

321

3.6 Estimation of the K_i value for abiraterone with purified CYP21A2

323 For the determination of a K_i value for abiraterone by the Dixon plot [44], reconstituted *in*

324 *vitro* assays using recombinant CYP21A2 were performed with three different 17OH-

325 progesterone concentrations (1, 2.5 and 5 µM) and the addition of increasing amounts of

326 abiraterone (0.25, 0.5, 1, 2.5 and 5 µM). For each 17OH-PROG concentration, the reciprocal

327 reaction velocity (v = nmol product/nmol CYP/min) was plotted against the respective

328 concentration of abiraterone, resulting in three linear fits, whose interface showed a K_i value

329 of 2.26 µM. The estimated K_i value agrees with the determined dissociation constant of 6.3

330 µM which was also measured with the recombinant CYP21A2. The K_i value obtained using

331 recombinant enzyme was higher when compared to results from cell culture experiments. This

332 could be due to the differences in the two systems and methods used, e.g. purification and

333 reconstitution steps involved in use of bacterially expressed CYP21A2.

334

4 Discussion

Abiraterone acetate was developed as an inhibitor of CYP17A1 to block androgen production. The effect of abiraterone has been demonstrated successfully in the treatment of metastatic castrate resistant prostate cancers (mCRPC) [29]. Abiraterone was approved as a treatment of mCRPC with co-administration of prednisolone or prednisone by U.S. Food and Drug Administration (FDA) and also by European Medicines Agency's (EMA) Committee for Medicinal Products for Human Use. The small doses of prednisone or prednisolone positively reduced side effects of abiraterone acetate administration which was associated with increased levels of adrenocorticotropic hormone and steroids upstream of CYP17A1 along with suppression of serum testosterone, downstream androgenic steroids, and estradiol in all patients [55]. Abiraterone is known to have strong (CYP1A2, CYP2D6 and CYP2C8) to moderate (CYP2C9, CYP2C19, CYP3A4, CYP3A5) inhibitory effect on several hepatic drug metabolizing cytochrome P450 enzymes and is a substrate of CYP3A4 *in vitro* (https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/202379s004lbl.pdf). Some studies have indicated reduced activity of HSD3B1 and HSD3B2 [56]. Abiraterone is also known to bind to the androgen receptor and produces a dose-dependent decrease in AR levels [57, 58]. However, surprisingly, information about effects of abiraterone on steroid metabolizing enzymes is lacking. Considering the inhibitory effect of abiraterone on multiple hepatic cytochrome P450 enzymes, in addition to its inhibitory effect on CYP17A1, effects of abiraterone on other similar steroid metabolizing cytochrome P450 enzymes should have been investigated. However, information regarding such studies is not available at either manufacturer's web site (www.zytiga.com) or from the FDA drug safety documents (https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/202379s004lbl.pdf) .

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14

359 We have previously performed a preliminary analysis of the steroid hydroxylation reactions

360 of the adrenal carcinoma cell line treated by CYP17A1 inhibitors [26]. The analysis indicated

361 low androgen (DHEAS and testosterone) as well as cortisol production under the abiraterone

362 treatment. Moreover, the steroid profiling, which provided the CYP21A2 activity as a ratio of

363 17OHP and 11 DOC conversion, showed complete inhibition of CYP21A2 in case of 1μM

364 concentration of abiraterone [26]. In our current study, different concentrations of abiraterone

365 were used to treat the NCI-H295R adrenal carcinoma cell line. Abiraterone caused a dose

366 dependent reduction of CYP21A2 activity. A significantly lower CYP21A2 activity was

367 observed at 0.03 μM and higher concentrations of abiraterone with an IC_{50} value of 25 nM. A

368 whole cell steroid analysis performed to observe the global changes in steroid patterns upon

369 abiraterone treatment showed a wide range of altered steroid metabolites (Table 1). In

370 addition to testosterone and dehydroepiandrosterone reduction as expected, we saw changes

371 in corticosterone and cortisol metabolites by abiraterone treatment (Figure 6). Based on these

372 results, we can conclude that abiraterone causes a complex pattern of changes in steroid

373 metabolites due to its inhibition of CYP21A2 activities in addition to inhibition of 17α-

374 hydroxylase and 17,20 lyase activities of CYP17A1.

375

376 Further studies were performed to elucidate the binding of abiraterone to CYP21A2. The

377 computational docking of abiraterone into the CYP21A2 crystal structure showed that

378 abiraterone binds closer to the central heme of CYP21A2, which was similar to the binding of

379 abiraterone to the CYP17A1, as observed in crystal structures and spectral binding studies

380 [54, 59]. The predicted model of CYP21A2 and abiraterone binding was confirmed by

381 spectral binding analysis. A complex formation between abiraterone and CYP21A2 was

382 observed as a type II spectral shift upon displacement of a water molecule by the coordination

383 of a nitrogen-containing ligand to the P450 heme iron. Binding of steroid substrates to

384 CYP21A2 results in a type I P450 spectra, indicating the effects of substrate binding on the

385 heme iron spin state equilibrium [54]. In our study, the K_d Value of the complex of bacterially

386 produced recombinant CYP21A2 with abiraterone was 6.3 ± 0.2 μM indicating a strong

387 affinity. Further experiments were then performed to detect the pattern of enzymatic

388 inhibition of CYP21A2 by abiraterone. A Dixon plot of enzymatic analysis using variable

389 concentrations of substrate as well as inhibitor revealed a competitive / simple mixed

390 inhibition pattern for inhibition of CYP21A2 by abiraterone.

391

392 Our studies provide the detailed analysis of the inhibitory effects of abiraterone on CYP21A2

393 activity. These results indicate that the cortisol production in patients with CRPC who are

394 treated with abiraterone may be affected not only by the inhibition of CYP17A1 but also by

395 the CYP21A2 inhibition. In addition, use of abiraterone in non-cancerous hyperandrogenic

396 disorders like polycystic ovary syndrome requires further caution as inhibition of both the

397 CYP17A1 as well as the CYP21A2, may potentially result in complications associated with

398 lower cortisol levels.

399

5 Conflict of Interest

401 *The authors declare that the research was conducted in the absence of any commercial or*

402 *financial relationships that could be construed as a potential conflict of interest.*

6 Author Contributions

404 Participated in research design: Malikova, Brixius-Anderko, Udhane, Parween, Dick,
405 Bernhardt, Pandey
406 Conducted experiments: Malikova, Brixius-Anderko, Udhane, Dick, Pandey
407 Contributed new reagents or analytical tools: Bernhardt
408 Performed data analysis: Malikova, Brixius-Anderko, Udhane, Parween, Dick, Bernhardt,
409 Pandey
410 Overall supervision of the project: Pandey
411 Wrote or contributed the writing of the manuscript: Malikova, Brixius-Anderko, Udhane,
412 Parween, Dick, Bernhardt, Pandey

9 References

[1]   C.E. Flück, A.V. Pandey, Testicular Steroidogenesis, in: M. Simoni, I. Huhtaniemi (Eds.), Endocrinology of the Testis and Male Reproduction, Springer International Publishing, Cham, 2017. pp. 1-29.

[2]   C.E. Flück, A.V. Pandey, Steroidogenesis of the testis -- new genes and pathways, Ann Endocrinol (Paris) 75(2) (2014) 40-47.

[3]   B.C. Chung, K.J. Matteson, R. Voutilainen, T.K. Mohandas, W.L. Miller, Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta, Proc Natl Acad Sci U S A 83(23) (1986) 8962-8966.

[4]   S. Nakajin, J.E. Shively, P.M. Yuan, P.F. Hall, Microsomal cytochrome P-450 from neonatal pig testis: two enzymatic activities (17 alpha-hydroxylase and c17,20-lyase) associated with one protein, Biochemistry 20(14) (1981) 4037-4042.

[5]   B.C. Chung, J. Picado-Leonard, M. Haniu, M. Bienkowski, P.F. Hall, J.E. Shively, W.L. Miller, Cytochrome P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues, Proc Natl Acad Sci U S A 84(2) (1987) 407-411.

[6]   T. Sakaki, M. Akiyoshi-Shibata, Y. Yabusaki, K. Manabe, H. Murakami, H. Ohkawa, Progesterone metabolism in recombinant yeast simultaneously expressing bovine cytochromes P450c17 (CYP17A1) and P450c21 (CYP21B1) and yeast NADPH-P450 oxidoreductase, Pharmacogenetics 1(2) (1991) 86-93.

[7]   T. Yamazaki, K. Nawa, S. Kominami, S. Takemori, Cytochrome P-450(17 alpha,lyase)-mediating pathway of androgen synthesis in bovine adrenocortical cultured cells, Biochim Biophys Acta 1134(2) (1992) 143-148.

[8]   W.L. Miller, R.J. Auchus, The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders, Endocr Rev 32(1) (2011) 81-151.

[9]   M.X. Zuber, E.R. Simpson, M.R. Waterman, Expression of bovine 17 alpha-hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS 1) cells, Science 234(4781) (1986) 1258-1261.

[10]   S. Nakajin, M. Shinoda, M. Haniu, J.E. Shively, P.F. Hall, C21 steroid side chain cleavage enzyme from porcine adrenal microsomes. Purification and characterization of the

17

455 17 alpha-hydroxylase/C17,20-lyase cytochrome P-450, J Biol Chem 259(6) (1984) 3971-
456 3976.
457 [11]   J. Lindholm, S. Juul, J.O. Jorgensen, J. Astrup, P. Bjerre, U. Feldt-Rasmussen, C.
458 Hagen, J. Jorgensen, M. Kosteljanetz, L. Kristensen, P. Laurberg, K. Schmidt, J. Weeke,
459 Incidence and late prognosis of cushing's syndrome: a population-based study, J Clin
460 Endocrinol Metab 86(1) (2001) 117-123.
461 [12]   O.M. Dekkers, E. Horváth-Puhó, J.O.L. Jørgensen, S.C. Cannegieter, V. Ehrenstein,
462 J.P. Vandenbroucke, A.M. Pereira, H.T. Sørensen, Multisystem Morbidity and Mortality in
463 Cushing's Syndrome: A Cohort Study, The Journal of Clinical Endocrinology & Metabolism
464 98(6) (2013) 2277-2284.
465 [13]   G. Öcal, M. Berberoğlu, Z. Sıklar, Z. Aycan, B. Hacıhamdioglu, Ş.S. Erdeve, E.
466 Çamtosun, P. Kocaay, H.I. Ruhi, B.G. Kılıç, A. Tukun, Clinical Review of 95 Patients with
467 46,XX Disorders of Sex Development Based on the New Chicago Classification, Journal of
468 Pediatric and Adolescent Gynecology 28(1) (2015) 6-11.
469 [14]   C.E. Flück, M. Meyer-Boni, A.V. Pandey, P. Kempna, W.L. Miller, E.J. Schoenle, A.
470 Biason-Lauber, Why boys will be boys: two pathways of fetal testicular androgen
471 biosynthesis are needed for male sexual differentiation, Am J Hum Genet 89(2) (2011) 201-
472 218.
473 [15]   E. Carmina, F. Rosato, A. Jannì, M. Rizzo, R.A. Longo, Relative Prevalence of
474 Different Androgen Excess Disorders in 950 Women Referred because of Clinical
475 Hyperandrogenism, The Journal of Clinical Endocrinology & Metabolism 91(1) (2006) 2-6.
476 [16]   A. Kumar, K.S. Woods, A.A. Bartolucci, R. Azziz, Prevalence of adrenal androgen
477 excess in patients with the polycystic ovary syndrome (PCOS), Clinical Endocrinology 62(6)
478 (2005) 644-649.
479 [17]   R. Azziz, L.A. Sanchez, E.S. Knochenhauer, C. Moran, J. Lazenby, K.C. Stephens, K.
480 Taylor, L.R. Boots, Androgen Excess in Women: Experience with Over 1000 Consecutive
481 Patients, The Journal of Clinical Endocrinology & Metabolism 89(2) (2004) 453-462.
482 [18]   N. Krone, W. Arlt, Genetics of congenital adrenal hyperplasia, Best Practice &
483 Research Clinical Endocrinology & Metabolism 23(2) (2009) 181-192.
484 [19]   E.N. Gönç, Z.A. Özön, M.D. Çakır, A. Alikaşifoğlu, N. Kandemir, Need for
485 Comprehensive Hormonal Workup in the Management of Adrenocortical Tumors in Children,
486 Journal of Clinical Research in Pediatric Endocrinology 6(2) (2014) 68-73.
487 [20]   M. Fleseriu, F. Castinetti, Updates on the role of adrenal steroidogenesis inhibitors in
488 Cushing's syndrome: a focus on novel therapies, Pituitary 19(6) (2016) 643-653.
489 [21]   X. Bertagna, R. Pivonello, M. Fleseriu, Y. Zhang, P. Robinson, A. Taylor, C.E.
490 Watson, M. Maldonado, A.H. Hamrahian, M. Boscaro, B.M.K. Biller, LCI699, a Potent 11β-
491 hydroxylase Inhibitor, Normalizes Urinary Cortisol in Patients With Cushing's Disease:
492 Results From a Multicenter, Proof-of-Concept Study, The Journal of Clinical Endocrinology
493 & Metabolism 99(4) (2014) 1375-1383.
494 [22]   M. Fleseriu, R. Pivonello, J. Young, A.H. Hamrahian, M.E. Molitch, C. Shimizu, T.
495 Tanaka, A. Shimatsu, T. White, A. Hilliard, C. Tian, N. Sauter, B.M.K. Biller, X. Bertagna,
496 Osilodrostat, a potent oral 11β-hydroxylase inhibitor: 22-week, prospective, Phase II study in
497 Cushing's disease, Pituitary 19 (2016) 138-148.
498 [23]   B. Montgomery, M.A. Eisenberger, M.B. Rettig, F. Chu, R. Pili, J.J. Stephenson, N.J.
499 Vogelzang, A.J. Koletsky, L.T. Nordquist, W.J. Edenfield, K. Mamlouk, K.J. Ferrante, M.-E.
500 Taplin, Androgen Receptor Modulation Optimized for Response (ARMOR) Phase I and II
501 Studies: Galeterone for the Treatment of Castration-Resistant Prostate Cancer, Clinical
502 Cancer Research 22(6) (2016) 1356-1363.
503 [24]   K. Fizazi, R. Jones, S. Oudard, E. Efstathiou, F. Saad, R.d. Wit, J.D. Bono, F.M. Cruz,
504 G. Fountzilas, A. Ulys, F. Carcano, N. Agarwal, D. Agus, J. Bellmunt, D.P. Petrylak, S.-Y.

Lee, I.J. Webb, B. Tejura, N. Borgstein, R. Dreicer, Phase III, Randomized, Double-Blind, Multicenter Trial Comparing Orteronel (TAK-700) Plus Prednisone With Placebo Plus Prednisone in Patients With Metastatic Castration-Resistant Prostate Cancer That Has Progressed During or After Docetaxel-Based Therapy: ELM-PC 5, Journal of Clinical Oncology 33(7) (2015) 723-731.

[25]   T. Kaku, T. Hitaka, A. Ojida, N. Matsunaga, M. Adachi, T. Tanaka, T. Hara, M. Yamaoka, M. Kusaka, T. Okuda, S. Asahi, S. Furuya, A. Tasaka, Discovery of orteronel (TAK-700), a naphthylmethylimidazole derivative, as a highly selective 17,20-lyase inhibitor with potential utility in the treatment of prostate cancer, Bioorganic & medicinal chemistry 19(21) (2011) 6383-6399.

[26]   S.S. Udhane, B. Dick, Q. Hu, R.W. Hartmann, A.V. Pandey, Specificity of anti-prostate cancer CYP17A1 inhibitors on androgen biosynthesis, Biochem Biophys Res Commun 477(4) (2016) 1005-1010.

[27]   I. Schuster, R. Bernhardt, Inhibition of cytochromes p450: existing and new promising therapeutic targets, Drug Metab Rev 39(2-3) (2007) 481-499.

[28]   T. Hakki, R. Bernhardt, CYP17- and CYP11B-dependent steroid hydroxylases as drug development targets, Pharmacol Ther 111(1) (2006) 27-52.

[29]   B.A. Gartrell, F. Saad, Abiraterone in the management of castration-resistant prostate cancer prior to chemotherapy, Therapeutic advances in urology 7(4) (2015) 194-202.

[30]   L. Ramudo Cela, J. Balea Filgueiras, J.R. Vizoso Hermida, I. Martin Herranz, Study of cases of abiraterone discontinuation due to toxicity in pre-chemotherapy after 1 year's experience, J Oncol Pharm Pract (2016).

[31]   Z. Li, M. Alyamani, J. Li, K. Rogacki, M. Abazeed, S.K. Upadhyay, S.P. Balk, M.-E. Taplin, R.J. Auchus, N. Sharifi, Redirecting abiraterone metabolism to fine-tune prostate cancer anti-androgen therapy, Nature 533(7604) (2016) 547-551.

[32]   S. Bonomo, C.H. Hansen, E.M. Petrunak, E.E. Scott, B. Styrishave, F.S. Jorgensen, L. Olsen, Promising Tools in Prostate Cancer Research: Selective Non-Steroidal Cytochrome P450 17A1 Inhibitors, Sci Rep 6 (2016) 29468.

[33]   N. Sharifi, Prostate cancer: CYP17A1 inhibitor failure-lessons for future drug development, Nature reviews. Urology 12(5) (2015) 245-246.

[34]   A.V. Pandey, C.E. Flück, NADPH P450 oxidoreductase: structure, function, and pathology of diseases, Pharmacol Ther 138(2) (2013) 229-254.

[35]   P.W. Speiser, R. Azziz, L.S. Baskin, L. Ghizzoni, T.W. Hensle, D.P. Merke, H.F.L. Meyer-Bahlburg, W.L. Miller, V.M. Montori, S.E. Oberfield, M. Ritzen, P.C. White, Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline, The Journal of Clinical Endocrinology & Metabolism 95(9) (2010) 4133-4160.

[36]   M. Janner, A.V. Pandey, P.E. Mullis, C.E. Flück, Clinical and biochemical description of a novel CYP21A2 gene mutation 962_963insA using a new 3D model for the P450c21 protein, Eur J Endocrinol 155(1) (2006) 143-151.

[37]   S. Brixius-Anderko, L. Schiffer, F. Hannemann, B. Janocha, R. Bernhardt, A CYP21A2 based whole-cell system in Escherichia coli for the biotechnological production of premedrol, Microb Cell Fact 14 (2015) 135.

[38]   L. Schiffer, S. Brixius-Anderko, F. Hannemann, J. Zapp, J. Neunzig, M. Thevis, R. Bernhardt, Metabolism of Oral Turinabol by Human Steroid Hormone-Synthesizing Cytochrome P450 Enzymes, Drug Metab Dispos 44(2) (2016) 227-237.

[39]   L.H. Zhang, H. Rodriguez, S. Ohno, W.L. Miller, Serine phosphorylation of human P450c17 increases 17,20-lyase activity: implications for adrenarche and the polycystic ovary syndrome, Proc Natl Acad Sci U S A 92(23) (1995) 10619-10623.

554 [40] A.V. Pandey, S.H. Mellon, W.L. Miller, Protein phosphatase 2A and phosphoprotein
555 SET regulate androgen production by P450c17, J Biol Chem 278(5) (2003) 2837-2844.
556 [41] A.V. Pandey, W.L. Miller, Regulation of 17,20 lyase activity by cytochrome b5 and
557 by serine phosphorylation of P450c17, J Biol Chem 280(14) (2005) 13265-13271.
558 [42] S. Parween, F. Roucher-Boulez, C.E. Flück, A. Lienhardt-Roussie, D. Mallet, Y.
559 Morel, A.V. Pandey, P450 Oxidoreductase Deficiency: Loss of Activity Caused by Protein
560 Instability From a Novel L374H Mutation, J Clin Endocrinol Metab 101(12) (2016) 4789-
561 4798.
562 [43] L. Michaelis, M.L. Menten, Die Kinetik der Invertinwirkung, Biochem Z 49 (1913)
563 333-369.
564 [44] M. Dixon, The determination of enzyme inhibitor constants, Biochem J 55(1) (1953)
565 170-171.
566 [45] C. Quattropani, B. Vogt, A. Odermatt, B. Dick, B.M. Frey, F.J. Frey, Reduced activity
567 of 11 beta-hydroxysteroid dehydrogenase in patients with cholestasis, J Clin Invest 108(9)
568 (2001) 1299-1305.
569 [46] C.H. Shackleton, Mass spectrometry in the diagnosis of steroid-related disorders and
570 in hypertension research, J Steroid Biochem Mol Biol 45(1-3) (1993) 127-140.
571 [47] E. Krieger, T. Darden, S.B. Nabuurs, A. Finkelstein, G. Vriend, Making optimal use
572 of empirical energy functions: force-field parameterization in crystal space, Proteins 57(4)
573 (2004) 678-683.
574 [48] G. Vriend, WHAT IF: a molecular modeling and drug design program, J Mol Graph
575 8(1) (1990) 52-56, 29.
576 [49] Y. Duan, C. Wu, S. Chowdhury, M.C. Lee, G. Xiong, W. Zhang, R. Yang, P. Cieplak,
577 R. Luo, T. Lee, J. Caldwell, J. Wang, P. Kollman, A point-charge force field for molecular
578 mechanics simulations of proteins based on condensed-phase quantum mechanical
579 calculations, Journal of Computational Chemistry 24(16) (2003) 1999-2012.
580 [50] W.L. Jorgensen, J. Tirado-Rives, Potential energy functions for atomic-level
581 simulations of water and organic and biomolecular systems, Proc Natl Acad Sci U S A
582 102(19) (2005) 6665-6670.
583 [51] O. Trott, A.J. Olson, AutoDock Vina: Improving the speed and accuracy of docking
584 with a new scoring function, efficient optimization, and multithreading, Journal of
585 Computational Chemistry 31(2) (2010) 455-461.
586 [52] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K1) and the
587 concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction,
588 Biochem Pharmacol 22(23) (1973) 3099-3108.
589 [53] B.T. Burlingham, T.S. Widlanski, An intuitive look at the relationship of K-i and
590 IC50: A more general use for the Dixon plot, J Chem Educ 80(2) (2003) 214-218.
591 [54] P.S. Pallan, L.D. Nagy, L. Lei, E. Gonzalez, V.M. Kramlinger, C.M. Azumaya, Z.
592 Wawrzak, M.R. Waterman, F.P. Guengerich, M. Egli, Structural and kinetic basis of steroid
593 17alpha,20-lyase activity in teleost fish cytochrome P450 17A1 and its absence in cytochrome
594 P450 17A2, J Biol Chem 290(6) (2015) 3248-3268.
595 [55] G. Attard, A.H. Reid, T.A. Yap, F. Raynaud, M. Dowsett, S. Settatree, M. Barrett, C.
596 Parker, V. Martins, E. Folkerd, J. Clark, C.S. Cooper, S.B. Kaye, D. Dearnaley, G. Lee, J.S.
597 de Bono, Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms
598 that castration-resistant prostate cancer commonly remains hormone driven, Journal of
599 clinical oncology : official journal of the American Society of Clinical Oncology 26(28)
600 (2008) 4563-4571.
601 [56] R. Li, K. Evaul, K.K. Sharma, K.H. Chang, J. Yoshimoto, J. Liu, R.J. Auchus, N.
602 Sharifi, Abiraterone inhibits 3beta-hydroxysteroid dehydrogenase: a rationale for increasing

20

drug exposure in castration-resistant prostate cancer, Clinical cancer research : an official journal of the American Association for Cancer Research 18(13) (2012) 3571-3579.

[57]   H.S. Soifer, N. Souleimanian, S. Wu, A.M. Voskresenskiy, F.K. Collak, B. Cinar, C.A. Stein, Direct regulation of androgen receptor activity by potent CYP17 inhibitors in prostate cancer cells, J Biol Chem 287(6) (2012) 3777-3787.

[58]   J. Richards, A.C. Lim, C.W. Hay, A.E. Taylor, A. Wingate, K. Nowakowska, C. Pezaro, S. Carreira, J. Goodall, W. Arlt, I.J. McEwan, J.S. de Bono, G. Attard, Interactions of abiraterone, eplerenone, and prednisolone with wild-type and mutant androgen receptor: a rationale for increasing abiraterone exposure or combining with MDV3100, Cancer Res 72(9) (2012) 2176-2182.

[59]   R. Yadav, E.M. Petrunak, D.F. Estrada, E.E. Scott, Structural insights into the function of steroidogenic cytochrome P450 17A1, Mol Cell Endocrinol 441 (2017) 68-75.

Tables

Table 1: Effect of abiraterone on steroid production by human adrenal cells.

Steroid Metabolite	Abbreviation	DMSO	Abiraterone
Androsterone	ANDRO	**0.390881**	**6.226052**
Etiocholanolone	ETIO	**0.109623**	**3.321062**
Androstenediol	5αAD3α17β	0.30697	0.465213
11-Oxo-Etiocholanolon	11-OXO-ETIO	N.A.	0.187621
11β-Hydroxy-Androsterone	11-OH-ANDRO	**0.188208**	**3.451147**
11β-Hydroxy-Etiocholanolone	11-OH-ETIO	**N.A.**	**2.134756**
Dehydroepiandrosterone	DHEA	**1.436706**	**0.144953**
5-Androstene-3β,17β-diol	5-AD-17β	0.340096	0.215782
16α-Hydroxy-DHEA	16α-OH-DHEA	2.154509	1.740921
5-Androstene-3β,16α,17β-triol	5-AT	0.234827	0.311715
5-Pregnene-3β, 16α,17β-triol	5-PT	1.371897	0.124343
Testosterone	TESTOSTERONE	**1.613952**	**0.413544**
5α-Dihydrotestosterone	5α-DIHYDROTEST	**0.182893**	**N.A.**
Estriol	ESTRIOL	0.07959	0.044161
17β-Estradiol	17β-ESTRADIOL	N.A.	0.027509
17-Hydroxypregnanolone	17-HP	**0.023376**	**1.030389**
Pregnanediol	PD	1.670818	0.715222
Pregnanetriol	PT	0.168326	3.385257
11-Deoxycortisol-Metabolite			
Tetrahydrosubstance S	THS	N.A.	0.263843
Corticosterone-Metabolite			
Tetrahydro DOC	THDOC	**2.087659**	**0.067438**
Tetrahydro dehydrocorticosterone	THA	N.A.	0.371549
Tetrahydrocorticosterone	THB	N.A.	0.780605
5α-Tetrahydrocorticosterone	5α-THB	**N.A.**	**1.132725**
18-Hydroxy-tetrahydrocompound A	18-OH-THA	N.A.	N.A.
Cortisol-Metabolite			
Cortison	CORTISONE	N.A.	0.5454
Tetrahydrocortison	THE	**0.192633**	**12.27504**
α-Cortolon	α-CORTOLONE	**0.006468**	**2.949182**
β-Cortolon	β-CORTOLONE	**N.A.**	**1.38639**
20α-Dihydrocortison	20α-DHE	N.A.	0.084738
20β-Dihydrocortison	20β-DHE	N.A.	0.195689
Cortisol	CORTISOL	**1.86521**	**0.747982**
Tetrahydrocortisol	THF	**0.095437**	**8.012946**
5α-Tetrahydrocortisol	5α-THF	**0.274516**	**7.184058**
α-Cortol	α-CORTOL	**N.A.**	**1.813959**
β-Cortol	β-CORTOL	**N.A.**	**1.653822**
20α-Dihydrocortisol	20α-DHF	0.315156	0.507297

Table 2: Computational binding energy, dissociation contacts and interacting residues for abiraterone binding with CYP21A2 compared to CYP17A1.

	Binding Energy (kcal/mol)	Dissociation constant (nM)	Contacting residues
CYP17A1 with PROG	10.6	14.66	ALA113 PHE114 **ASN202** ILE205 ILE206 LEU209 **ARG239** GLY297 ASP298 GLY301 ALA302 THR306 ALA367 ILE371 VAL482 VAL483 **HEME**
CYP21A2 with PROG	12.7	0.49	VAL101 ASP107 SER109 LEU110 VAL198 LEU199 **TRP202** LEU227 ILE231 **ARG234** MET284 VAL287 ASP288 ILE291 GLY292 THR296 VAL360 LEU364 VAL470 ILE471 **HEME**
CYP17A1 with Abiraterone	12.5	0.69	ALA113 PHE114 TYR201 **ASN202** ILE205 ILE206 LEU209 **ARG239** GLY297 ASP298 GLY301 ALA302 **GLU305** THR306 **VAL366** ALA367 LEU370 ILE371 VAL482 VAL483 **HEME**
CYP21A2 with Abiraterone	13.2	0.20	VAL101 ASP107 SER109 LEU110 VAL198 LEU199 **TRP202** LEU227 ILE231 **ARG234** MET284 VAL287 ASP288 ILE291 GLY292 THR296 VAL359 VAL360 LEU364 VAL470 ILE471 **HEME**

Figure legends

Figure 1. Pathway of steroid hormone production in humans with of **roles of CYP21A2 and CYP17A1 in humans.** Cholesterol is transported to mitochondrion by steroidogenic acute regulatory protein (StAR), where CYP11A1 converts it to pregnenolone. The pregnenolone metabolized in the endoplasmic reticulum to 17OHPreg, DHEA by CYP17A1 and androstenedione or androstenediol and this process continues to production of testosterone. The alternate pathway of steroid metabolism proceeds from 17OHPreg to 17OHProg, 17OH-DHP, 17OH-Allo, androsterone, androstanediol (A'diol) and then to DHT in the testis. DHEA is converted to androstenedione and then to testosterone, which is further metabolized to estrogens.

Abbreviations: CYP11A1 (P450scc, cholesterol side-chain cleavage enzyme), **StAR** (steroidogenic acute regulatory protein), **FDX1**, Adrenodoxin; **FDXR**, NADPH adrenodoxin oxidoreductase; **CYP17A1** (P450c17, 17α-hydroxylase/17,20-lyase), **HSD3B2** (3βHSD2, 3β-hydroxysteroid dehydrogenase, type 2), **CYB5**, cytochrome b$_5$; **POR**, P450 oxidoreductase; **HSD17B3** (17βHSD3, 17β-hydroxysteroid dehydrogenase, type 3), and **SRD5A2** (5α-reductase, type 2). The alternative pathway has four additional enzymes: **SRD5A1** (5α-reductase, type 1); **AKR1C2** (Aldo-keto reductase 1C2, 3αHSD3) and **AKR1C4** Aldo-keto reductase 1C4, 3αHSD1) for reductive 3αHSD activity; and **HSD17B6** (17βHSD6, 17β-hydroxysteroid dehydrogenase, type 6) and/or **AKR1C2/4** for oxidative 3αHSD activity. Full steroid names: **17OHPreg**, 17-hydroxypregnenolone; **17OHProg**, 17-hydroxyprogesterone; **17OH-DHP**, 17-hydroxydihydroprogesterone (5α-pregnan-3α,17α-ol-20-one); **17OH-Allo**, 17-hydroxyallopregnanolone (5α-pregnan-3α,17α-diol-20-one; P'diol); **DHEA**, dehydroepiandrosterone.

657 **Figure 2.** Inhibition of CYP21A2 activity by abiraterone in human adrenal cells. The 21-

658 hydroxylase activity was monitored in H295R cells treated with control (DMSO) and 0.001 to

659 1 μM abiraterone with two different concentrations of the substrate for 24 h. CYP21A2

660 activities were measured by monitoring the conversion of [^{3}H] 17OH-PROG (17α-

661 hydroxyprogesterone) to 11-deoxycortisol. Data are presented as mean ± SD of three

662 independent experiments. **A.** A dose response curve showing the effect of increasing

663 concentrations of abiraterone on CYP21A2 activity. **B.** A Dixon plot for calculating the Ki

664 values of abiraterone for inhibition of CYP21A2 activity. A K_i value of 23 nM was obtained

665 for inhibition of CYP21A2 activity by abiraterone. 17OH-PROG, 17α-hydroxyprogesterone;

666 11-DOC, 11-deoxycortisol.

667

668 **Figure 3.** Computational docking of abiraterone into the human CYP21A2 crystal structure.

669 **A.** Abiraterone docking into CYP21A2 structure. Abiraterone was docked into the structure of

670 CYP21A2 and found to bind like its native substrate, progesterone. **B.** A close up of

671 abiraterone docked into CYP21A2 compared to its substrate. **C.** Abiraterone binds to heme

672 through nitrogen-iron co-ordination. **D.** A close up of abiraterone bound to CYP17A1.

673 Binding of abiraterone to CYP21A2 is similar to CYP17A1. These data are in line with our

674 findings of the inhibitory effects of abiraterone on CYP21A2 in addition to inhibition of

675 CYP17A1 activities.

676

677 **Figure 4.** Binding spectra of abiraterone with CYP21A2. To confirm the inhibition in cell

678 experiments and computational binding, we carried out spectral binding analysis for the

679 interaction of abiraterone with CYP21A2. Titration of CYP21A2 with increasing

680 concentrations of abiraterone showed a type II shift indicating nitrogen-iron complex as

681 indicated by an absorption decrease at 410 nm and an increase at 424 nm (inset). The

25

682   difference of the absorbance maximum and minimum plotted against the respective ligand

683   concentration of each titrating step results in a hyperbolic regression curve revealing a K_d

684   value of 6.3 ± 0.2 μM.

685

686   **Figure 5.** Inhibition of recombinant human CYP21A2 activity by abiraterone. To further

687   verify the results obtained from cell experiments, we used recombinant bacterially expressed

688   CYP21A2 for determining the inhibition parameters of abiraterone on CYP21A2. A Dixon

689   Plot (1/v vs inhibitor concentration) at three different concentrations of substrate is shown.

690   Abiraterone inhibited the recombinant CYP21A2 activity with an estimated Ki value of 2.26

691   μM.

692

693   **Figure 6.** A schematic representation of the effect of abiraterone on steroidogenesis. Steroid

694   metabolites changes are based on data in Table 1.

Figure 1
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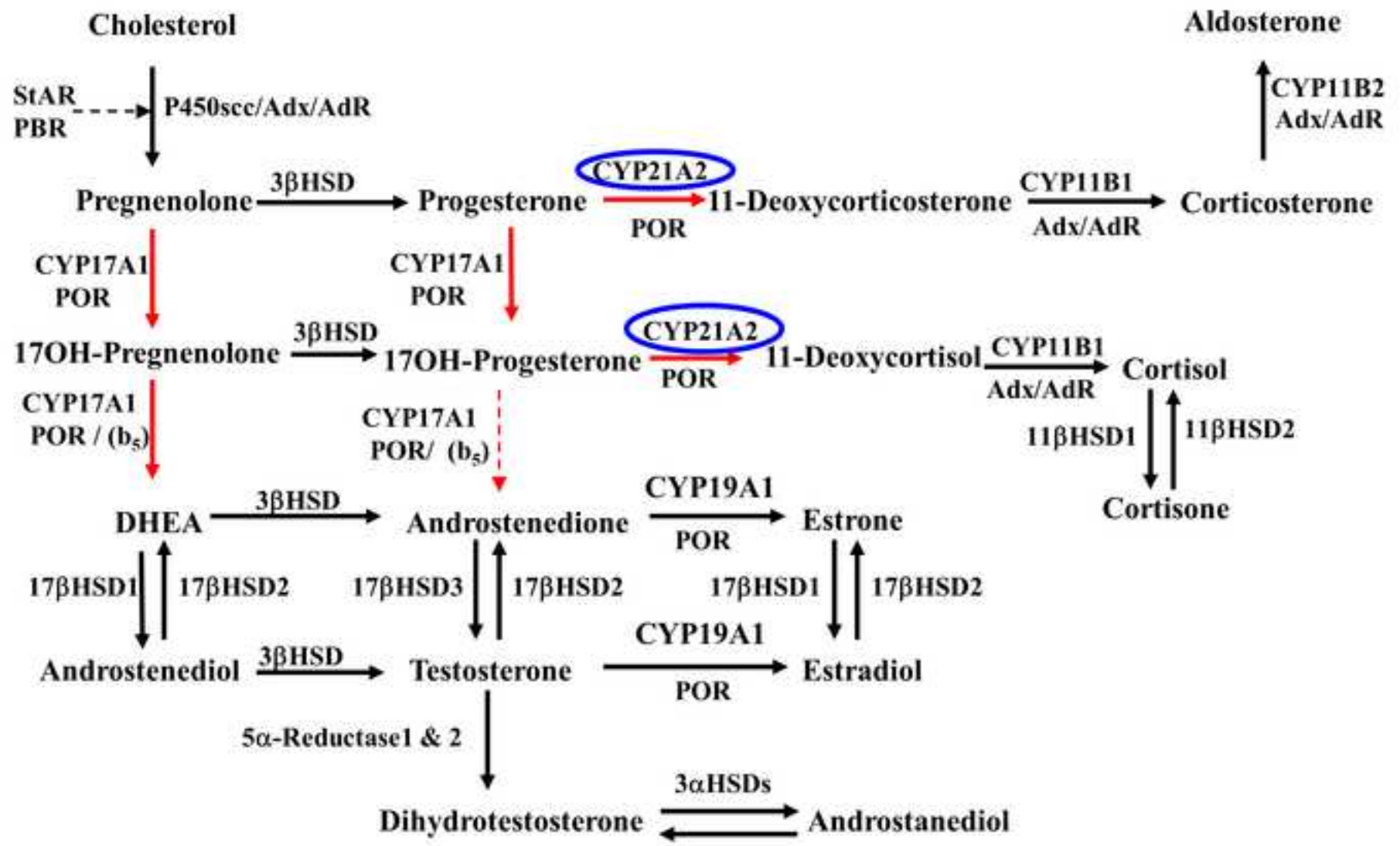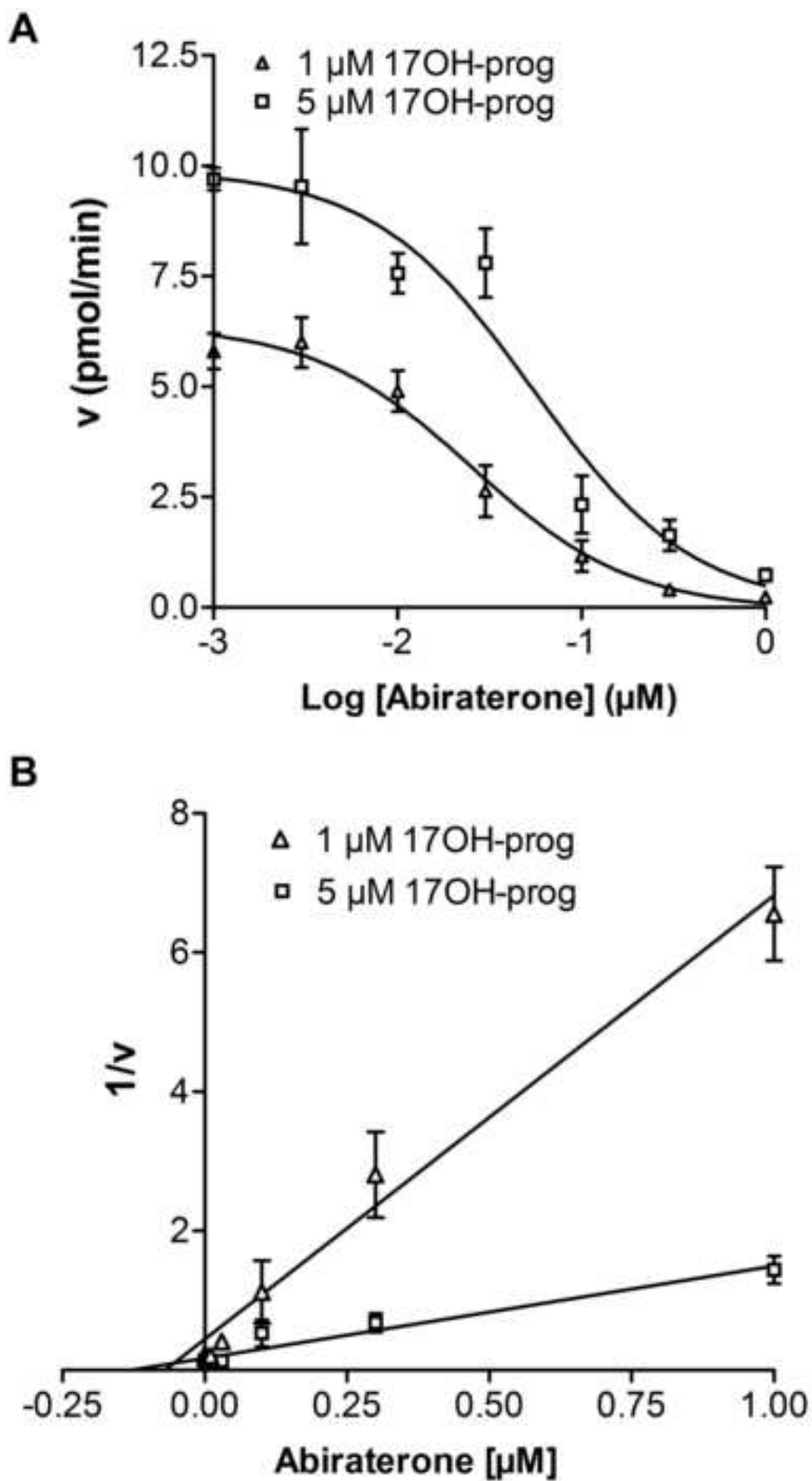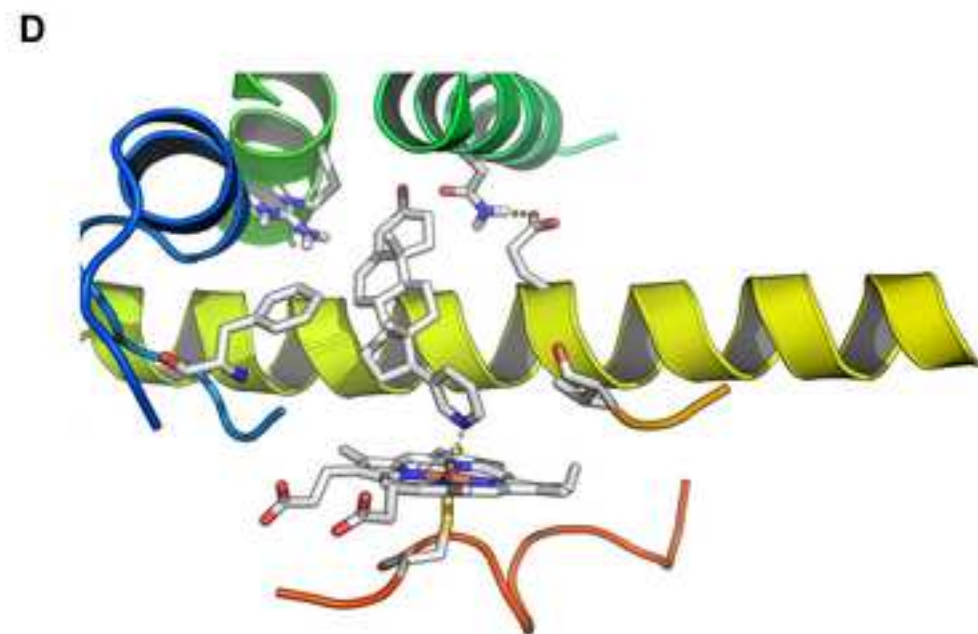
Figure 2
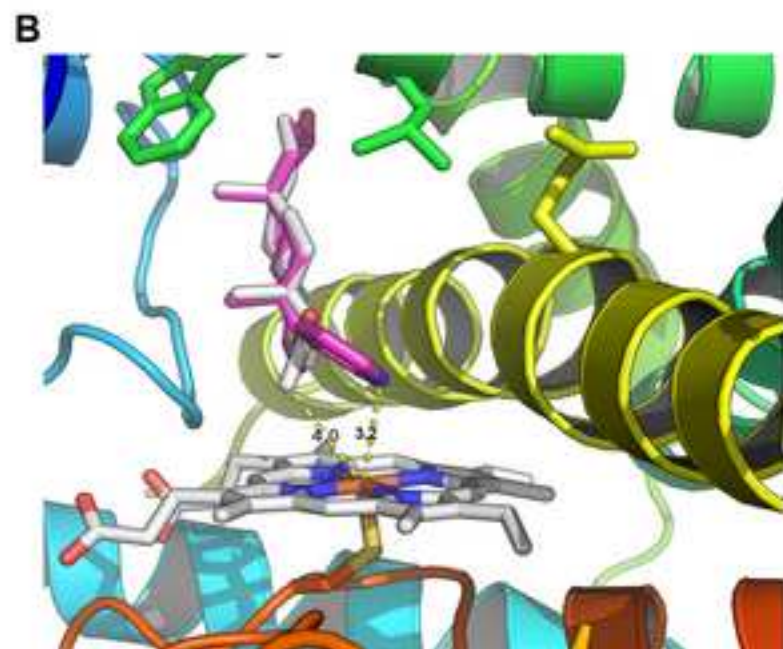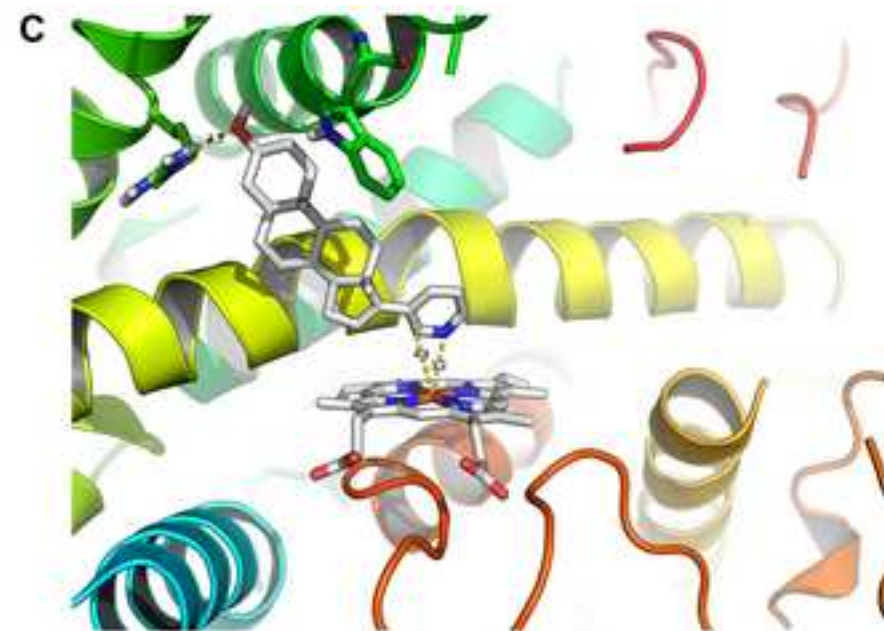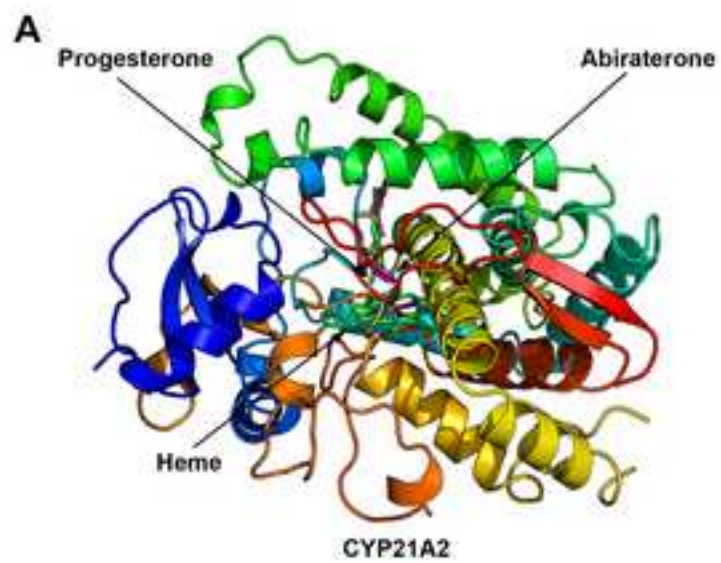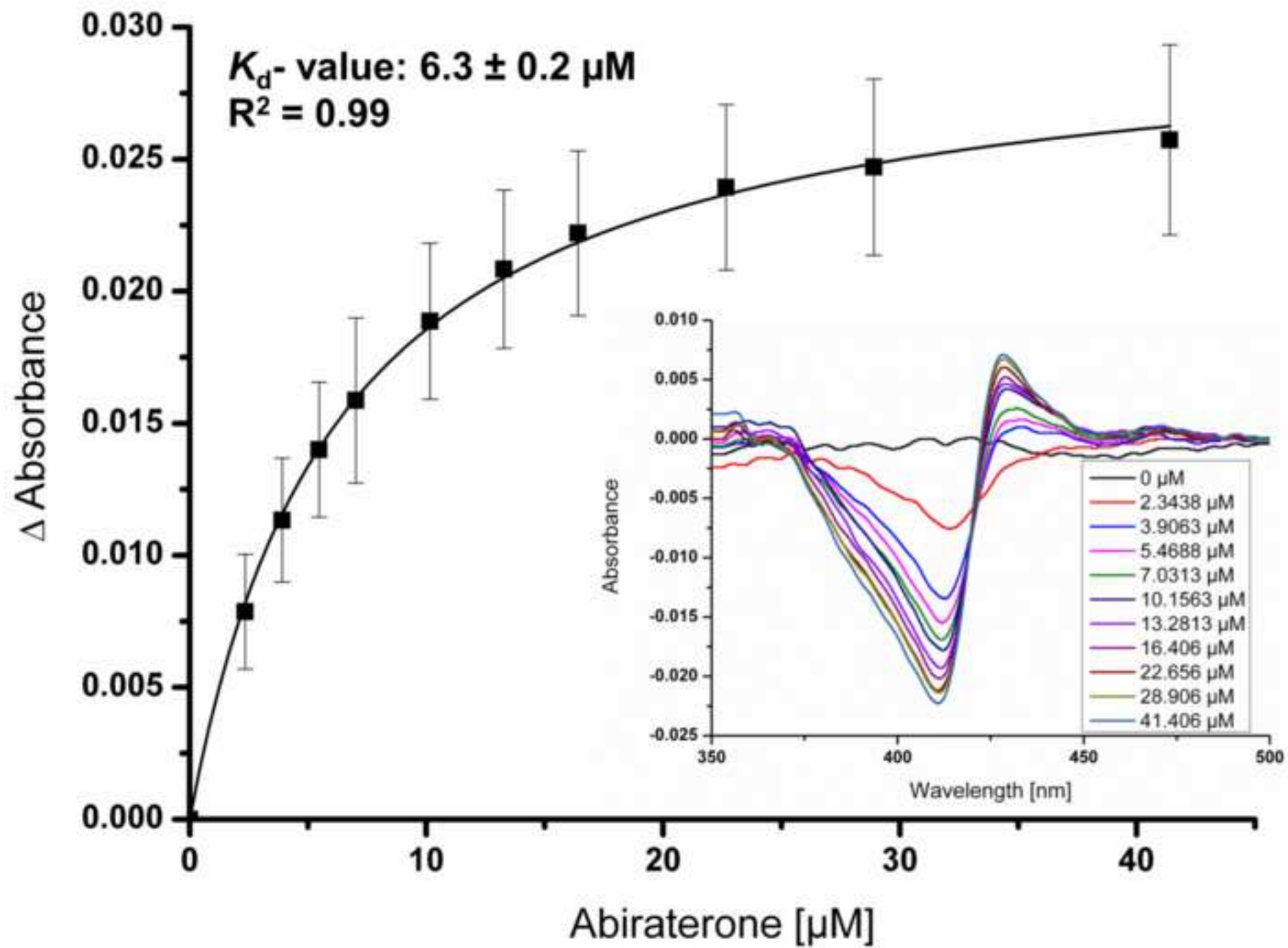**Click here to download high resolution image**

Figure 4
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Figure 4

K_d- value: 6.3 ± 0.2 µM
R^2 = 0.99

Figure 5

Figure 6
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