Osilodrostat Is a Potential Novel Steroidogenesis Inhibitor for the Treatment of Cushing Syndrome: An *In Vitro* Study

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Context: Metyrapone and ketoconazole, frequently used steroidogenesis inhibitors for treatment of Cushing syndrome, can be associated with side effects and limited efficacy. Osilodrostat is a CYP11B1 and CYP11B2 inhibitor, with unknown effects on other steroidogenic enzymes.

Objective: To compare the effects of osilodrostat, metyrapone, and ketoconazole on adrenal steroidogenesis, and pituitary adenoma cells *in vitro*.

Methods: HAC15 cells, 17 primary human adrenocortical cell cultures, and pituitary adenoma cells were incubated with osilodrostat, metyrapone, or ketoconazole (0.01 to 10 μM). Cortisol and ACTH were measured using chemiluminescence immunoassays, and steroid profiles by liquid chromatography-mass spectrometry.

Results: In HAC15 cells, osilodrostat inhibited cortisol production more potently (IC50: 0.035 μM) than metyrapone (0.068 μM; $P < 0.0001$), and ketoconazole (0.621 μM; $P < 0.0001$). IC50 values of osilodrostat and metyrapone for basal cortisol production varied with a 25- and 18-fold difference, respectively, with comparable potency. Aldosterone production was inhibited more potently by osilodrostat vs metyrapone and ketoconazole. Osilodrostat and metyrapone treatment resulted in strong inhibition of corticosterone and cortisol, 11-deoxycortisol accumulation, and modest effects on adrenal androgens. No pituitary-directed effects of osilodrostat were observed.

Conclusions: Under our study conditions, osilodrostat is a potent cortisol production inhibitor in human adrenocortical cells, comparable with metyrapone. All steroidogenesis inhibitors showed large variability in sensitivity between primary adrenocortical cultures. Osilodrostat might inhibit CYP11B1 and CYP11B2, in some conditions to a lesser extent CYP17A1 activity, and a proximal step in the steroidogenesis. Osilodrostat is a promising treatment option for Cushing syndrome, and *in vivo* differences with metyrapone are potentially driven by pharmacokinetic differences. (***J Clin Endocrinol Metab*** **104: 3437–3449, 2019)**

Cushing syndrome (CS) is characterized by chronic exposure to excess glucocorticoids, resulting in substantial multisystem morbidity and, when untreated, increased mortality (1). ACTH-dependent CS can be caused by a corticotroph pituitary adenoma [Cushing disease (CD)] or, more rarely, by ectopic ACTH secretion by a neuroendocrine tumor (ectopic ACTH syndrome). ACTH-independent CS is in most cases caused by a

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Abbreviations: 11-DOC, 11-deoxycortisol; 17-OHP, 17-hydroxyprogesterone; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; CD, Cushing disease; CS, Cushing syndrome; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; IC50, half maximal inhibitory concentration; UFC, urinary free cortisol.

unilateral cortisol-producing adrenocortical adenoma (ACA) and less frequently by an adrenocortical carcinoma (ACC) or bilateral adrenal hyperplasia (1, 2). The first-line treatment modality in all types of CS is surgery (3). There are several conditions, however, such as surgical failure, metastatic or occult disease, or high surgical risk, in which other treatment modalities such as medical therapy are indicated (3). Traditionally, medical treatment options for CS can be divided into three categories: (i) pituitary-targeting drugs (*i.e.*, pasireotide and cabergoline); (ii) glucocorticoid receptor blockers (*e.g.*, mifepristone); and (iii) adrenocortical steroidogenesis inhibitors that directly suppress cortisol production via inhibition of steroidogenic enzymes (3).

Two of the most frequently used steroidogenesis inhibitors are metyrapone and ketoconazole. Although it is known that metyrapone selectively inhibits the last step in the cortisol biosynthesis via inhibition of CYP11B1 (11β-hydroxylase), it also inhibits CYP11B2 (aldosterone synthase) (4, 5). More recently, *in vitro* data obtained in a rodent model even suggested that metyrapone has greater potency to inhibit CYP11B2, a feature not previously recognized (6). Ketoconazole, originally developed as an antifungal agent, is known to inhibit several steps in adrenal steroid synthesis (7–9). Although several medical therapies are currently available, not all patients respond and many patients experience side effects (3). Metyrapone can cause hypertension, edema, hypokalemia, acne, and hirsutism resulting from an increase of mineralocorticoid precursors and adrenal androgens (5). The most important adverse events of ketoconazole include hepatotoxicity and gastrointestinal symptoms (10–13).

Osilodrostat (LCI699) is an adrenal-blocking drug that, based on preclinical *in vitro* studies (14), was thought to specifically inhibit CYP11B2 and at higher concentrations also CYP11B1 (15). It was originally developed for its inhibitory effects on aldosterone production and blood pressure–lowering abilities (15–17). In these clinical studies, however, a blunted cortisol response to synthetic ACTH and increased levels of 11-deoxycorticosterone were observed. In 14 patients with primary hyperaldosteronism, basal cortisol levels remained unchanged under treatment, but morning ACTH levels were approximately twofold higher than baseline values (17). Doses that were used varied between 1 and 2 mg osilodrostat per day. Recently, an extended phase 2 study using a dose escalation schedule of osilodrostat starting at 4 mg/d showed normalized urinary free cortisol (UFC) or ≥50% decrease of UFC from baseline in 79% of the 19 patients with CD after 22 weeks of treatment (18). Plasma levels of cortisol and aldosterone decreased, whereas levels of their precursors, 11-deoxycortisol and 11-deoxycorticosterone, increased (18). Most common adverse events were nausea, diarrhea, asthenia, and adrenal insufficiency ($n = 6$ for each). Hirsutism and/or acne resulting from increased testosterone levels were reported in 4 of 14 female patients (18). Initial results of a phase 3 study investigating the effects of osilodrostat showed that 53% ($n = 72$) of patients had normalized UFC without uptitration after 12 weeks. Of these patients, 86% randomized to continue osilodrostat treatment for 8 weeks showed normalized UFC compared with 29% of patients in the placebo group (19).

The aim of the current study is to further explore the effects of osilodrostat on basal and ACTH-stimulated cortisol production and adrenocortical steroidogenesis in human adrenocortical cells, compared with those of metyrapone and ketoconazole. In addition, we examined possible pituitary-directed effects of osilodrostat on cell amount and ACTH secretion in corticotroph pituitary adenoma cells.

Materials and Methods

Cell culture and compounds

Cortisol and aldosterone-producing human adrenocortical carcinoma HAC15 (a kind gift by Dr. W. Rainey) and AtT20 mouse corticotroph tumor cells (ATCC number CRL-1795) were used. Short tandem repeat profiling of HAC15 showed a genetic profile identical to H295R, which is consistent with a previous report that HAC15 is a clone of H295R (20). Cells were routinely cultured in 75-cm^2 flasks at 37°C in a humidified incubator at 5% CO_2, harvested with trypsin (0.05%)-EDTA (0.53 mM), and resuspended in culture medium, as previously described (21). HAC15 cells were cultured in DMEM/F12 containing 5% fetal calf serum, whereas AtT20 cells were cultured in DMEM supplemented with 10% fetal calf serum. Both media were supplemented with L-glutamine (2 mmol/L) and penicillin (10^5 U/L; Bristol-Meyers Squibb, Utrecht, Netherlands). Media and supplements, except penicillin, were obtained from Fisher Scientific, Landsmeer, Netherlands.

Stock solution of osilodrostat, metyrapone (both Novartis Pharma, Arnhem, Netherlands), and ketoconazole (Sigma-Aldrich, Zwijndrecht, Netherlands) were dissolved in 0.01N hydrochloric acid, distilled water, and absolute ethanol, respectively, according to manufacturer's instructions, and stored at −20°C at a stock concentration of 10^{-2} M. At the start of each experiment, osilodrostat, metyrapone, and ketoconazole were diluted to the correct concentration in the same solution as it was dissolved in. Synacten (synthetic ACTH, Novartis Pharma) stock concentration was stored at 4°C and diluted in culture medium at the day of use. Angiotensin II (Sigma-Aldrich) stock concentration was stored at −20°C and diluted in distilled water at the day of use. The concentrations of ACTH and angiotensin II used were based on a dose-response curve performed in HAC15 cells on cortisol and aldosterone production, respectively, and according to previously reported studies (22, 23). One day after seeding the cells, incubations were started. Control cells were vehicle treated. For HAC15, cells were plated at a density of 100,000 cells per well in 0.5 mL medium. Osilodrostat, metyrapone, or ketoconazole (0.01 to 5 μM) were added for 3 days, with or without 10 nM ACTH or 100 nM angiotensin II, for evaluation of the effects on the

steroid profile, and aldosterone production, respectively. To assess the effect of osilodrostat on mouse corticotroph pituitary cells, AtT20 cells were incubated with osilodrostat for 1, 3, and 7 days (0.01 to 10 μM), to evaluate potential effects of the drug in multiple conditions (different incubation times and higher concentrations). For 7-day experiments, medium and compounds were refreshed after 3 days.

Cells and media were collected at the end of experiments and stored at −20°C until analysis. At the end of both the AtT20 and primary corticotroph pituitary adenoma culture experiments, media were collected and supplemented with the protease inhibitor Trasylol (final concentration 5 IU/mL, Sigma-Aldrich) to prevent degradation of ACTH. All cell culture experiments were carried out in quadruplicate at least twice. DNA measurement was performed using the bisbenzimide fluorescent dye (Hoechst 33258, Sigma-Aldrich), as previously described (24). In case compounds had an effect on cell number, steroid levels were corrected for total DNA per well as a measure of cell number.

Processing of adrenocortical and pituitary tissue

Adrenocortical tissues (ACA, hyperplasia, ACC) and ACTH-secreting corticotroph pituitary adenomas were collected during surgery at the Department of Surgery at Erasmus MC from January 2015 until February 2018. The study was approved by the Medical Ethics Committee of Erasmus MC and informed consent was obtained from all patients. Directly after surgery, the adrenal specimens were minced into small fragments of about 2 to 5 mm^3 and washed twice with culture medium (Fisher Scientific). The fragments were dissociated for 2 hours at 37°C, using collagenase type 1 (2 mg/mL; Sigma-Aldrich). The suspension was filtered through a sterile gauze (single layer) to obtain single-cell suspensions, and subsequently Ficoll density gradient separation was used to remove any remaining cell debris. Cell viability was determined by trypan blue exclusion, visually counted using Türk solution, and plated at a density of 10^5 cells per well. Corticotroph pituitary adenoma tissues from patients with CD ($n = 2$) were available after transsphenoidal surgery. Single-cell suspensions of the pituitary adenoma tissues were prepared as previously described (25). Primary human adrenal and pituitary adenoma culture experiments were similar to experiments in HAC15 and AtT20 cells, with small adjustments: ACTH was used at a concentration of 85 pM, angiotensin II was used at a concentration of 10 nM, treatment was started 3 to 4 days after plating the cells and preceded by medium refreshment, and in primary corticotroph pituitary adenoma cultures, osilodrostat was only tested at a concentration of 1 μM. The concentrations of ACTH and angiotensin II used in the primary cultures were lower compared with those in HAC15 cells because of the generally higher sensitivity to these compounds in primary cultures. Owing to a limited number of cells obtained from some specimens, not all experiments could be carried out in every primary culture.

Measurement of steroid hormone concentrations

For the dose-response curves, both cortisol and ACTH were measured in supernatants using an Immulite 2000 XPi immunoassay analyzer (Siemens Medical Solutions USA, Inc). In addition, in selected conditions, androstenedione, corticosterone, cortisol, 11-deoxycortisol (11-DOC), dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), progesterone, 17-hydroxyprogesterone (17-OHP), and testosterone were measured simultaneously using a Waters® Acquity™ UPLC HSS T3 1.8 μm column and a Waters XEVO-TQ-S system (Waters, Milford, MA) equipped with an ESI source operating in the electrospray positive mode except for DHEAS (negative ESI). Intra- and interassay coefficients of variation for the steroid assays were <7% and <8% for androstenedione, <8% and <4% for corticosterone, <6% and <6% for cortisol, <10% and <6% for 11-deoxycortisol, <7% and <8% for DHEA, <8% and <13% for DHEAS, <6% and <7% for progesterone, <6% and <6% for 17-OHP, and <6% and <9% for testosterone. Aldosterone was measured by mass spectrometry equipped with an ESI source operating in the electrospray negative mode using a protein precipitation method with a mixture of methanol, zinc sulfate, and phosphoric acid. Intra- and interassay coefficients of variation were 6.6% and 10.8%, respectively.

Multiple reaction monitoring was applied for the detection of the analytes using both quantifiers and qualifiers. Samples for liquid chromatography/tandem mass spectometry steroid measurements were those closest to 50% inhibition or maximal inhibition of cortisol as determined by the immunoassay.

Statistical analysis

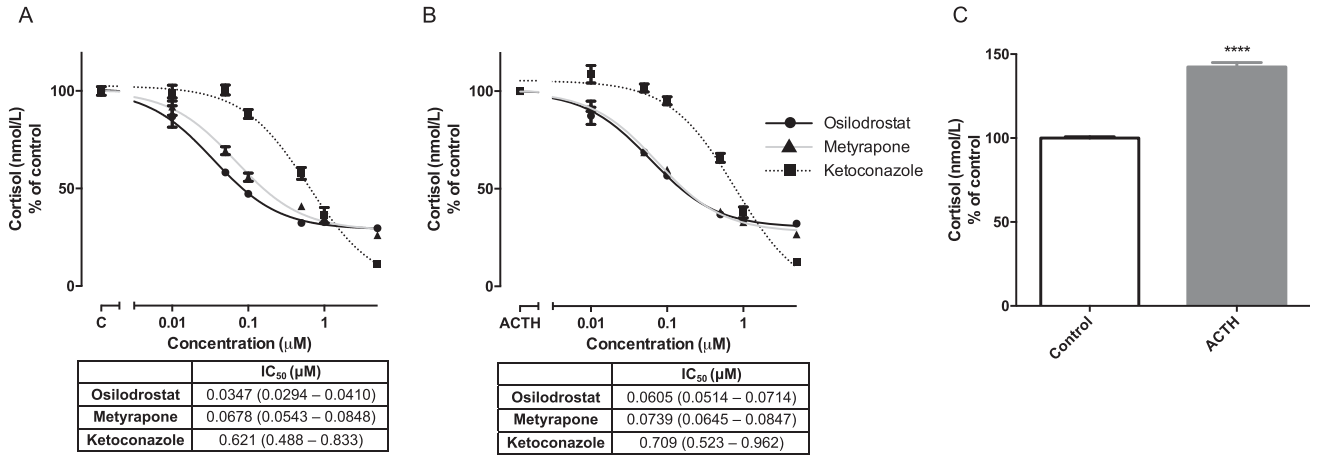
Statistical analysis was performed using GraphPad Prism 6.0. Nonlinear regression curve fitting program was used to calculate the half maximal inhibitory concentration (IC50 in μM) of the steroidogenesis inhibitors for cortisol and aldosterone production. Effects of the three compounds on the components of the steroid profile compared with control were compared using Student *t* test, and in case of multiple concentrations using ANOVA with the Tukey multiple comparisons test. Student *t* test was used to compare the effects of similar concentrations of osilodrostat with metyrapone and ketoconazole on the steroid profile. Significance was accepted at the 0.05 level of probability. Data are presented as mean ± SEM.

Results

Effects of osilodrostat, metyrapone, and ketoconazole on basal and ACTH-stimulated cortisol production *in vitro*

HAC15 cell line

After 3 days, osilodrostat inhibited cortisol production at significantly lower concentrations (IC50 0.0347 μM; 95% CI, 0.0294 to 0.0410) than metyrapone (0.0678 μM; 95% CI, 0.0543 to 0.0848; $P < 0.0001$), and ketoconazole (0.621 μM; 95% CI, 0.488 to 0.833; $P < 0.0001$) (Fig. 1A). Mean cortisol stimulation by ACTH was 42% (±4%) in all experiments (Fig. 1C). For osilodrostat, the IC50 value increased 1.7-fold when HAC15 cells were stimulated with ACTH ($P < 0.0001$ vs basal condition), whereas potency under ACTH stimulation did not significantly change for metyrapone and ketoconazole. Comparing the three compounds under ACTH stimulation, osilodrostat inhibited cortisol production as potently as metyrapone (IC50 0.0605 μM; 95% CI, 0.0514 to 0.0714 vs IC50 0.0739 μM; 95% CI,



	IC_{50} (μM)
Osilodrostat	0.0347 (0.0294 – 0.0410)
Metyrapone	0.0678 (0.0543 – 0.0848)
Ketoconazole	0.621 (0.488 – 0.833)

	IC_{50} (μM)
Osilodrostat	0.0605 (0.0514 – 0.0714)
Metyrapone	0.0739 (0.0645 – 0.0847)
Ketoconazole	0.709 (0.523 – 0.962)

Figure 1. Dose-dependent effects of osilodrostat (black solid lines, ●), metyrapone (gray solid lines, ▲), and ketoconazole (black dotted lines, ■), on (A) basal and (B) 10 nM ACTH-stimulated cortisol production in HAC15 cells after 72 hours of incubation. (C) Effects of 10 nM ACTH after 72 hours of incubation in HAC15 cells. The different concentrations tested are 0.01, 0.05, 0.1, 0.5, 1, and 5 μM, but not all concentrations could be tested in every primary culture. Controls represent (A) vehicle-treated or (B) vehicle with 10 nM ACTH-treated HAC15 cells. Values are depicted as mean ± SEM and as percentage of control. ****$P < 0.0001$ vs control. C, control.

0.0645 to 0.0847; $P = 0.0669$), and more potently compared with ketoconazole (IC50 0.709 μM; 95% CI, 0.523 to 0.962; $P < 0.0001$). Addition of the inhibitors of steroidogenesis did not affect cell amounts.

Primary adrenocortical cultures

Effects of osilodrostat, metyrapone, and ketoconazole were also assessed in 17 primary cultures of human adrenocortical tissue: 8 cortisol-producing ACA, 3 ACTH-dependent adrenal hyperplasia, 2 ACTH-independent adrenal hyperplasias, 2 cortisol-producing ACCs, and 2 Conn syndrome–associated adrenal hyperplasias. Patient and tissue characteristics are outlined in Table 1. IC50 values of osilodrostat, metyrapone, and ketoconazole for cortisol production in primary adrenocortical cultures are listed in Table 2; dose-response curves are displayed in Fig. 2 and (26). DNA measurement was performed in 37 of the 58 adrenal culture plates

Table 1. Clinical and Pathological Characteristics of Patients From Whom a Primary Culture Was Obtained

Patient No.	Sex	Side	Age at Surgery, y	Size of Lesion, cm	Weiss Score	Steroid Production
Cortisol-producing adrenocortical adenoma						
1	F	Left	51	2.9	0	Cortisol
2	M	Left	45	6	2	Cortisol
3	M	Right	52	4.8	1	Cortisol
4	F	Left (bilateral)	65	6	0	Cortisol
5	F	Left (bilateral)	57	4.5	0	Cortisol
6	F	Right	64	2.5	0	Cortisol
7	F	Right (bilateral)	62	4	0	Cortisol
8	F	Right	66	3.9	0	Cortisol
ACTH-dependent adrenal hyperplasia						
1	F	Bilateral	79	—	—	Cortisol
2	F	Right (bilateral)	75	—	—	Cortisol
3	F	Left (bilateral)	69	—	—	Cortisol
ACTH-independent hyperplasia						
1	M	Right (bilateral)	70	—	—	Cortisol
2	F	Left (bilateral)	50	—	—	Cortisol
Cortisol-producing adrenocortical carcinoma						
1	F	Right	61	5	5	Cortisol
2	M	Left	64	18.5	6	Cortisol
Conn syndrome						
1	F	Left	57	—	—	Aldosterone
2[a]	F	Left	44	1.5	—	Aldosterone

Tissue diagnosis was based on the pathology report. ACTH-dependent adrenal hyperplasias are all based on ectopic ACTH syndrome.

Abbreviations: bilateral, the lesion was bilateral, but only one side was used to obtain the primary culture; F, female; M, male.

[a]Recurrent Conn syndrome, adrenal hyperplasia with dominant 1.5-cm nodule.

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Table 2. Efficacy of Osilodrostat, Metyrapone, and Ketoconazole for Cortisol Production by Human Primary Adrenocortical Cultures

			Basal Condition			ACTH-Stimulated Cortisol, % Change	ACTH-Stimulated Condition		
			Osilodrostat	Metyrapone	Ketoconazole		Osilodrostat	Metyrapone	Ketoconazole
Cortisol-Producing ACA	No. 1	●	0.0519 (0.0296–0.0910)	NT	2.198 (0.375–1.290)	**+737**	0.0251 (0.00939–0.0700)	NT	0.499 (0.0875–2.851)
	No. 2	■	0.206 (0.127–0.333)	0.0632 (0.0541–0.0739)[a]	1.392 (0.912–2.127)[a]	**+538**	0.988 (0.641–1.524)	0.440 (0.323–0.600)[b]	1.315 (0.753–2.296)
	No. 3	◆	0.053 (0.019–0.15)	NT	0.570 (0.13–2.55)[c]	NT	NT	NT	NT
	No. 4	○	0.044 (0.032–0.059)	NT	NT 0.027 (0.016–0.045)	NT	NT	NT	NT
	No. 5	□	0.395 (0.270–0.576)	NT	1.826 (0.551–6.05)[a]	NT	NT	NT	NT
	No. 6	◊	0.534 (0.360–0.793)	0.412 (0.258–0.667)	2.270 (0.776–6.64)[a]	NT	NT	NT	NT
	No. 7	▼	0.0217 (0.0102–0.0461)	NT	0.138 (0.0705–0.269)[a]	NT	NT	NT	NT
	No. 8	▽	NT	0.0226 (0.0164–0.0310)	NT	**+207**	NT	0.0557 (0.0391–0.0793)	0.0328 (0.0213–0.0504)
ACTH-Dependent Adrenal Hyperplasia	No. 1	●	0.0311 (0.0242–0.0399)	0.105 (0.0658–0.168)[a]	0.0702 (0.0423–0.117)[d]	**+343**	0.0887 (0.0371–0.212)	0.138 (0.0644–0.296)	0.138 (0.0578–0.329)
	No. 2	■	NT	NT	NT	**+127**	0.194 (0.118–0.321)	0.263 (0.123–0.533)	0.799 (0.289–1.206)[b]
	No. 3	◆	0.0232 (0.0146–0.0369)	NT	0.0509 (0.0300–0.0863)[c]	NT	NT	NT	NT
ACTH-Independent Adrenal Hyperplasia	No. 1	○	NT	NT	NT	**+92**	0.0602 (0.0377–0.0960)	NT	0.826 (0.112–6.074)[d]
	No. 2	□	NT	NT	NT	**+48**	0.369 (0.224–0.610)	0.430 (0.318–0.581)	1.476 (0.568–3.837)[d]
ACC	No. 1	●	0.100 (0.0446–0.226)	NT	0.0728 (0.0376–0.141)	NT	NT	NT	NT
	No. 2	□	0.0431 (0.0328–0.0568)	0.0266 (0.0179–0.0396)[c]	0.107 (0.0609–0.188)[d]	NT	NT	NT	NT

IC50 values are presented in micromolar units. ACTH (85 pM) stimulated cortisol represents the mean percentage increase of cortisol production compared with control, with $P < 0.0001$ vs control shown in boldface. Column 3 includes the symbols used in Fig. 2 and (26).

Abbreviation: NT, not tested.

[a] $P < 0.0001$ compared with the IC50 of osilodrostat.

[b] $P < 0.001$ compared with the IC50 of osilodrostat.

[c] $P < 0.05$ compared with the IC50 of osilodrostat.

[d] $P < 0.01$ compared with the IC50 of osilodrostat.

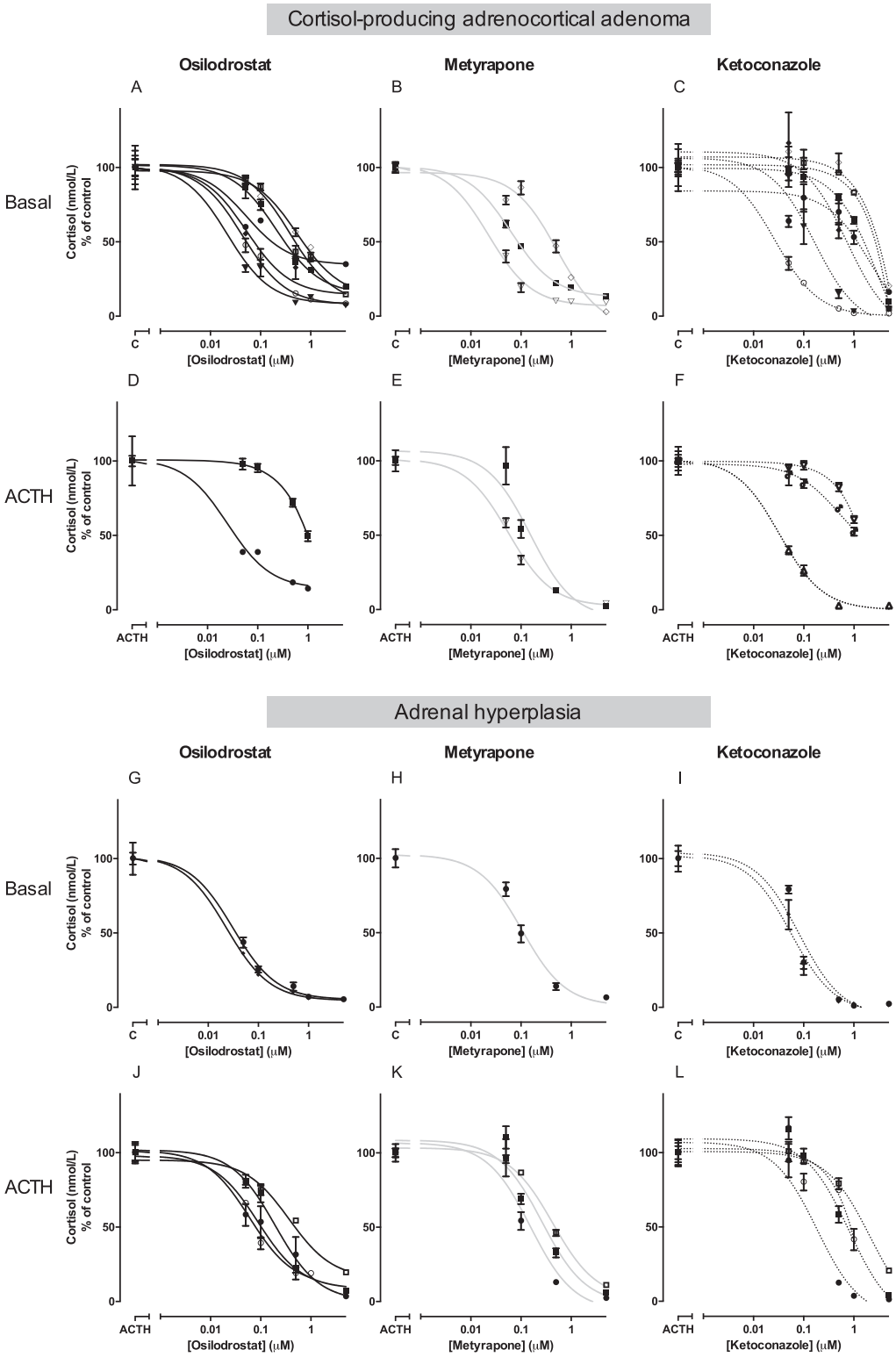

Figure 2. Effects of (left) osilodrostat, (middle) metyrapone, and (right) ketoconazole on basal and ACTH-stimulated cortisol production in primary human adrenocortical cultures. The upper panels represent cortisol-producing adrenocortical adenoma cultures; lower panels represent primary adrenal hyperplasia cultures, both ACTH-dependent and ACTH-independent. The different concentrations tested are 0.01, 0.05, 0.1, 0.5, 1, and 5 μM, but not all concentrations could be tested in every primary culture. Controls represent vehicle treatment (A-C, G-I) without or (D-F, J-L) with ACTH stimulation (85 pM). Symbols are presented in Table 2. Values are depicted as mean ± SEM and as percentage of control.

in which dose responses of the compounds on either cortisol or aldosterone were assessed, and showed no effects of any of the drugs on cell number in these cultures. The 85 pM ACTH-induced cortisol increase varied from 48% to 737% in primary adrenocortical cultures (Table 2).

In unstimulated primary ACA cultures, IC50 values of osilodrostat for cortisol production varied with a 25-fold difference (Table 2, Fig. 2A; 0.0217, 95% CI, 0.0102 to 0.0461; 0.534, 95% CI, 0.360 to 0.793), whereas there was an 18-fold difference for metyrapone, and 84-fold difference for ketoconazole. The mean IC50 of osilodrostat in ACA was higher ($n = 7$; 0.104 μM; 95% CI, 0.0716 to 0.151) compared with the mean IC50 in adrenal hyperplasia ($n = 2$; 0.0269 μM, 95% CI, 0.0210 to 0.0346; $P < 0.0001$ vs ACA), and not statistically significantly different from the IC50 of ACC ($n = 2$; 0.0644 μM; 95% CI, 0.0419 to 0.0988; $P = 0.1889$ vs ACA), although groups were small. The mean IC50 of osilodrostat was lower in adrenal hyperplasia compared with ACC ($P = 0.0007$). In eight conditions (basal or ACTH-stimulated), a direct comparison between osilodrostat and metyrapone could be made (Table 2). Metyrapone inhibited cortisol production more potently in three conditions compared with osilodrostat ($P < 0.05$), whereas osilodrostat inhibited cortisol more potently in ACTH-dependent adrenal hyperplasia no. 1 ($P < 0.0001$). Osilodrostat inhibited cortisol more potently compared with ketoconazole in 8 of the 11 cultures in which efficacy of both compounds were studied in the basal condition ($P < 0.05$ to $P < 0.0001$). Under ACTH stimulation, a lower IC50 was found for osilodrostat in two of the six primary cultures compared with ketoconazole ($P < 0.01$ and $P < 0.001$).

Efficacy of osilodrostat changed in two of the three cultures in which efficacy was compared in the basal and ACTH simulated conditions, with in one culture a higher and in the other culture a lower potency in the ACTH-stimulated condition ($P < 0.01$ and $P < 0.05$, respectively).

Effects of osilodrostat, metyrapone, and ketoconazole on aldosterone production in human adrenocortical cells

In angiotensin II–stimulated HAC15 cells (Fig. 3D; mean increase of aldosterone, 282%; $P < 0.0001$), osilodrostat inhibited aldosterone levels at more than 10 times lower concentrations compared with metyrapone (Fig. 3A; IC50, 0.0354 μM; 95% CI, 0.0269 to 0.0465 vs 0.413 μM; 95% CI, 0.306 to 0.557; $P < 0.0001$). The aldosterone concentration in unstimulated HAC15 cells was too low to adequately assess the inhibitory effect of the compounds. Osilodrostat also inhibited aldosterone production much more potently compared with metyrapone in an aldosterone-producing adrenal hyperplasia causing Conn syndrome (Figure 3B; IC50, 0.00281 μM; 95% CI, 0.000910 to 0.00866 vs 0.822 μM; 95% CI, 0.471 to 1.433; $P < 0.0001$). In a second aldosterone-producing adrenal hyperplasia, no differences were observed in suppressive effects of osilodrostat and metyrapone on basal aldosterone concentrations in two concentrations tested (0.1 and 5 μM, data not shown). In ACTH-dependent adrenal hyperplasia no. 1 in the basal condition, osilodrostat inhibited aldosterone significantly more potent compared with metyrapone (Fig. 3C; IC50, 0.00469 μM; 95% CI, 5.516E-5 to 0.398 vs 0.364 μM; 95% CI, 0.05515 to 2.397; $P < 0.0001$), and ketoconazole (0.315 μM; 95% CI, 0.0516 to 1.916; $P < 0.0001$ vs osilodrostat). In this primary culture, osilodrostat inhibited aldosterone production at significantly lower concentrations compared with those needed for cortisol inhibition (IC50 aldosterone, 0.00469 μM; 95% CI, 5.516E-5 to 0.398 vs cortisol, 0.0311 μM; 95% CI, 0.0242 to 0.0399; $P = 0.0164$).

Effects of osilodrostat, metyrapone, and ketoconazole on the steroid hormone profile in adrenocortical cells

HAC15 cell line

To examine other effects of osilodrostat on steroidogenesis next to inhibition of cortisol and aldosterone production, multisteroid analysis was carried out using liquid chromatography/tandem mass spectometry in culture media in several conditions [Fig. 4 and (26)]. HAC15 cells were studied in the basal and ACTH-stimulated condition. ACTH increased the production of all steroids (range, 11% to 216%), except for DHEA and DHEAS, of which the concentrations decreased. Under ACTH stimulation, osilodrostat only induced a strong decrease in cortisol and corticosterone concentrations (−51 nmol/L, −87%; $P < 0.0001$; −4.7 nmol/L, −84%, $P = 0.0005$; respectively), accompanied by accumulation of 11-DOC (+316.4 nmol/L, +12%, $P = 0.0005$). When focusing on differences between osilodrostat and metyrapone, metyrapone had a slightly stronger percentual inhibitory effect on 17-OHP ($P < 0.0001$), 11-DOC ($P < 0.0001$), cortisol ($P < 0.05$), androstenedione ($P < 0.0001$), and testosterone ($P < 0.0001$; all vs osilodrostat; Fig. 4). Both in the basal and the ACTH-stimulated condition, ketoconazole strongly blocked production of all steroids (all >79% decrease), except progesterone, which accumulated.

To evaluate the overall effects of the compounds on the steroid hormone profile and possible inhibitory effects on the proximal steroidogenic enzymes in the adrenal cortex, the absolute changes of the steroids were added together. In total, in the basal condition, all

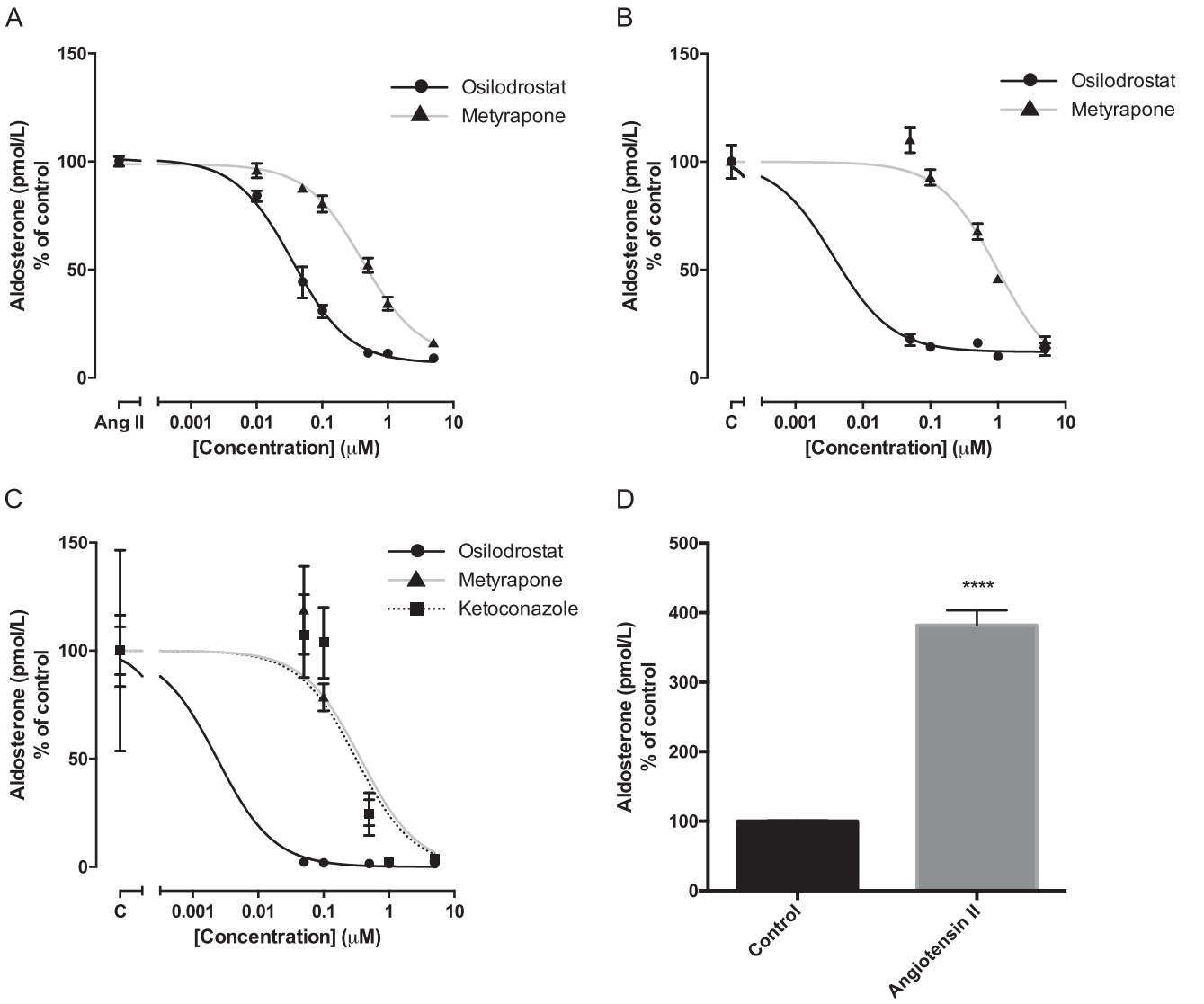

Figure 3. Effects of osilodrostat (black solid lines, ●) and metyrapone (gray solid lines, ▲) on (A) 100 nM angiotensin II–stimulated HAC15 cells, (B) on basal aldosterone production in aldosterone-producing adrenocortical hyperplasia no. 1, and (C) of ketoconazole (black dotted lines, ■) in ACTH-dependent adrenal hyperplasia no. 1 after 72 hours of incubation. (D) Effect of 100 nM angiotensin II in HAC15 cells after 72 hours of incubation. The different concentrations tested are 0.01, 0.05, 0.1, 0.5, 1, and 5 μM, but not all concentrations could be tested in every primary culture. Values are depicted as mean ± SEM and as percentage of control. ****$P < 0.0001$ vs control. Ang II, angiotensin II.

measured steroids together were inhibited with 1414 nmol/L by osilodrostat, whereas this was 976 nmol/L for metyrapone. Under ACTH stimulation, this was reversed, with a total increase of concentration of steroids of 171 nmol/L under osilodrostat, and a decrease of 900 nmol/L by metyrapone. In both conditions, the total decrease of steroids was strongest under treatment with ketoconazole, with a decrease of 3727 and 4376 nmol/L in the basal and ACTH-stimulated condition, respectively.

ACTH-dependent adrenal hyperplasia

The steroid hormone profiles were analyzed in two primary cultures of ACTH-dependent adrenal hyperplasia [Fig. 4 and (26)]. In the first primary culture, osilodrostat significantly suppressed the levels of corticosterone (−89 nmol/L, −98%, $P = 0.0003$), cortisol (−200 nmol/L, −99%, $P = 0.0003$), and androstenedione (−15 nmol/L, −47%, $P = 0.026$), accompanied by accumulation of progesterone (+1.9 nmol/L, +58%, $P = 0.0009$). Metyrapone predominantly showed the same percentual effects on the steroid profile as osilodrostat, except for a stronger accumulative effect on progesterone by osilodrostat (+58% vs +9%; $P < 0.01$), and a stronger inhibition of 17-OHP (−2% vs −38%; $P < 0.0001$; Fig. 4). In both conditions, ketoconazole strongly inhibited production of all steroids except progesterone, which increased. Besides progesterone, ACTH stimulated the concentration of all steroids. In the ACTH-stimulated condition, no differences in percentual change were observed between osilodrostat and metyrapone. The total inhibition of measured steroids in the basal condition was similar for osilodrostat

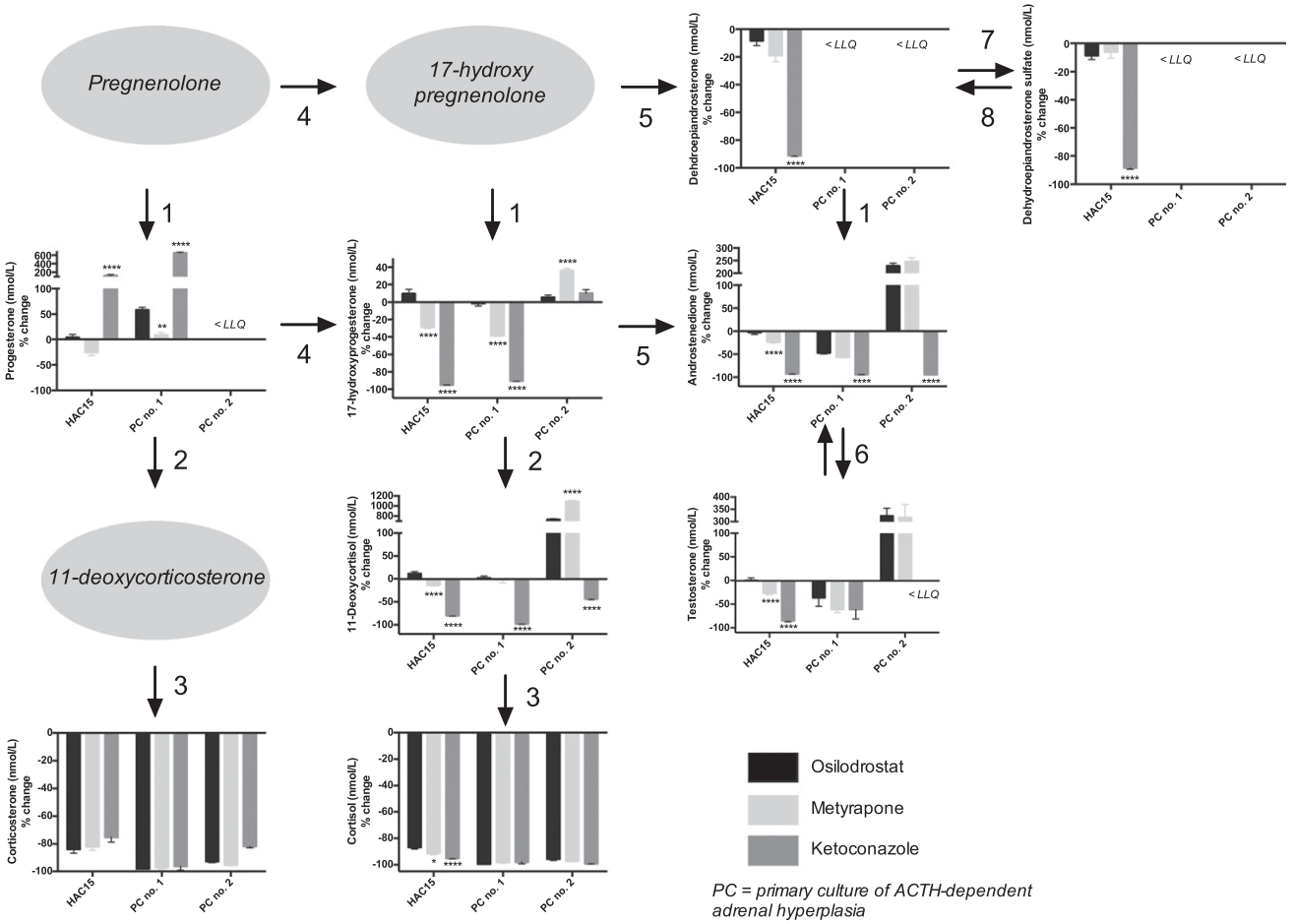

Figure 4. Effects of 5 μM osilodrostat (black bars), metyrapone (light gray bars), and ketoconazole (dark gray bars) on the steroid hormone profile in three different adrenocortical cultures. PCs represent ACTH-dependent adrenal hyperplasia. The displayed conditions were chosen based on the most pronounced differences among the three compounds and were under ACTH stimulation (HAC15 and ACTH-dependent adrenal hyperplasia no. 2), and the basal condition (ACTH-dependent adrenal hyperplasia no. 1). Numbers of the primary cultures correspond to the numbers in Tables 1 and 2. Arrows represent steroidogenic enzymes: (1) 3β-hydroxysteroid dehydrogenase, (2) CYP21A2, (3) CYP11B1, (4) CYP17A1 hydroxylase, (5) CYP17A1 lyase, (6) 17β-hydroxysteroid dehydrogenase III, (7) sulfotransferase, and (8) steroid sulfatase. Values are depicted as percentage change ± SEM compared with ACTH stimulation (HAC15 and ACTH-dependent adrenal hyperplasia no. 2) or vehicle-treated control (ACTH-dependent adrenal hyperplasia no. 1). Note the differences in scales of the y-axes. LLQ, lower limit of quantitation; PC, primary culture. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ vs the effect of osilodrostat.

and metyrapone, and under ACTH stimulation a decrease of 758 nmol/L was found for osilodrostat, and of 438 nmol/L for metyrapone. Ketoconazole inhibited the total amount of steroids more potently compared with osilodrostat and metyrapone in the basal and ACTH-stimulated condition, with inhibition of 598 and 1748 nmol/L, respectively.

In ACTH-dependent adrenal hyperplasia culture no. 2, three concentrations of the compounds (0.1, 0.5, and 5 μM) were tested only in the ACTH-stimulated condition [Fig. 4 and (26)]. Progesterone, DHEA, and DHEAS were below the limit of quantitation. ACTH increased the levels of all steroids. Osilodrostat induced a dose-dependent decrease in both cortisol and corticosterone (at 5 μM: −427 nmol/L, −96%, $P < 0.0001$; −55 nmol/L, −93%, $P < 0.0001$, respectively), accompanied by a dose-dependent accumulation of 11-DOC (at 5 μM: +218 nmol/L, +728%, $P < 0.0001$). Androstenedione and testosterone also showed an increase under osilodrostat treatment (both $P < 0.0001$). The percentual increases in 17-OHP (+36% vs +5%; $P < 0.0001$), and 11-DOC (+1086% vs +728%; $P < 0.0001$) were stronger by metyrapone compared with osilodrostat. At 5 μM, there was a slightly greater negative balance of the total steroids of 257 nmol/L by osilodrostat compared with 182 nmol/L by metyrapone. In this primary culture, ketoconazole strongly inhibited all steroids, except 17-OHP. At 5 μM, the total decrease in steroids was 687 nmol/L for ketoconazole.

Cortisol-producing ACA

The effects of the compounds were additionally studied in two primary cortisol-producing ACA cultures (26). When specifically focusing on the difference

between osilodrostat and metyrapone (in ACA primary culture no. 2 only) in the ACTH-stimulated condition, 17-OHP, 11-DOC and the adrenal androgens androstenedione and testosterone accumulated more strongly by metyrapone (all $P < 0.0001$), and corticosterone and cortisol decreased more strongly ($P < 0.01$ and $P < 0.0001$, respectively) (+100% vs −6%; $P < 0.001$). In the basal condition however, Total reduction in concentration of measured steroids was strongest for ketoconazole (basal: −573 nmol/L; ACTH: −1474 nmol/L), followed by metyrapone (basal: −283 nmol/L; ACTH: −946 nmol/L) and osilodrostat (basal: −116 nmol/L; ACTH: −48 nmol/L).

Effects of osilodrostat on cell growth and ACTH secretion by pituitary tumor cells

No effects of osilodrostat were observed on cell amount and ACTH production by mouse pituitary AtT20 cells after 1, 3, and 7 days of treatment with osilodrostat (0.1 to 10 μM) (26). In addition, no inhibitory effects were observed of osilodrostat (1 μM) on both cell growth and ACTH production in two primary human corticotroph pituitary adenoma cultures after 7 days of treatment (26).

Discussion

Medical therapy for CS is indicated in case of surgical failure or when surgery is contraindicated. In this respect, it is very important to aim at complete normalization of cortisol production to reduce morbidity and mortality. Pharmacotherapy for CS can, however, be associated with limited efficacy and severe side effects restricting prolonged use. Therefore, additional therapeutic options are urgently needed. In this study, we show the effects of osilodrostat, a steroidogenesis inhibitor, on human adrenocortical steroidogenesis *in vitro*, and compared the potency of osilodrostat on steroid inhibition with those of metyrapone and ketoconazole. In addition, we evaluated potential pituitary-targeting effects of the drug *in vitro*.

In HAC15 cells, osilodrostat inhibits cortisol production approximately 2 times more potently compared with metyrapone, and 18 times more potently compared with ketoconazole. The effects of osilodrostat and metyrapone on cortisol production in primary cultures were largely similar, whereas osilodrostat did in general inhibit cortisol production at significantly lower concentrations compared with ketoconazole.

Sensitivity to steroidogenesis inhibitors appeared to be highly variable between adrenal patient tissues, with IC50 values varying by a factor of 25, 18, and 84, for osilodrostat, metyrapone, and ketoconazole, respectively. A direct comparison between the compounds is difficult, because these IC50 values are partly based on different primary cultures. The primary cultures that responded to a lesser extent to osilodrostat, also seemed to respond less strongly to metyrapone and ketoconazole. A higher mean IC50 of osilodrostat was found for ACA (0.104 μM), followed by ACC (0.0644 μM); the lowest mean IC50 was found in ACTH-dependent adrenal hyperplasia (0.0269 μM). These differences between tissue entities have to be interpreted with caution, considering the relative low number of primary cultures. Our study suggests that clinically observed differences in effects of steroidogenesis inhibitors between patients may be caused by differences in drug sensitivity on tissue level as well, rather than (only) pharmacokinetic differences. Compounds could thereby be metabolized or inactivated by the adrenal cortex, causing lower concentrations of steroidogenesis inhibitors in certain primary cultures. In patients with CS, a single-nucleotide polymorphism in the CYP17A1 gene has been shown to be associated with the response to ketoconazole and metyrapone (27). Considering the small sample size and individual dose titration schemes, these results have to be interpreted with caution and confirmed in larger populations. Sensitivity might also depend on basal enzyme expression levels, differences in genotype, or cell-specific uptake and/or outward transport. Further research could focus on elucidating this issue in an attempt to make the first step toward selecting patients in which specific steroidogenesis inhibitors might be effective.

Osilodrostat is known to inhibit CYP11B2 more potently compared with CYP11B1 (15). In ACTH-dependent adrenal hyperplasia no. 1, we indeed found an almost sevenfold higher IC50 value for the effect on cortisol production compared with the IC50 value for suppressing aldosterone production. This study reports the effects of osilodrostat on human adrenocortical steroidogenesis *in vitro*. Because ACTH-dependent cortisol-producing adrenal hyperplasias are suggested to contain no molecular alterations, these specimens are considered most useful to investigate the effects of the compounds on the steroid profile. This contrasts with, for example, cortisol-producing ACA, where recurrent activating mutations in PRKACA, encoding the catalytic subunit α of protein kinase A, have been identified in 35% to 65% of cases (28). Measurement of the steroid profile showed a clear inhibition of CYP11B1 by osilodrostat, as demonstrated by a strong decrease of cortisol levels and no effect on or even accumulation of its precursor 11-DOC. An increase of 11-DOC was also found in the serum of patients treated with osilodrostat (18). In HAC15 and ACTH-dependent adrenal hyperplasia no. 1, there might also be a block of CYP17A1 lyase as

demonstrated by a stronger absolute inhibition of androstenedione compared with 17-OHP. This was not observed for metyrapone. Because we did not observe a strong accumulation equal to the total inhibition of steroids, there might also be inhibition of (one of the) proximal steps of the steroid biosynthetic pathway, such as cholesterol side-chain cleavage enzyme and/or the StAR protein. We hypothesize that the extent of this absolute proximal inhibition might be variable between patients, considering for example the different balance of the absolute change in steroids between the two ACTH-dependent adrenal hyperplasias at 5 μM osilodrostat treatment (−758 vs −257 nmol/L). In the two ACA cultures in the basal condition, osilodrostat might result in less strong absolute proximal inhibition as demonstrated by a less pronounced sum of balance in change of steroids (+51 and −116 nmol/L). A comparison in relative inhibition between cultures from different patients cannot be made. The upstream inhibition was variable between osilodrostat and metyrapone, with proximal inhibition that was alternately higher for osilodrostat or metyrapone. Ketoconazole might inhibit the proximal steps in the adrenal steroidogenesis more strongly compared with both osilodrostat and metyrapone, given the increased negative balance in the total amount of steroids in the different primary cultures. We do have to acknowledge that we did not measure all steroids of the profile. To study to what exact extent the separate enzymes are inhibited, cell lines transfected with the respective enzymes treated with osilodrostat could be used. In general, the effects of osilodrostat and metyrapone on the steroid profile were highly comparable, with subtle differences that were not comparable in every culture.

In this *in vitro* study, we observed in some cultures a slight decrease in levels of adrenal androgens under osilodrostat treatment, but not as strong as that of mineralocorticoids and glucocorticoids. *In vivo* however, an increase in testosterone might be expected because of (compensatory) ACTH stimulation. This assumes that osilodrostat treatment, such as metyrapone, might in female patients be limited by testosterone-related side effects. Correspondingly, increased levels of testosterone were observed in 4 of the 14 female patients included in the clinical trial investigating osilodrostat (18). In HAC15 cells, androstenedione and testosterone were inhibited more strongly by metyrapone, whereas metyrapone caused slightly stronger accumulation of adrenal androgens in ACTH-dependent adrenal hyperplasia no. 2 and ACA no. 2. This indicates that patients may respond slightly different to osilodrostat and metyrapone with respect to the levels of adrenal androgens, although the clinical relevance of this difference is yet unknown.

As expected, ketoconazole had a distinctive effect on the steroid profile compared with osilodrostat and metyrapone as a result of inhibition of multiple steroidogenic enzymes (7–9). In some conditions, ketoconazole caused accumulation of corticosterone. In ACTH-dependent adrenal hyperplasia no. 2, the direction of change of corticosterone was dose dependent. In a previous study, in which human adrenal tissue slices were incubated with ketoconazole, it was shown that the different steroidogenic enzymes are inhibited with distinct IC50 values of ketoconazole (9).

For the steroidogenesis inhibitor ketoconazole, direct effects on corticotroph pituitary adenoma cells have been shown (29). Therefore, we aimed to examine potential effects of osilodrostat on pituitary adenoma cells as well. However, no effects of osilodrostat were observed on both cell growth and ACTH secretion of pituitary adenomas in this *in vitro* study. Corresponding to this, in the clinical trial using osilodrostat, diameter changes of less than 2.0 mm were observed at week 22 in the 6 patients in whom the pituitary tumor size could be followed, which is a change that is considered not clinically meaningful (18, 30).

Taking the effects on the production of cortisol and the steroid profile together, it is concluded that direct effects of osilodrostat and metyrapone on adrenocortical cells are highly comparable, with no clear higher potency for cortisol inhibition of one of both compounds. However, despite the similarities of *in vitro* potency of osilodrostat and metyrapone, clinically it appears that there is a large difference in the administered dosage between the compounds, in order to obtain a response (normalization of UFC or ≥50% decrease of baseline) in patients with CD. The median metyrapone dosage for normalization of UFC in patients with CD appears to be 1375 mg (range, 500 to 3500 mg) per day (4), whereas dosages of 4 to 100 mg osilodrostat per day were administered in the phase 2 clinical trial to normalize UFC (31). Furthermore, in the extended phase 2 study, osilodrostat dosages were escalated to a maximum of 60 mg/d (18). Initial results of the phase 3 study investigating osilodrostat demonstrated the use of a mean dose of approximately 8 to 15 mg/d in CD patients (19). Based on our *in vitro* study, *in vivo* differences between efficacy of osilodrostat and metyrapone seem to be driven particularly by pharmacokinetic differences between both compounds. Indeed, it has been suggested that osilodrostat has an approximately twofold longer half-life compared with metyrapone (~4 to 5 vs ~2 hours) (31–33). This may result in more stable plasma and tissue levels of osilodrostat. Regarding pharmacokinetics, the plasma concentration of osilodrostat reached a maximum of 204 ng/mL (0.9 μM), measured at day 70,

12 hours after the final administration, in a patient who was uptitrated to the maximum dosage of 100 mg/d for 14 days (31). Little is known about the plasma concentration of metyrapone and its variance, although 4 hours after administration, a plasma concentration of 500 ng/mL (2.2 μM) may be reached (750 mg single dose, www.accessdata.fda.gov/scripts/cder/daf). The conditions in which the concentrations were measured are not comparable, making a solid comparison between the plasma levels of both compounds impossible. Given the lower required dose, there is a rationale of reduced side effects and a favorable safety profile for osilodrostat compared with metyrapone. Until now, however, this has not been shown in clinical trials, and no head-to-head comparison has been made.

In the proof-of-concept study, patients needed highly variable dosages of osilodrostat to achieve normalized UFC (range, 4 to 100 mg/day; factor 25 difference); this dose corresponded to the plasma levels (0.34 to 204 ng/mL) (31). Metyrapone varied only from 500 to 3500 mg/d (factor 7 difference) to achieve normalization of UFC in patients with CD (4). Furthermore, hypocortisolism related adverse events were reported in 32% to 51% (n/N = 6/19 and n/N = 70/137) of the patients treated with osilodrostat (18, 19), compared with 7% of patients with CD in a retrospective study on metyrapone treatment (4). The highly variable necessary dosages to achieve eucortisolism and the occurrence of adrenal insufficiency in 25% of patients both stress the need for careful uptitration of osilodrostat in patients with CD to prevent hypoadrenalism.

In conclusion, we show that osilodrostat in pharmacological concentrations is a potent inhibitor of *in vitro* cortisol and aldosterone secretion in human adrenocortical cells. We demonstrate highly variable sensitivity to steroidogenesis inhibitors with respect to cortisol production between adrenal tissues of patients, which together with differences in pharmacokinetics, potentially explain clinically observed differences between patients treated with the same compound. Under the conditions of our study, effects of osilodrostat and metyrapone on the steroid profile are highly comparable, where osilodrostat seems to block CYP11B1 and CYP11B2, in some conditions to a lesser extent CYP17A1 lyase activity, and a proximal step in the steroidogenesis pathway. Differences between osilodrostat and metyrapone *in vivo* are potentially the result of pharmacokinetic differences rather than the pharmacodynamic effects on the adrenal cortex. These data indicate that osilodrostat is a promising treatment option for patients with CS. Additional information from phase 3 trials will provide important further data on efficacy and safety of osilodrostat.

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