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Specificity of anti-prostate cancer CYP17A1 inhibitors on androgen biosynthesis

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ABSTRACT

The orteronel, abiraterone and galeterone, which were developed to treat castration resistant prostate cancer, inhibit 17,20 lyase activity but little is known about their effects on adrenal androgen biosynthesis. We studied the effect of several inhibitors and found that orteronel was selective towards 17,20 lyase activity than abiraterone and galeterone. Gene expression analysis showed that galeterone altered the expression of HSD3B2 but orteronel did not change the expression of HSD3B2, CYP17A1 and AKR1C3. The CYP19A1 activity was not inhibited except by compound IV which lowered activity by 23%. Surprisingly abiraterone caused complete blockade of CYP21A2 activity. Analysis of steroid metabolome by gas chromatography – mass spectrometry revealed changes in steroid levels caused by different inhibitors. We can conclude that orteronel is a highly specific inhibitor of 17,20 lyase activity. The discovery of these specific drug actions on steroidogenic enzyme activities would be valuable for understanding the regulation of androgens.

1. Introduction

Androgens are essential for sexual differentiation and reproduction in both male and female. Androgen production in humans occurs mainly in the testis, ovaries and the adrenal glands. The initial steps of steroidogenesis in both the adrenal and the gonads are same and use similar enzymes for steroid biosynthesis pathways [1]. Both the adrenal and the ovary produce dehydroepiandrosterone (DHEA) as the key precursor of androgens and estrogens. Premature adrenarche and polycystic ovary syndrome (PCOS) are common hyperandrogenic disorders. During adrenarche (functional activation of adrenal zona reticularis) production of adrenal androgens (DHEA and DHEA sulfate) is at a higher level [1]. In case of premature adrenarche, the concentration of circulating adrenal androgens increases at an earlier age. Several studies link premature adrenarche with post pubertal adrenal and ovarian hyperandrogenism [2,3]. Premature adrenarche is also linked to the hyperandrogenism in PCOS [4]. In hyperandrogenic women with PCOS, androgen production from both the ovaries and the adrenals is higher [5]. Changes in cytochrome P450 17A1 (P450c17, CYP17A1) activities could explain the increase in androgen secretion that causes hyperandrogenic disorders [1].

The CYP17A1 plays a vital role in regulating adrenal androgen production (Suppl Fig. 1). CYP17A1 is localized in the endoplasmic reticulum and responsible for 17α-hydroxylase and 17,20 lyase reactions. The 17,20 lyase activity regulates androgen production and is dependent on cytochrome P450 oxidoreductase (POR) [6], cytochrome b_5 (CYB5) [7] and phosphorylation [7,8]. Treatment of androgen dependent prostate cancer is by androgen deprivation therapy, androgen receptor (AR) antagonists or anti androgen agents and CYP17A1 inhibitors that block androgen production [9]. Hormonal therapy such as using a gonadotrophin hormonal analogue for treatment of hyperandrogenic disorders may not be

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specific enough to control the extra-gonadal and the gonadal androgen production. Drugs targeting androgen production are being used for treatment of prostate cancer and to decrease the androgen levels in hyperandrogenic women [10,11]. But anti androgen treatment can have side effects on sexuality of women with hyperandrogenism [12]. One example is flutamide, a non-steroidal anti androgen drug used to treat hyperandrogenic women [13–16]. Flutamide has many side effects like diarrhea, gynecomastia, muscle cramps, hematological alterations, and hepatotoxicity [17,18].

CYP17A1 inhibitors are designed for the treatment of the androgen dependent prostate cancer patients (Suppl Fig. 2). Abiraterone prolonged the survival of castration-resistant prostate cancer (CRPC) patients in phase III clinical trials [19]. Abiraterone also has modest affinity towards the androgen receptor [20]. In the hyperandrogenic disorders like premature or exaggerated adrenarche and PCOS, use of total CYP17A1 inhibitors will also block the 17α-hydroxylase activity. New inhibitors of CYP17A1, orteronel and galeterone were also designed for treatment of castration-resistant prostate cancer [21,22]. Effect of abiraterone, orteronel and galeterone on adrenal androgen production is not clear.

Here we are reporting the effects of CYP17A1 inhibitors on the adrenal androgen production. We compared CYP17A1 inhibitors orteronel, galeterone, abiraterone and recently reported compounds I and IV and studied the specificity of their action. We found that orteronel is a potent inhibitor of 17,20 lyase activity with no impact on other cytochrome P450 enzymes involved in steroid hormone biosynthesis. We also analyzed the impact of these inhibitors on the steroid metabolome, focusing on C19 steroids. Use of specific 17,20 lyase inhibitors can be a treatment option for hyperandrogenic disorders for the control of androgen production.

2. Materials and methods

2.1. Cell culture and treatment

Human adrenocortical NCI-H295R cells obtained from American Type Culture Collection (ATCC; CRL-2128) were cultivated in DMEM/Ham's F-12 medium with additives as described in supplementary material. Orteronel, galeterone and abiraterone were purchased from the Selleckchem (Houston, TX, USA). CYP17A1 inhibitors compound I and IV were described earlier [23].

2.2. Steroid profiling

Steroid profiling was performed with the cells grown on 6 well plates by adding 100,000 cpm [^{3}H] pregnenolone (preg) to the culture medium for 90 min. To study specific CYP17A1 enzyme activities, cells were first treated with 1 μM or 2 μM CYP17A1 inhibitors. After 24 h of inhibitor treatment, cells were treated with 1 μM trilostane (a specific blocker of HSD3B) for 90 min before adding [^{3}H] preg. For CYP21A2 activity, cells were treated with 1 μM of different inhibitors for 24 h and then labeled with [^{3}H] 17α-hydroxyprogesterone (17-OH Prog) for 60 min to monitor the conversion of 11-deoxycortisol. Steroids were extracted from cell supernatants and separated by thin layer chromatography (TLC) as previously described [24]. Steroid conversion was calculated as percentage of total radioactivity incorporated into specific products.

2.3. Quantitative real time PCR (qRT-PCR)

The H295R cells were treated with inhibitors for 72 h. After the treatment, total RNA was isolated and qRT-PCR was done as previously described [24]. Details are in supplementary material.

2.4. CYP19A1 enzyme activity using titrated water release assay

Human Placental JEG3 cells were grown on 12 well plates and treated with CYP17A1 inhibitors for 24 h before aromatase activity was assayed. Aromatase activity was assayed with ^{3}H labeled androstenedione ([1β-^{3}H(N)]-androstene-3,17-dione; ~100,000 cpm/well). Androstenedione was added to the treated cells for 6 h. Aromatase activity was then assessed by the titrated water release assay as previously described [25,26].

2.5. GC-MS measurement of steroid metabolites

The measurement of steroid hormone metabolites in human adrenal H295R cells was performed by gas chromatography – mass spectrometry (GC-MS) as previously described [27]. 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 of CYP17A1 inhibitors in medium without NU-I serum for 24 h. After 12 h of treatment, 1 μM of pregnenolone was added. At the end, 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.

3. Results and discussion

3.1. CYP17A1 inhibitors modulate the adrenal androgen production

CYP17A1 is required for androgen production in humans and control of its enzyme activity is essential for treatment of hyperandrogenic disorders. Abiraterone, galeterone and orteronel are inhibitors of CYP17A1 (Suppl Fig. 2). Abiraterone is an effective active site-directed inhibitor, designed for the treatment of CRPC patients to suppress the androgen production by inhibition of CYP17A1 activities [28,29]. The galeterone and orteronel were designed to selectively inhibit 17,20 lyase activity of CYP17A1 for the treatment of CRPC [21,22]. There have been suggestions that galeterone is more specific towards the 17,20 lyase activity of CYP17A1 than abiraterone but this effect is not clear [9,30]. Functional studies on the effects of orteronel *in vivo* (cynomolgus monkey) and *in vitro* (human adrenal tumor cells) showed that orteronel is a potent inhibitor of 17,20 lyase activity [31]. In addition to these well-known inhibitors, Hartmann group synthesized several inhibitors of steroid biosynthesis [23]. They observed compound IV was a more potent inhibitor of 17,20 lyase activity compared to its effects on 17α-hydroxylase activity [23]. However, the effect of these drugs on adrenal androgen production remains unknown.

We used the steroidogenic human adrenal cortex H295R cells to study the effect of CYP17A1 inhibitors (orteronel, galeterone, compound I and IV and abiraterone). We found that orteronel and galeterone drastically decreased the DHEA and androstenedione production (Fig. 1A). Compounds I and IV decreased DHEA, androstendione and 11-DOC/11-deoxycortisol production. In the cells treated with abiraterone, we only observed the conversion of preg to prog and other products in the pathway were completely blocked (Fig. 1B). This suggests that abiraterone inhibits CYP17A1 and possibly other enzymes that are involved in steroidogenesis.

3.2. Orteronel is a specific inhibitor of the CYP17A1 17,20 lyase activity

We studied the effect of CYP17A1 inhibitors in more detail for the specificity of the inhibition of CYP17A1 enzyme activities. Cells

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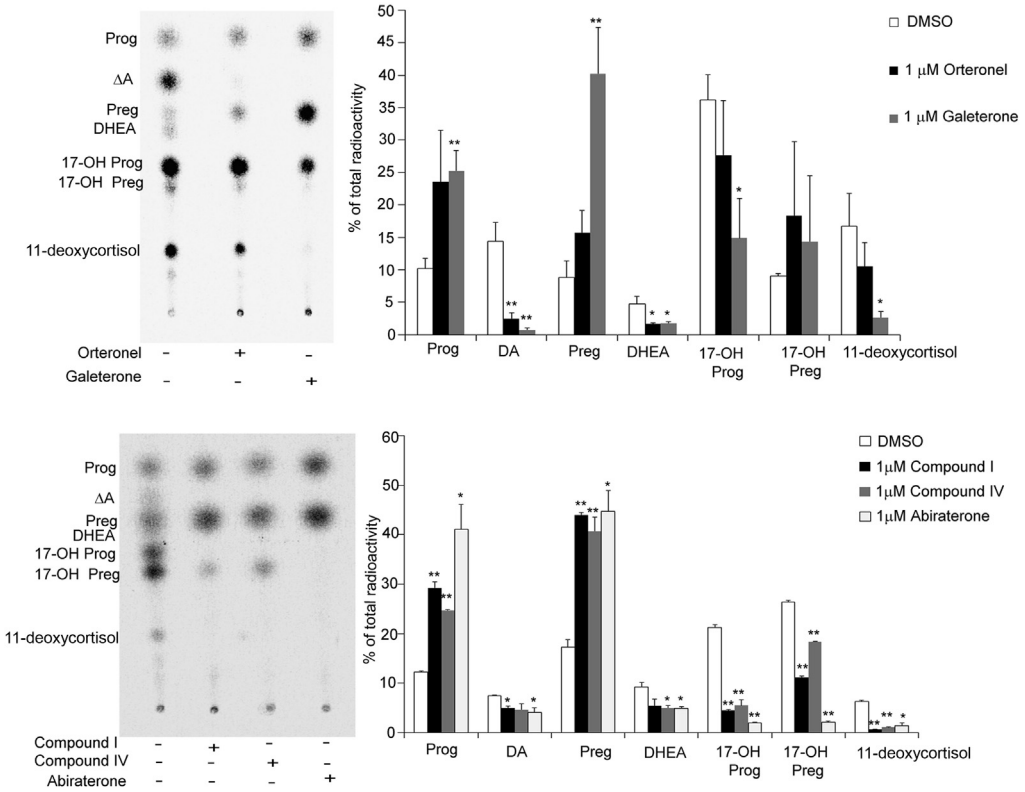

Fig. 1. Effect of the CYP17A1 inhibitors on adrenal steroid profile. For the steroid profiling H295R cells were treated with 1 μm orteronel, galeterone, compound I and IV and abiraterone for 24 h. Steroid production was labeled with [^{3}H] preg for 90 min. Steroids were extracted and resolved by TLC. A) Steroid profiling of adrenal H295R cells treated with DMSO and 1 μM of orteronel or galeterone B) Steroid profiling of adrenal H295R cells treated with 1 μM of compound I, compound IV and abiraterone. Representative TLCs are depicted on the left, summaries and quantifications of the results are given on the right. Prog, progesterone; Δ4A, androstenedione; Preg, pregnenolone; DHEA, dehydroepiandrosterone; 17-OH Preg, 17α-hydroxypregnenolone; 17-OH Prog, 17α-hydroxy progesterone; Data are presented as mean ± SD of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.

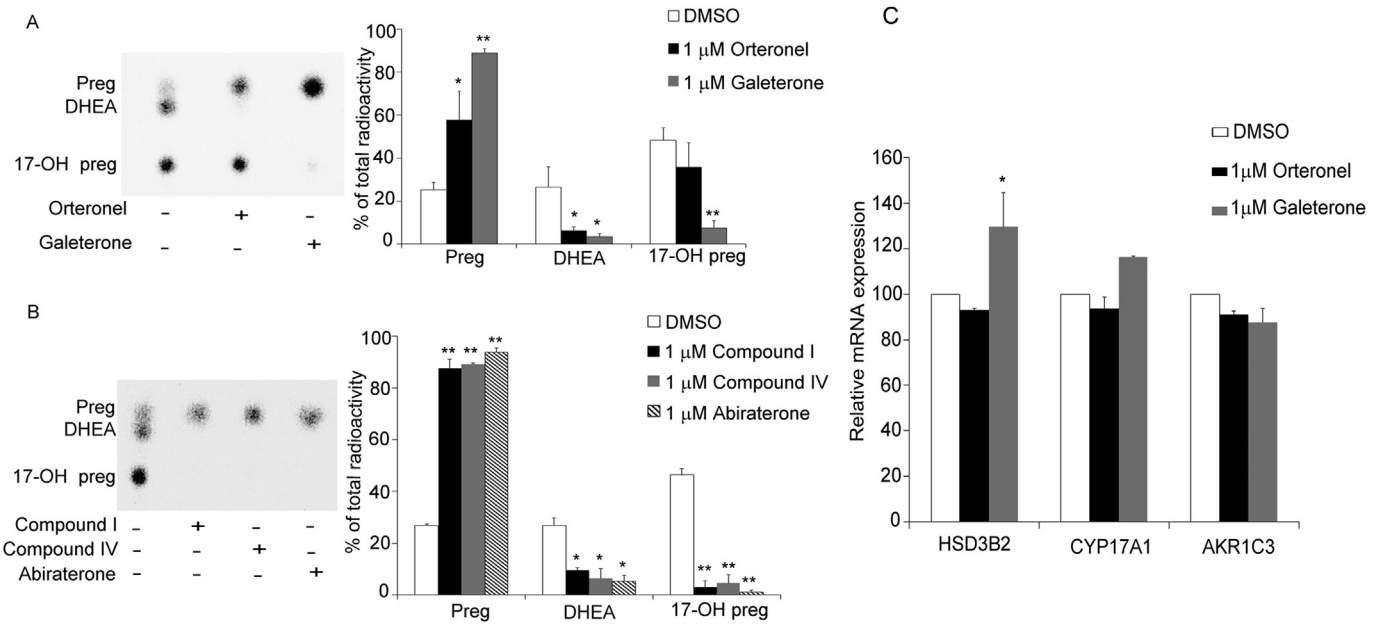

Fig. 2. Effects of CYP17A1 inhibitors on NCI-H295 cells. A and B . Effect of inhibitors on CYP17A1 enzyme activity. The enzyme activities of CYP17A1 were checked in H295R cells treated with control (DMSO) and 1 μM of orteronel, galeterone, compound I, compound IV and abiraterone. CYP17A1 activities were assessed by the conversion of [^{3}H] preg to 17-OH preg for 17α-hydroxylase activity and 17-OH preg to DHEA for 17,20 lyase activity. Representative TLCs are depicted on the left, summaries and quantifications of the results are given on the right. Data are given as mean ± SD of three independent experiments. Preg, pregnenolone; DHEA, dehydroepiandrosterone; 17-OH Preg, 17α-hydroxypregnenolone. $^*p < 0.05$,$^{**}p < 0.01$. **C. Impact of CYP17A1 inhibitors on genes involved in androgen biosynthesis**. H295R cells were treated with control (DMSO) or 1 μM orteronel and galeterone for 72 h and then RNA was isolated and transcribed to cDNA. Results of qRT-PCR validation of HSD3B2, CYP17A1 and AKR1C3 genes relative to the housekeeping gene cyclophilin A. Expression of the genes was analyzed by SYBR Green real-time PCR. Analysis of relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method. $^*p < 0.05$.

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were treated with inhibitors and then with 1 μM trilostane (a specific blocker of HSD3B) for 90 min before labeling with [^{3}H] preg. We found that the orteronel decreased only DHEA production, suggesting selectivity of inhibition towards the 17,20 lyase activity of CYP17A1 (Fig. 2A). Treatments of galeterone, abiraterone, compound I and IV showed a decrease in the conversion from preg to 17-OH preg and to DHEA which suggests these compounds inhibited both the hydroxylase as well as lyase activity of CYP17A1 (Fig. 2A–B). These data indicate that orteronel is a specific inhibitor of 17,20 lyase activity, as observed in previous studies [31,32].

3.3. Effect of CYP17A1 inhibitors on genes involved in androgen biosynthesis

Abiraterone and galeterone are steroidal structures that also act as androgen receptor (AR) antagonist. Orteronel is a non-steroidal CYP17A1 inhibitor. Abiraterone has been reported to alter the androgen regulated gene expression in prostate cancer cells through the AR-mediated signaling [33]. Since, both the orteronel and galeterone have shown potential as potent CYP17A1 inhibitors, we conducted further experiments to find out if these inhibitors have any impact on gene transcription. The H295R cells were treated with control (DMSO) or 1 μM orteronel and galeterone for 72 h. Gene expressions were studied by relative quantification PCR (qRT-PCR). We studied the expression of three key genes CYP17A1, HSD3B2 and AKR1C3 that are involved in androgen biosynthesis. Interestingly, we found a slight increase in HSD3B2 gene expression upon treatment with galeterone (Fig. 2C). In presence of orteronel no significant changes in gene expression were observed (Fig. 2C).

3.4. Influence of CYP17A1 inhibitors on aromatase activity

To further study the specificity of the CYP17A1 inhibitors, we determined their impact on other related steroidogenic enzyme activities. First, we studied the effect of inhibitors on aromatase (CYP19A1) activity. The activity and expression of aromatase differs between different cell types depending on the need of cells for estrogens. Aromatase is localized in endoplasmic reticulum and catalyzes the last step of estrogen biosynthesis from androgens (converts androstenedione to estrone and testosterone to estradiol). The orteronel, galeterone, compound I and abiraterone showed no effect of on aromatase activity (Fig. 3A). Compound IV treatment resulted in a decrease of aromatase activity by 23% (Fig. 3A), which was in accordance with its reported modest inhibition of aromatase *in vitro* (IC_{50} = 228 nM) [34].

3.5. Abiraterone severely inhibits 21-hydroxylase (CYP21A2) activity

We then studied the effect of these inhibitors on the 21-hydroxylase (CYP21A2) enzyme activity. The 21-hydroxylase plays a role in producing glucocorticoids (cortisol) and mineralocorticoid (aldosterone). Cortisol helps maintain blood sugar levels, protects the body from stress and suppresses inflammation. Maintaining the cortisol level is required for the gonadal function, as disruption in cortisol production may affect fertility by the regulation of hypothalamic-pituitary-adrenal (HPA) and gonadal (HPG) axis [35]. CYP21A2 activities were assessed by checking the conversion of [^{3}H] 17-OH Prog to 11-deoxycortisol. Orteronel, galeterone, compound I and IV treatment did not change 11-deoxycortisol production, suggesting no effect on CYP21A2 activity. Surprisingly, abiraterone was found to completely inhibit the CYP21A2 activity (Fig. 3B). This suggests that abiraterone is not a specific inhibitor of CYP17A1 and can also inhibit CYP21A2 activity. Protein sequence comparison of CYP17A1 and CYP21A2 shows an identity of around

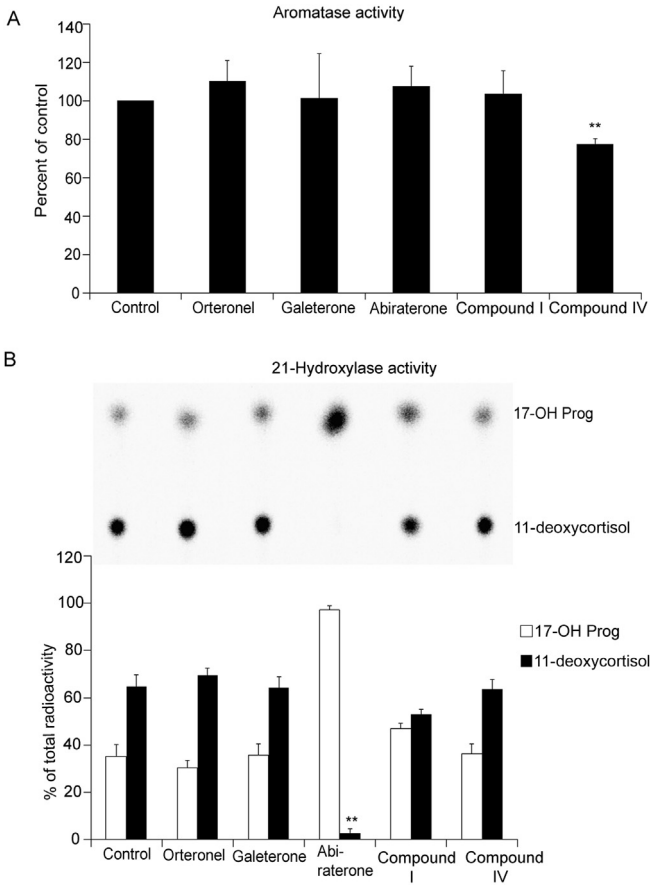

Fig. 3. Influence of CYP17A1 inhibitors on other steroid metabolizing cytochrome P450 activity. The effect of inhibitors on P450 aromatase (CYP19A1) and 21-hydroxylase (CYP21A2). **A) Effect of inhibitors on aromatase**. Effect of inhibitors was studied on aromatase activity in human placental JEG-3 cells. Cells were treated with control (DMSO) and 1 μM of orteronel, galeterone, abiraterone, compound I and IV for 24 h before aromatase activity was tested. Aromatase activity was assayed with androstenedione using 2 μM cold substrate and ^{3}H labeled androstenedione ([1β-3H(N)]-androstene-3,17-dione; ~100,000 cpm/well) as radioactive tracer. Androstenedione was added to the treated cells for 6 h. Aromatase activity was measured by observing the conversion of ^{3}H-androstenedione to estrone using the titrated water release assay. Data are presented as mean ± SEM of two independent experiments. **B) Effect on CYP21A1 activity**. The 21-hydroxylase activity was checked in H295R cells treated with control (DMSO) and 1 μM of orteronel, galeterone, abiraterone, compound I and compound IV for 24 h. CYP21A2 activities were measured by observing the conversion of [^{3}H] 17-OH prog to 11-deoxycortisol. Representative TLCs are depicted in the upper panel, summaries and quantifications of the results are given in the lower panel. Data are presented as mean ± SD of three independent experiments. $^{**}p < 0.01$.

28% but structures of CYP17A1 and CYP21A2 have many similarities and may retain similar binding sites. The inhibition of CYP21A2 could be a serious problem since using this drug inhibits whole adrenal androgen biosynthesis. Use of abiraterone for androgen lowering effects, where inhibition of CYP21A2 is not desired, will create additional complications.

3.6. Impact of CYP17A1 inhibitors on the metabolomics of C19 steroids by GC-MS

The CYP17A1 inhibitors orteronel, galeterone and abiraterone showed significant decrease in potent androgens like testosterone and dihydrotestosterone (Table 1). Galeterone lowered all classical and backdoor pathway metabolites of androgens in human adrenal cells. However, orteronel and abiraterone treatment lead to an increase of androgen metabolite androsterone, which is involved in

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Table 1
The table shows the GC-MS measurement of secreted steroid metabolites in the human adrenal H295R cells in the untreated vs the treated condition. The change in metabolites are indicated based on untreated levels which are set as hundred percent compared to CYP17A1 inhibitor treatments. *nd: Not determined; +: Increased metabolite.

Metabolites	Untreated	Orteronel	Galeterone	Abiraterone
Androgen metabolites				
Androsterone	100	448	30	1593
Etiocholanolone	100	3915	51	3030
Androstenediol	100	328	nd	152
11β-Hydroxy-Androsterone	100	104	94	1834
11β-Hydroxy-Etiocholanolone	nd	+	nd	+
Dehydroepiandrosterone	100	48	30	10
5α androstendiol	100	80	66	63
16α-Hydroxy-DHEA	100	74	84	81
5α androstenetriol	100	108	39	133
5-Pregnenetriol	100	36	71	9
Testosterone	100	50	29	26
5α-Dihydrotestosterone	100	nd	nd	nd
Estrogen metabolites				
Estriol	100	103	58	55
17-β Estradiol	nd	+	+	+
Progesterone metabolites				
17α-OH-Pregnanolone	100	4162	56	4408
Pregnanetriol	100	802	49	2011
Corticosterone metabolites				
Tetrahydrocorticosterone	nd	nd	+	+
5α-Tetrahydrocorticosterone	nd	nd	nd	+
Cortisol-metabolites				
Cortisone	nd	nd	nd	+
Tetrahydrocortisone	100	90	96	6372
α-Cortolone	100	873	374	45,600
β-Cortolone	nd	+	+	+
20α-Dihydrocortisone	nd	nd	nd	+
20β-Dihydrocortisone	nd	nd	nd	+
Cortisol	100	22	22	40
TH-Cortisol	100	nd	83	2617
5α-Tetrahydrocortisol	100	nd	83	2617
α-Cortol	nd	+	+	+
β-Cortol	nd	nd	+	+
20α-Dihydrocortisol	nd	120	162	161

the backdoor pathway of dihydrotestosterone biosynthesis. One possible explanation is that abiraterone can influence backdoor pathway by elevating CYP17A1 expression and induce progesterone accumulation [36]. The progesterone accumulation can compete against abiraterone for binding to CYP17A1 leading to induction of androgen metabolites involved in backdoor pathway [36]. Orteronel seems to be more specific for inhibiting the 17,20 lyase activity in the classical androgen biosynthesis pathway and might not influence the androgen metabolism via backdoor pathway. Additionally, we found that orteronel and abiraterone treatment also lead to an increase in other androgen metabolites like etiocholanolone and 11β-hydroxy-etiocholanolone. For the first time the data here show the effect of CYP17A1 inhibitors on the human steroid metabolome especially on androgen metabolites, in adrenal H295R cells. These data indicate that orteronel, galeterone and abiraterone can influence or alter different steroid metabolites in human adrenal cells apart from blocking the CYP17A1 activity.

Based on our results, we can conclude that orteronel is a more specific inhibitor for CYP17A1 17,20 lyase activity compared to other inhibitors tested here. Orteronel had no effect on other tested enzymes that are involved in steroid hormone biosynthesis. Hence, it can be considered as a treatment option for hyperandrogenic disorders like polycystic ovary syndrome. Using abiraterone for lowering androgen production in hyperandrogenic disorders may create additional undesired complications in patients because of its inhibitory effect on CYP21A2 activities. For the first time we showed the effect of CYP17A1 inhibitors abiraterone, orteronel and galeterone on steroid metabolome in human adrenal H295R cells. Our data provide evidence for the significantly altered steroids metabolites by CYP17A1 inhibitors. The steroid metabolome model may be used as an efficient diagnostic tool for further studies on urine or serum samples of hyperandrogenic patients treated with CYP17A1 inhibitors. Our observations that abiraterone is also a very potent inhibitor of CYP21A2, suggests that abiraterone should not be used to treat hyperandrogenic disorders when inhibition of CYP21A2 is an undesirable side effect. Orteronel may be tried for a more specific inhibition of CYP17A1 activities without affecting other steroid metabolizing enzymes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.07.019.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.07.019.

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