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Clinical Research Article

# Levoketoconazole, the 2S,4R Enantiomer of Ketoconazole, a New Steroidogenesis Inhibitor for Cushing's Syndrome Treatment

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## Abstract

**Introduction:** Racemic ketoconazole (RK) is a steroidogenesis inhibitor used for treatment of Cushing's syndrome. Levoketoconazole (COR-003), the pure 2S,4R enantiomer, is potentially more potent and safe compared to RK. We compared in vitro effects of levoketoconazole and RK on adrenocortical and pituitary adenoma cells.

**Materials and methods:** HAC15 cells and 15 primary human neoplastic adrenocortical cultures (+/– ACTH), and murine (AtT20) and human corticotroph adenoma cultures were incubated with levoketoconazole or RK (0.01–10  $\mu$ M). Cortisol and ACTH were measured using a chemiluminescence immunoassay system, and steroid profiles by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Results:** In HAC15, levoketoconazole inhibited cortisol at lower concentrations ( $IC_{50}$ : 0.300  $\mu$ M) compared to RK (0.611  $\mu$ M;  $P < 0.0001$ ).  $IC_{50}$  values of levoketoconazole for basal cortisol production in primary adrenocortical cultures varied over a 24-fold range (0.00578–0.140  $\mu$ M), with 2 patients having a higher sensitivity for levoketoconazole vs RK (2.1- and 3.7-fold). LC-MS/MS analysis in selected cases revealed more potent inhibition of cortisol and other steroid profile components by levoketoconazole vs RK. In AtT20, levoketoconazole inhibited cell growth and ACTH secretion (10  $\mu$ M: –54% and –38%, respectively), and levoketoconazole inhibited cell number in 1 of 2 primary human corticotroph pituitary adenoma cultures (–44%,  $P < 0.001$ ).

**Conclusion:** Levoketoconazole potently inhibits cortisol production in adrenocortical cells, with a variable degree of suppression between specimens. Levoketoconazole inhibits adrenal steroid production more potently compared to RK and might also inhibit ACTH secretion and growth of pituitary adenoma cells. Together with previously

reported potential advantages, this indicates that levoketoconazole is a promising novel pharmacotherapy for Cushing's syndrome.

**Key Words:** Levoketoconazole, COR-003, Cushing's syndrome, racemic ketoconazole, cortisol, LC-MS/MS

Endogenous Cushing's syndrome (CS) is characterized by chronic glucocorticoid excess and is associated with significant comorbidities potentially leading to increased mortality (1). CS can be caused by adrenocorticotrophic hormone (ACTH) overproduction by a pituitary adenoma or nonpituitary tumors or by autonomous cortisol production by an adrenal tumor or hyperplasia (1). The primary treatment modality of CS is surgical resection of the underlying cause (1). Medical therapy can be applied as pretreatment before surgery, in case of surgical failure, in the acute setting with complications of (severe) hypercortisolism or in patients with inoperable neuroendocrine or adrenocortical tumors (2). Medical therapy can be divided into pituitary-targeting drugs, adrenal steroidogenesis inhibitors, and glucocorticoid receptor antagonists (2). The most important adrenal blocking drugs include ketoconazole, metyrapone, mitotane, and etomidate.

Ketoconazole, originally developed as an antifungal agent, is one of the most widely used cortisol lowering drugs for the treatment of CS. It is commercially manufactured as a racemic mixture containing 2 cis enantiomers (2S,4R and 2R,4S) (2,3). One of the severe side effects is hepatotoxicity (3,4). Levoketoconazole (COR-003) is the purified 2S,4R enantiomer of ketoconazole. Based on early in vitro analyses, levoketoconazole is thought to inhibit CYP11B1, CYP17A1, and CYP21A2 enzymes 15- to 25-fold more potently compared to the 2R,4S enantiomere (5,6). Increased potency was also shown in a preclinical study in rats, where levoketoconazole more potently inhibited serum corticosterone, the main glucocorticoid in rats, compared to 2R,4S ketoconazole (6). This may allow for a lower dose of levoketoconazole compared to racemic ketoconazole to achieve the same efficacy and thus an increased therapeutic index. In vivo studies in rats suggest that levoketoconazole may have a favorable safety profile compared to racemic ketoconazole, based on less potent inhibition of CYP7A, compared to the 2R,4S enantiomer, although this assumption needs to be confirmed (7). Decreased CYP7A activity may lead to decreased bile acid production and functional cholestasis, which may cause hepatotoxicity. A clinical study in patients with type 2 diabetes mellitus showed decreased low-density lipoprotein cholesterol levels after 14 days of treatment with levoketoconazole 200 to 600 mg (8), suggesting that levoketoconazole may have beneficial metabolic effects. In a comparative study in 24 healthy subjects, levoketoconazole (400 mg daily) inhibited serum cortisol

slightly more potently compared to racemic ketoconazole (6). Besides, levoketoconazole plasma levels appeared to be 3-fold higher compared to those of the 2R,4S enantiomer (6), suggesting a lower hepatic metabolism of levoketoconazole. Headache and nausea were the most commonly reported adverse events (6,8). Results of the first prospective, open-label, phase III maintenance-of-benefit study (SONICS) investigating levoketoconazole resulted in normalized urinary free cortisol in 31% (n/N = 29/94) of CS patients without dose increase after a 6 months maintenance phase (9). Currently, 2 multicenter phase 3 trials are being conducted to further assess the efficacy and safety of levoketoconazole in patients with elevated urinary free cortisol concentrations due to CS (NCT03277690 and NCT03621280).

Taken together, levoketoconazole might inhibit cortisol synthesis more potently, might have a reduced hepatic metabolism and may have less hepatotoxic effects compared to racemic ketoconazole. The aim of this study is to compare the direct effects of pharmacological concentrations of levoketoconazole on basal and ACTH-stimulated adrenocortical steroid production to those of racemic ketoconazole. In vitro studies were performed in HAC15 cells and in primary adrenocortical cultures by assessing the concentrations of steroid hormones in the supernatant after treatment with both compounds. Finally, we assessed the pituitary-directed effects of both levoketoconazole and racemic ketoconazole on cell amount and ACTH secretion in pituitary corticotroph cells.

## Materials and Methods

### Cell culture and compounds

Human adrenocortical carcinoma HAC15 (kind gift by Dr. W. Rainey) and mouse corticotroph AtT20 cells (ATCC number: CRL-1795) were used. Dulbecco's Modified Eagle Medium F12 containing 5% fetal calf serum was used for HAC15 cells, whereas AtT20 cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Both media were supplemented with L-glutamine (2 mmol/L) and penicillin ( $10^5$  U/L). Medium and supplements were obtained from Fisher Scientific (Landsmeer, the Netherlands), except penicillin, which was obtained from Bristol-Meyers Squibb (Woerden, the Netherlands). HAC15 and AtT20 cells were cultured in 75 cm<sup>2</sup> flasks at 37°C in a humidified incubator (Greiner

Bio-One, Alphen a/d Rijn, the Netherlands) at 5% CO<sub>2</sub>. Short tandem repeat profiling using a Powerplex Kit (Promega, Leiden, the Netherlands) of HAC15 cells provided results consistent with the ATCC database, confirming the identity of the cell line. Once a week, cells were harvested with trypsin (0.05%)-EDTA (0.53 mM) and resuspended in culture medium. Levoketoconazole and racemic ketoconazole (both from Cortendo AB, Savedalen, Sweden) were dissolved in absolute ethanol according to manufacturer's instructions and stored at -20°C at a stock concentration of 10<sup>-2</sup> M. At the start of each experiment, both drugs were freshly diluted in absolute ethanol to the correct concentrations. Synacten (synthetic ACTH, Novartis Pharma, Arnhem, the Netherlands) stock solution was stored at 4°C and diluted in culture medium on the day of use. The final concentration of ACTH was chosen based on a dose-response curve in HAC15 cells and on previously reported studies (10). For HAC15, 200 000 and 100 000 cells were plated in 0.5 mL medium in 24-wells plates for experiments of 1 and 3 days, respectively. One day after seeding the HAC15 cells, medium was refreshed and cells were treated 1 or 3 days in quadruplicate with levoketoconazole or racemic ketoconazole (0.05-5 μM), with or without 10 nM ACTH (10). To study effects of both compounds on pituitary AtT20 cells, concentrations of 0.1 to 10 μM of both drugs were used and incubations were performed for 1, 3, and 7 days. For 7-days experiments, medium and compounds were refreshed after 3 days. Controls were vehicle treated. If compounds had an effect on cell number, steroid levels were corrected for total amount of DNA per well as a measure of cell number. DNA concentrations were determined using the bisbenzimidazole fluorescent dye (Hoechst 33258, Sigma-Aldrich, Zwijndrecht, the Netherlands), as previously described (11). Media were collected at the end of the experiments and stored at -20°C until analysis. Regarding AtT20 and primary pituitary adenoma culture experiments, media were supplemented with the protease inhibitor Trasylol (final concentration 5 IU per ml, Sigma-Aldrich, Zwijndrecht, the Netherlands) before storage to prevent degradation of ACTH. All cell line culture experiments were carried out at least twice in quadruplicate.

### Processing of human tissues

To obtain primary cultures, adrenal specimens (adrenocortical adenomas [ACA], adrenal hyperplasias, and adrenocortical carcinomas [ACC]) were collected after adrenalectomy at the Erasmus University Medical Center, Rotterdam, the Netherlands, between April 2016 and May 2018. The study was conducted under guidelines that have been approved by the Medical Ethics

Committee of the Erasmus Medical Center. Furthermore, informed written consent was obtained from all patients. Immediately after surgery, the specimens were processed as previously described (12). Briefly, specimens were minced, washed in culture medium, centrifuged, and stored overnight in culture medium at 4°C. The next day, the specimens were centrifuged again, after which the supernatant was removed. Dissociation of the fragments was performed using collagenase type 1 (10-25 mL; 2 mg/mL; Sigma-Aldrich, Zwijndrecht, the Netherlands), followed by incubation at 37°C for up to 2 h. We used Ficoll (GE healthcare, Eindhoven, the Netherlands) density gradient separation once or twice as required to separate contaminating red blood cells from the adrenal cells. Cell viability was determined by trypan blue exclusion and visually counted using Türk solution. Dissociated cells were plated at a density of 10<sup>5</sup> cells per well in a 24-wells plate in 0.5 mL medium. ACTH-secreting corticotroph pituitary adenoma tissue was available after transsphenoidal surgery from 2 patients with Cushing's disease. Single-cell suspensions of the pituitary adenoma tissues were prepared as previously described (13).

Culture conditions for primary cultures were similar as described in the previous section Cell Culture and Compounds, but with small adjustments: ACTH was used at a concentration of 85 pM (250 pg/mL), treatment was started 3 to 4 days after plating of the cells and cells were incubated for 3 days. For pituitary primary cultures, levoketoconazole and racemic ketoconazole were only tested at a concentration of 5 μM. Owing to a limited number of cells obtained from the specimens, not all experiments could be performed in every primary culture.

### Measurement of steroid hormone concentrations

For construction of the dose-response curves, cortisol and ACTH were measured in the culture media of adrenal and pituitary cultures, respectively, using an Immulite 2000 XPI immunoassay analyzer (Siemens Medical Solutions USA, Inc). Samples for liquid chromatography-tandem mass spectrometry (LC-MS/MS) steroid measurements were those closest to 50% inhibition or maximal inhibition of cortisol as determined by the immunoassay. In these selected culture conditions, androstenedione, corticosterone, cortisol, 11-deoxycortisol (11-DOC), dehydroepiandrosterone (DHEA), DHEA-sulphate (DHEAS), progesterone, 17-hydroxyprogesterone (17-OHP), and testosterone were simultaneously measured using a Waters® Acquity™ UPLC HSS T3 1.8 μm column and a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) equipped with an electrospray ionization source operating in the electrospray positive mode except for

DHEAS (negative electrospray ionization). Intra- and inter-assay coefficients of variation for the steroid assays were <7% and <8% for androstenedione, <4 % and <8% for corticosterone, <6% and <6% for cortisol, <6% and <10% for 11-DOC, <7% and <8% for DHEA, <8% and <13% for DHEAS, <6% and <7% for progesterone, <6% and <6% for 17-OHP, and <65 and <9% for testosterone. Multiple reaction monitoring was applied for the detection of the analytes using both quantifiers and qualifiers.

### Statistical analysis

Statistical analysis was performed using Graphpad Prism 6.0 (Graphpad Software, San Diego, CA, USA). The nonlinear regression curve fitting program was used to calculate the half maximal inhibitory concentrations ( $IC_{50}$ ).  $IC_{50}$  values were only calculated when the curve reached a clear bottom and the top of the curve did not extend 100%. Effects of both compounds on the steroid profile were measured as absolute change compared to control. The effects were compared using the Student's *t*-test or 1-way analysis of variance with Tukey's multiple comparison test in case multiple concentrations were tested in the same experiment. When assessing differences between effects of both compounds, the percentage change was evaluated and compared to correct for differences in the vehicle treated control cells. Values of  $P < 0.05$  were considered statistically significant and data are presented as mean  $\pm$  SEM.

## Results

### Effects of racemic ketoconazole and levoketoconazole on cortisol production in vitro

#### HAC15 cells

After 3 days of treatment, levoketoconazole more potently suppressed cortisol production in HAC15 cells compared to racemic ketoconazole, with an approximate 2-fold lower  $IC_{50}$  value (Fig. 1D; 3 days  $IC_{50}$  0.300  $\mu$ M, 95% confidence interval (CI) 0.221-0.407 vs 0.611  $\mu$ M, 95% CI 0.425-0.878,  $P < 0.0001$ ).  $IC_{50}$  values of both compounds did not significantly change when HAC15 cells were treated for 1 day (Fig. 1A) or were stimulated with ACTH (Fig. 1B and 1E). ACTH stimulation resulted in a mean increase in cortisol of 34% and 61% after 1 and 3 days of incubation, respectively (Fig. 1C and 1F; both  $P < 0.0001$ ). In the conditions as previously mentioned, no effects on cell amounts were observed.

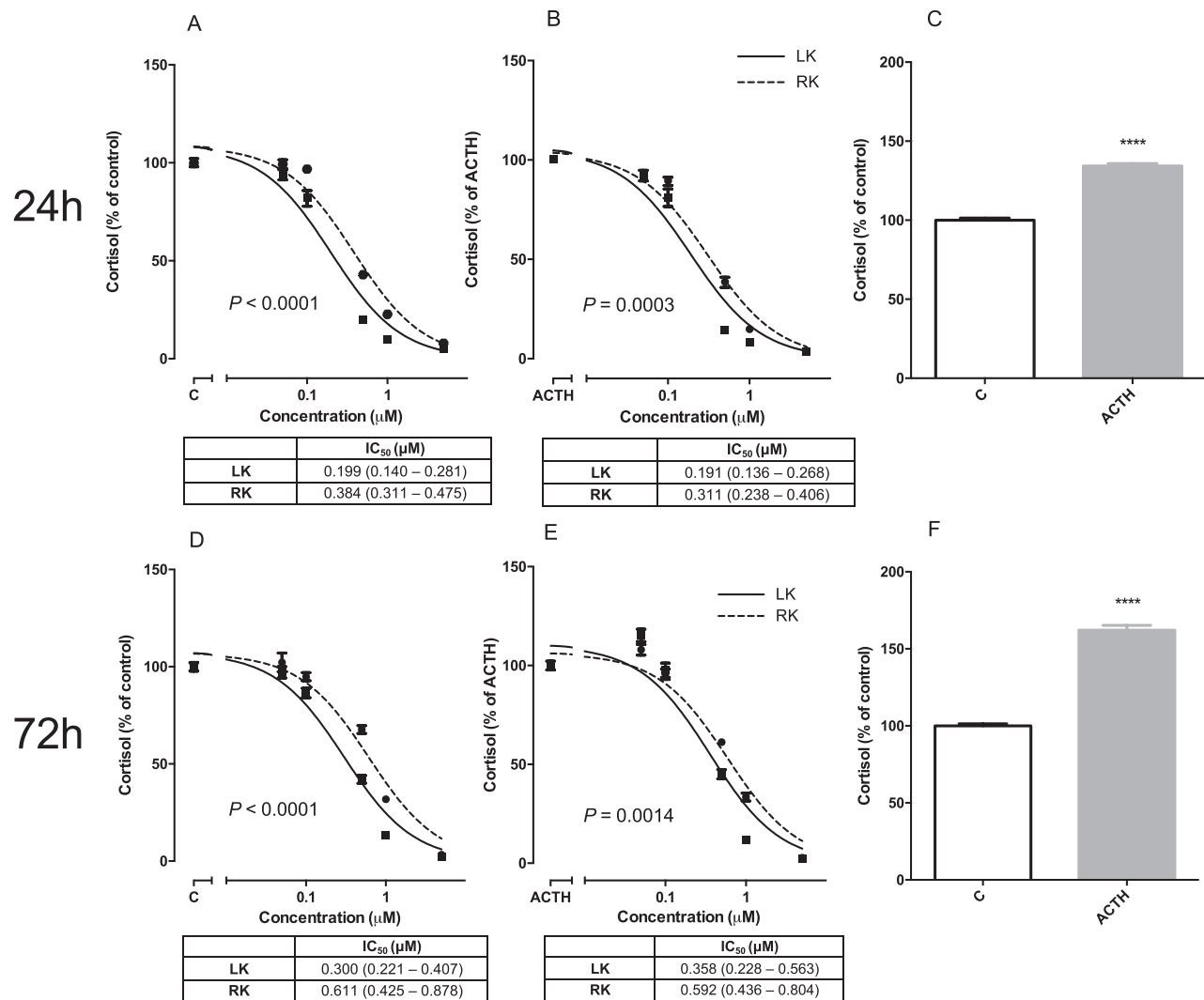
### Primary adrenocortical cultures

Characteristics of patients of whom a primary culture was obtained are listed in Table 1, with corresponding numbers that will be used to refer to throughout the Results section. Effects of levoketoconazole and racemic ketoconazole were assessed in 15 primary cultures of human adrenocortical tissue: 6 cortisol-producing ACA, 3 ACTH-dependent adrenal hyperplasias, 3 ACTH-independent adrenal hyperplasias and 3 cortisol-producing ACC. Measurement of DNA concentration (as a measure of cell amount) was performed in 28 of 37 primary adrenal culture plates and showed no effects of the drugs on cell number in these cultures at any of the concentrations tested.

$IC_{50}$  values and dose-response curves for cortisol production of both compounds in the different primary adrenocortical cultures are listed in Table 2 and shown in Fig. 2 and Supplementary Figure 1 (14).  $IC_{50}$  values for levoketoconazole in the basal condition in ACA primary cultures varied between 0.0631 and 0.140  $\mu$ M, whereas in ACTH-dependent adrenal hyperplasia the 3  $IC_{50}$  values varied between 0.0220 and 0.179  $\mu$ M. In the basal condition, the mean  $IC_{50}$  of levoketoconazole was 0.110  $\mu$ M (95% CI 0.0867-0.139) in ACA ( $n = 4$ ), 0.0562  $\mu$ M (95% CI 0.0336-0.0940;  $P = 0.0014$  vs ACA) in ACTH-dependent adrenal hyperplasia ( $n = 3$ ), and 0.0383  $\mu$ M (95% CI 0.0253-0.0578;  $P < 0.0001$  vs ACA) in ACC ( $n = 3$ ). In 8 of the 11 conditions in which a direct comparison between levoketoconazole and racemic ketoconazole could be made in the same patient, higher  $IC_{50}$  values were observed of racemic ketoconazole compared to levoketoconazole (mean percentage increase in  $IC_{50}$  vs levoketoconazole 116%, range 29%-303%). The difference, however, only reached statistical significance in 3 cultures corresponding to 2 patients (ACA no. 2 and ACTH-dependent adrenal hyperplasia no. 3). In the 3 remaining cultures,  $IC_{50}$  values were highly comparable between both compounds (mean difference in  $IC_{50}$  4%, range 1%-8%). Levoketoconazole also inhibited cortisol production in ACC cultures (Supplementary Figure 1 (14)). Cortisol production significantly increased in 9 of the 11 primary cultures with ACTH stimulation, varying from 34% to 2239% (Table 2). In 1 of the 6 primary cultures in which basal and ACTH stimulated levoketoconazole  $IC_{50}$  values could be compared, a lower  $IC_{50}$  value was observed under ACTH stimulation ( $P = 0.0095$ , ACC no. 3).

### Effects of racemic ketoconazole and levoketoconazole on the steroid hormone profile on adrenocortical cells

To determine the effects of levoketoconazole and racemic ketoconazole on steroid precursors and adrenal androgens,



**Figure 1. Dose-dependent effects of levoketoconazole and racemic ketoconazole on cortisol production by HAC15 cells in the basal condition and when stimulated with 10 nM ACTH after 24 h and 72 h of incubation.** Levoketoconazole (solid lines, ■) and racemic ketoconazole (dotted lines, ●) in the basal condition (A, D) and when stimulated with 10 nM ACTH (B, E) after 24 h (A, B) and 72 h (D, E) of incubation. Effects of ACTH after 24 h (C) or 72 h (F) of treatment. IC<sub>50</sub> values are depicted in micromolar with 95% confidence interval. P-value compares IC<sub>50</sub> value of levoketoconazole and racemic ketoconazole. Values are depicted as mean ± SEM and as percentage of vehicle treated control or ACTH stimulated HAC15 cells. \*\*\*\*P < 0.0001 vs control. Abbreviations: ACTH, adrenocorticotropic hormone; C, control LK, levoketoconazole; RK, racemic ketoconazole.

multiteroid analysis was carried out using LC-MS/MS (Fig. 3, Supplementary Tables 1 and 2 (14)).

#### HAC15 cells

In both the basal and ACTH-stimulated condition of HAC15, effects of treatment with levoketoconazole on the steroid profile were comparable. Except for DHEA, DHEAS, and testosterone, the production of all steroids statistically significantly increased under ACTH stimulation, varying from an increase of 7% of androstenedione to a 359% increase of corticosterone. In the ACTH stimulated condition, levoketoconazole and racemic ketoconazole significantly inhibited the production of all steroids, including cortisol (–22 nmol/L,

–65%) and 11-DOC (Fig. 3; –1524 nmol/L, –47%). In both conditions, levoketoconazole inhibited almost all steroids to a slightly greater extent compared to racemic ketoconazole (Fig. 3, HAC15; all P < 0.05), except DHEA, which was more strongly inhibited by racemic ketoconazole (Fig. 3, HAC15; P < 0.01). DHEAS was only inhibited more strongly in the basal condition under levoketoconazole (P < 0.05). To evaluate the overall effects of the compounds on the steroid profile, absolute changes were added together. The total sum of decrease of steroids was stronger under levoketoconazole compared to racemic ketoconazole treatment (basal: –3007 vs –1920 nmol/L; ACTH: –2813 vs –1590 nmol/L).

**Table 1.** Clinical and tumor characteristics of patients of whom a primary culture was obtained

Patient no.	Sex	Side	Age at surgery (yrs)	Size of lesion (cm)	Weiss score	Steroid production
<b>Cortisol-producing adrenocortical adenoma</b>						
No. 1	F	Left	57	2.5	0	Cortisol
No. 2	F	Left	67	4	0	Cortisol
No. 3	F	Left (Bilateral)	63	4.2	0	Cortisol
No. 4	F	Right	66	3.9	0	Cortisol
No. 5	M	Right	55	6.8	2	Cortisol
No. 6	F	Left	38	3.3	0	Cortisol
<b>ACTH-dependent adrenal hyperplasias</b>						
No. 1	F	Bilateral	79	-	-	Cortisol
No. 2	F	Left (Bilateral)	69	-	-	Cortisol
No. 3	F	Bilateral	32	-	-	Cortisol
<b>ACTH-independent hyperplasias</b>						
No. 1	F	Left (Bilateral)	50	-	-	Cortisol
No. 2	F	Left	73	-	-	Cortisol
No. 3	M	Left	66	-	-	Cortisol
<b>Adrenocortical carcinoma</b>						
No. 1	F	Right	61	5	9	Cortisol
No. 2	M	Left	64	18.5	7	Cortisol
No. 3	F	Right	67	15	8	Cortisol and androgens

ACTH-dependent adrenal hyperplasias are based on ectopic ACTH syndrome (no. 1 and 2) or an ACTH-secreting corticotroph pituitary adenoma (no. 3). (Bilateral) indicates that the lesion was bilateral, but only one side was used to obtain the primary culture.

Abbreviations: ACTH, adrenocorticotropic hormone; cm, centimeter; F, female patient; M, male patient; yrs, years.

### ACTH-dependent adrenal hyperplasia

The effects of both compounds on the steroid profile were tested in ACTH-dependent adrenal hyperplasia no. 1 and no. 3. In both cultures, the effects were studied in multiple concentrations, generally resulting in a dose-dependent effect on the components of the steroid profile (Supplementary Table 1 (14)). DHEA and DHEAS were below the limit of quantitation. In ACTH-dependent adrenal hyperplasia no. 1, ACTH stimulation resulted in an increase of corticosterone, 17-OHP, 11-DOC, cortisol, androstenedione, and testosterone (mean increase +96%; Supplementary Table 1 (14)). Progesterone levels decreased slightly (-11%). In the ACTH-stimulated condition at a concentration of 0.05  $\mu$ M (Fig. 3 Ectopic ACTH syndrome, EAS no. 1), levoketoconazole significantly inhibited cortisol (-235 nmol/L, -37%,  $P < 0.01$ ), androstenedione (-9.6 nmol/L, -50%;  $P < 0.0001$ ), and testosterone (-0.3 nmol/L, -45%,  $P < 0.01$ , all *vs* control). In contrast to the basal condition, corticosterone and 17-OHP accumulated after treatment with levoketoconazole under ACTH stimulation (+98 nmol/L, +29%,  $P = 0.01$ ; +8.3 nmol/L, +41%,  $P < 0.05$ ; respectively), whereas 11-DOC did not change (Fig. 3, EAS no. 1). When focusing on the difference between levoketoconazole and racemic ketoconazole in this condition, accumulation of progesterone (+167% *vs* +96%;  $P < 0.01$ ), and decrease of cortisol (-37% *vs* -11%;  $P < 0.05$ ) were stronger after exposure to levoketoconazole

(Fig. 3, EAS no. 1). In contrast, accumulation of 11-DOC was higher under racemic ketoconazole (+25% *vs* +1.6%;  $P < 0.01$  *vs* levoketoconazole). In the basal condition at 0.05  $\mu$ M, no statistically significant changes between both compounds were observed (Supplementary Table 1 (14)). The total change of steroids in the basal condition was roughly comparable between levoketoconazole and racemic ketoconazole (-343 *vs* -219 nmol/L, respectively), whereas under ACTH stimulation, the total sum of change of the steroids was a decrease of 139 nmol/L under levoketoconazole. In contrast, there was an increase of 125 nmol/L under racemic ketoconazole.

At 100 $\times$  higher concentration of 5  $\mu$ M of the drugs, all steroids except progesterone were strongly inhibited by both levoketoconazole and racemic ketoconazole (all decrease >66%; Supplementary Table 1 (14)) both in the basal- and the ACTH-stimulated condition. In both conditions, the total sum of change of the steroids was approximately comparable between levoketoconazole and racemic ketoconazole.

In ACTH-dependent adrenal hyperplasia no. 3, levoketoconazole 0.1  $\mu$ M decreased the concentrations of cortisol (-2975 nmol/L, -98%,  $P < 0.0001$ ), corticosterone (-165 nmol/L, -55%,  $P < 0.05$ ), 11-DOC (-209 nmol/L, -59%,  $P < 0.0001$ ), and the adrenal androgens androstenedione (-35 nmol/L, -98%,  $P < 0.0001$ ) and testosterone (-2.2 nmol/L, -96%,  $P < 0.0001$ ; Fig. 3, EAS

**Table 2.** Efficacy of levoketoconazole, and racemic ketoconazole on inhibition of cortisol production in human primary adrenocortical cultures

Diagnosis		Basal condition				ACTH simulated condition	
		Levoketoconazole	Racemic ketoconazole	ACTH stimulated cortisol (%)	Levoketoconazole	Racemic ketoconazole	
Cortisol-producing adrenocortical adenomas (ACA)	No. 1	■	0.116 (0.0762-0.177)	NT	NT	NT	NT
	No. 2	●	0.125 (0.0809-0.194)	0.266 (0.158-0.450) #	+145%****	0.188 (0.0937-0.378)	NT
	No. 3	◆	0.140 (0.0782-0.251)	0.138 (0.0700-0.274)	+66%****	0.187 (0.102-0.342)	0.241 (0.127-0.460)
	No. 4	○	0.0631 (0.0443-0.0899)	NT	+230****	0.0934 (0.0685-0.127)	0.0895 (0.0597-0.134)
	No. 5	□	NT	NT	+615****	NT	0.204 (0.116-0.360)
	No. 6	◇	NT	NT	+132%**	0.586 (0.272-1.267)	NT
ACTH-dependent hyperplasias	No. 1	■	0.0262 (0.00859-0.122)	0.0544 (0.0311-0.0951)	+228%****	0.0799 (0.0484-0.132)	0.106 (0.0262-0.428)
	No. 2	●	0.0220 (0.00986-0.0492)	0.0296 (0.00515-0.170)	NT	NT	NT
	No. 3	◇	0.179 (0.112-0.284)	0.661 (0.364-1.120)####	+6%	0.240 (0.176-0.326)	0.967 (0.326-2.87)####
ACTH-independent hyperplasias	No. 1	◆	Ambiguous	Ambiguous	+34%***	Ambiguous	Ambiguous
	No. 2	○	NT	NT	-14%*	Ambiguous	1.394 (0.2770-7.012)
	No. 3	□	NT	NT	+2239%****	0.117 (0.0763-0.180)	NT
ACC	No. 1	■	0.00578 (0.00270-0.0124)	NT	NT	NT	NT
	No. 2	○	0.0571 (0.0309-0.105)	0.0763 (0.0430-0.135)	NT	NT	NT
	No. 3	◆	0.0731 (0.0477-0.112)	0.0676 (0.0534-0.0857)	+67%*	0.0321 (0.0158-0.0651)	NT

IC<sub>50</sub> values are presented in micromolar (μM) after 3 days of treatment. ACTH (85 pM) stimulated cortisol represents the mean percentage increase of cortisol production compared to vehicle-treated control, with the applicable *P*-value. Column 3 represents the symbols used in Fig. 2 and Supplementary Figure 1 (14). Ambiguous means that the IC<sub>50</sub> value could not be calculated, because dose-response curves were not suitable. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001 vs vehicle treated control. #*P* < 0.05, ####*P* < 0.0001 vs IC<sub>50</sub> value of levoketoconazole in the same patient.

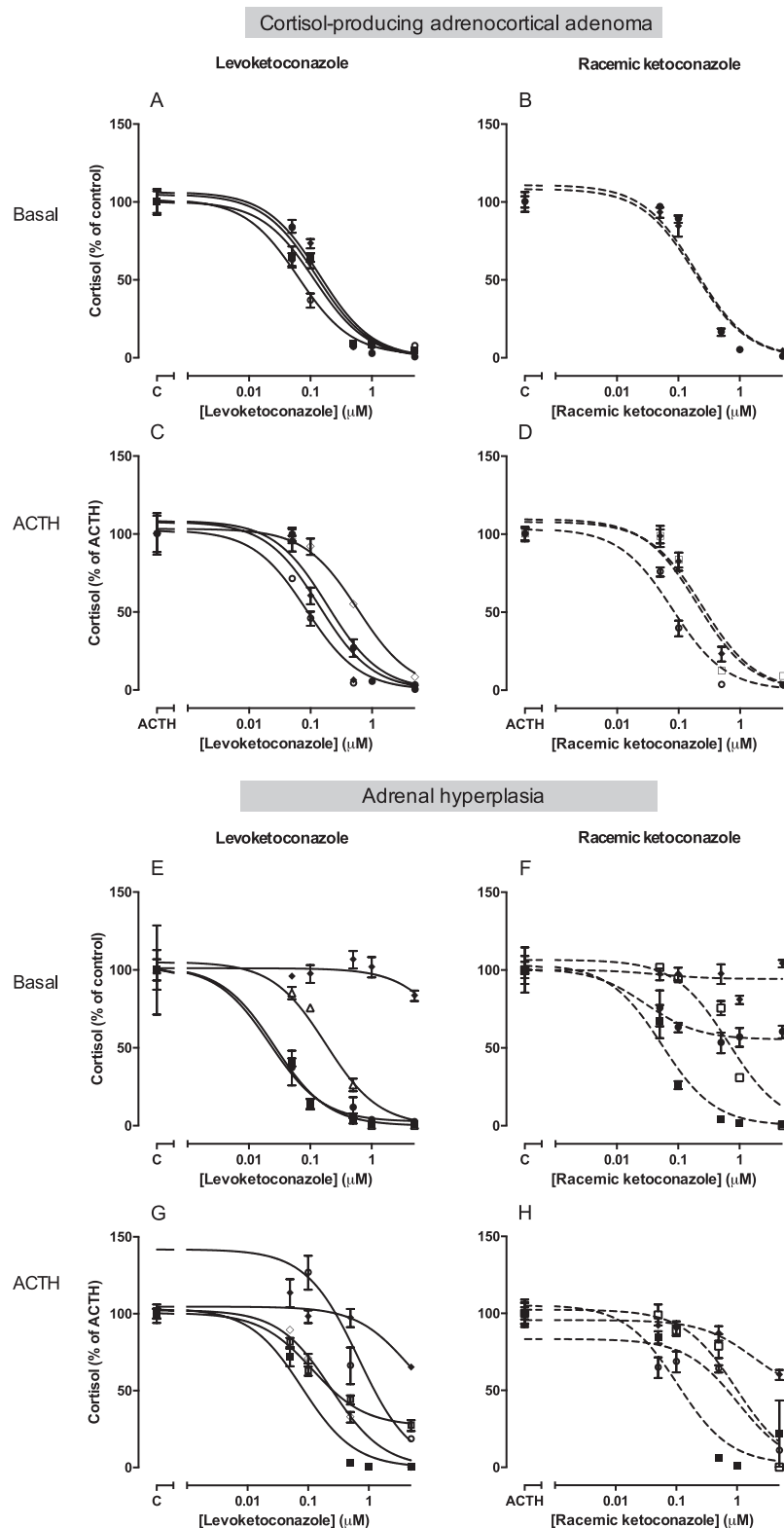
Abbreviations: ACC, adrenocortical carcinoma; ACTH, adrenocorticotropic hormone. NT, not tested.

no. 3). In contrast, progesterone and 17-OHP accumulated in this condition. Levoketoconazole suppressed the concentrations of cortisol (-98% vs -73%, *P* < 0.0001) and androstenedione (-98% vs -77%, *P* < 0.05) more strongly compared to racemic ketoconazole (Fig. 3, EAS no. 3; Supplementary Table 1 (14)). Racemic ketoconazole resulted in an increase in 11-DOC and corticosterone at this concentration compared to a decrease under treatment with levoketoconazole (Fig. 3, EAS no. 3). Accumulation of progesterone was furthermore stronger

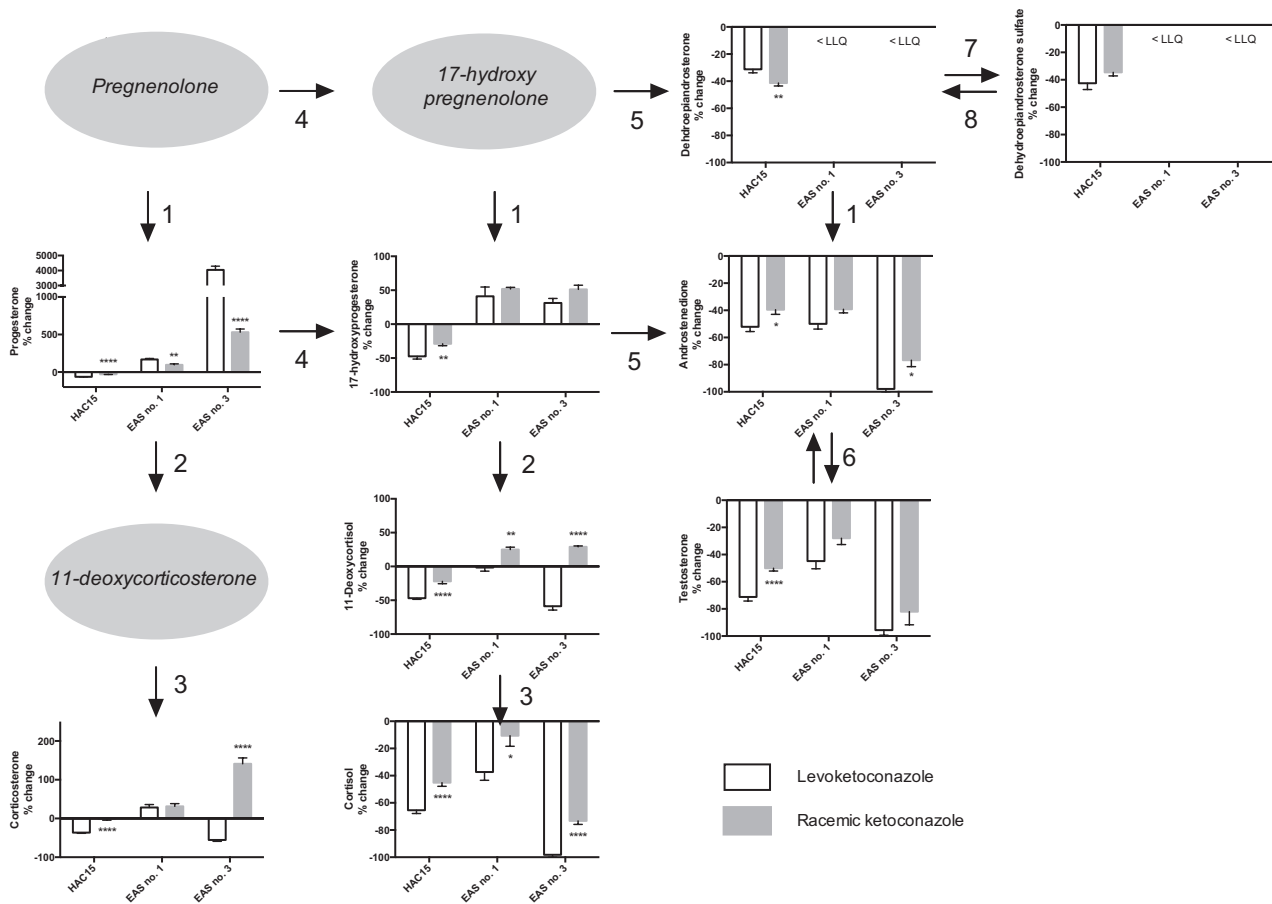
under treatment with levoketoconazole (+4041% vs +532%, *P* < 0.0001).

At lower concentrations, approximately the same tendency was observed, although differences between levoketoconazole and racemic ketoconazole were most pronounced at 0.1 μM (Supplementary Table 1 (14)). Besides, at lower concentrations of levoketoconazole, corticosterone accumulated instead of decreased. At all concentrations, the absolute decrease in concentration of steroids was stronger for levoketoconazole compared to racemic





**Figure 2. Dose-dependent effects of levoketoconazole and racemic ketoconazole on cortisol production in primary human adrenocortical cultures.** Levoketoconazole (left panel, solid lines) and racemic ketoconazole (right panel, dotted lines). Upper panel represents cortisol-producing adrenal adenoma cultures and lower panel primary adrenal hyperplasia cultures, both ACTH dependent and independent. No  $IC_{50}$  values were calculated from the dose-response curves that did not reach a bottom (E, F), or had a top of the curve above 100% (G). The symbols correspond to the symbols as presented in Table 2 and thus correspond to the same ACA or adrenal hyperplasia patient. Basal cultures represent dose-response curves compared to vehicle treated control (A, B, E, F). Panels C, D, G, H show results after ACTH stimulation (85 pM). Values are depicted as mean  $\pm$  SEM and as percentage of vehicle treated control. Abbreviations: ACTH, adrenocorticotropic hormone. C, control.



**Figure 3. Effects of levoketoconazole and racemic ketoconazole on the steroid hormone profile in three different adrenocortical cultures.** Levoketoconazole (white bars) and racemic ketoconazole (grey bars). The displayed conditions were chosen based on the most pronounced differences between levoketoconazole and racemic ketoconazole and were different for HAC15 (ACTH stimulation, concentration 0.5  $\mu$ M), ectopic ACTH syndrome associated (ACTH-dependent) adrenal hyperplasia no. 1 (EAS no. 1; ACTH stimulation, concentration 0.05  $\mu$ M), and no. 3 (EAS no. 3; basal condition, concentration 0.1  $\mu$ M). Numbers of the primary cultures correspond to the numbers in Tables 1 and 2. Arrows represent steroidogenic enzymes: (1)  $3\beta$ -hydroxysteroid dehydrogenase, (2) CYP21A2, (3) CYP11B1, (4) CYP17A1 hydroxylase, (5) CYP17A1 lyase, (6)  $17\beta$ -hydroxysteroid dehydrogenase III, (7) sulfotransferase, and (8) steroid sulfatase. Values are depicted as percentage change  $\pm$  SEM compared to ACTH stimulation (HAC15 and EAS no. 1) or vehicle treated control (EAS no. 3). Note the difference in scale of the y-axes. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, and \*\*\*\* $P$  < 0.0001 vs the effect of levoketoconazole. Abbreviations: ACTH, adrenocorticotropic hormone; LLQ, lower limit of quantitation.

ketoconazole (0.01  $\mu$ M,  $-925$  vs  $-251$  nmol/L; 0.05  $\mu$ M,  $-2020$  vs  $-466$  nmol/L; 0.1  $\mu$ M,  $-3272$  vs  $-1827$  nmol/L).

### Cortisol-producing adrenocortical adenoma

In ACA primary culture no. 2, two concentrations (0.1 and 0.5  $\mu$ M) of levoketoconazole and racemic ketoconazole were tested in the basal condition (Supplementary Table 2 (14)). At 0.1  $\mu$ M, levoketoconazole inhibited cortisol and androstenedione more potently compared to racemic ketoconazole ( $-40\%$  vs  $-14\%$ ,  $P$  < 0.01 and  $-79\%$  vs  $-66\%$ ,  $P$  < 0.05, respectively), while corticosterone accumulated more strongly under racemic ketoconazole ( $+213\%$  vs  $+54\%$ ,  $P$  < 0.0001). The total change of steroids at 0.1  $\mu$ M was a decrease of 229 nmol/L under levoketoconazole, whereas there was an increase of 56 nmol/L by racemic ketoconazole. At a 5 $\times$ -higher concentration of 0.5  $\mu$ M, the

same tendency was observed, although with a more pronounced absolute change of all steroids in both up- and downward directions by both compounds (Supplementary Table 2 (14)). No difference was observed in the total sum of change of the steroids between levoketoconazole and racemic ketoconazole.

### Effects of levoketoconazole and racemic ketoconazole on corticotroph pituitary cells

Levoketoconazole and racemic ketoconazole inhibited cell number after 3 and 7 days of treatment in corticotroph pituitary murine AtT20 cells (Fig. 4A and 4B), whereas no effect was seen after 24 h of treatment (data not shown). IC<sub>50</sub> values for inhibition of cell number after 7 days were 1.05  $\mu$ M (95% CI 0.576-1.91)

and 5.81  $\mu\text{M}$  (95% CI 0.948-35.5) for levoketoconazole and racemic ketoconazole, respectively ( $P = 0.0892$ ). Only levoketoconazole showed inhibition of ACTH secretion, corrected for cell amount, after 3 days of treatment ( $P = 0.0436$  vs racemic ketoconazole), where both levoketoconazole and racemic ketoconazole inhibited ACTH secretion after 7 days of treatment (Fig. 4E and 4F). Maximal inhibition of ACTH secretion after 7 days of treatment with 10  $\mu\text{M}$  was 38% and 34% for levoketoconazole and racemic ketoconazole, respectively (Fig. 4F).

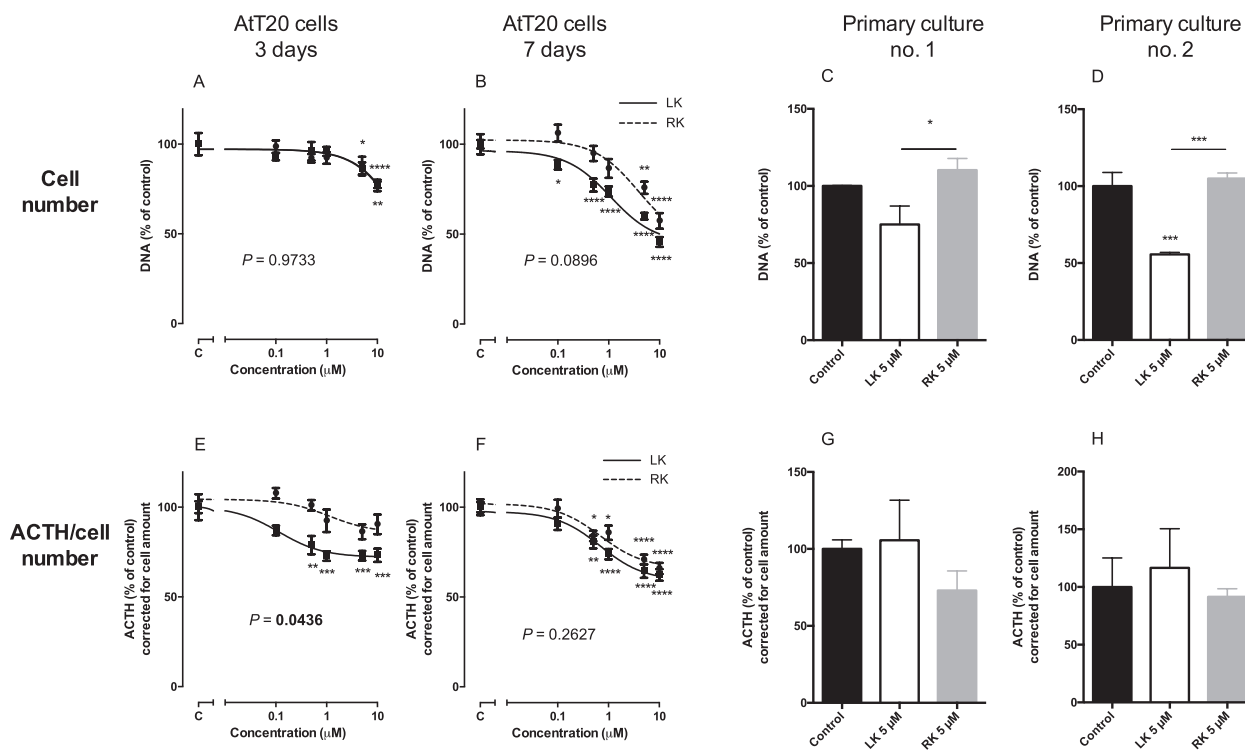
In 2 primary ACTH-secreting corticotroph pituitary adenoma cultures, the effects of levoketoconazole and racemic ketoconazole were examined on both cell amount and ACTH secretion after 7 days of treatment. In primary culture no. 2, levoketoconazole significantly inhibited cell number after 7 days of treatment ( $P < 0.001$  vs control; Fig. 4D). In both cultures, there was a significant difference between levoketoconazole and racemic ketoconazole, favoring a stronger effect by levoketoconazole (Fig. 4C and 4D). No effects were observed on ACTH secretion corrected for cell number

after 7 days of treatment in both primary cultures (Fig. 4G and 4H).

## Discussion

Ketoconazole is frequently used for medical treatment of CS, but this is often accompanied by serious adverse effects, including gastrointestinal complaints and hepatotoxicity. Levoketoconazole, the 2S,4R enantiomer of ketoconazole, might have a favorable toxicity profile, a higher potency, and a lower hepatic metabolism (6,7). To the best of our knowledge, this is the first study evaluating the direct effects of levoketoconazole on primary human adrenocortical cell cultures. We show that levoketoconazole is a potent inhibitor of cortisol secretion and might be more potent compared to racemic ketoconazole *in vitro*.

The basis for interest in this purified form of racemic ketoconazole involves a study of Rotstein et al, showing large differences in selectivity for inhibition of the cytochromes P450 involved in steroid synthesis by different stereoisomers of ketoconazole (7). In HAC15 cells, we found a 2-fold lower  $\text{IC}_{50}$  value for inhibition of cortisol



**Figure 4.** Effects of levoketoconazole and racemic ketoconazole on cell amount and ACTH secretion corrected for cell amount in mouse pituitary AtT20 cells and in 2 primary human corticotroph pituitary adenoma cultures. Effects of levoketoconazole (solid lines, ■) and racemic ketoconazole (dotted lines, ●) on cell amount (upper row, A-D) and ACTH secretion corrected for cell amount (bottom row, E-H). Primary cultures were incubated with treatment of levoketoconazole or racemic ketoconazole for 7 days. Values are depicted as mean  $\pm$  SEM and as percentage of vehicle treated control. P-values compare dose response curves of levoketoconazole and racemic ketoconazole in AtT20 cells. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$  vs control or as stated by the lines. Abbreviations: LK, levoketoconazole; RK, racemic ketoconazole.

production by levoketoconazole compared to racemic ketoconazole, indicating that only the 2S,4R enantiomer contributes to inhibition of cortisol. In primary human adrenocortical cultures, levoketoconazole also appears to be a potent inhibitor of cortisol secretion. Sensitivity to levoketoconazole seems to be slightly higher compared to racemic ketoconazole in primary cultures as well, although the difference only reached statistical significance in two patients. We also demonstrate that potency of levoketoconazole is highly variable between patients and tissue specimens with a 24-fold difference in  $IC_{50}$  value, indicating that there might also be heterogeneity in response to levoketoconazole in clinical studies, due to differences in sensitivity at the cellular level. Sensitivity to racemic ketoconazole varied with a 10-fold difference in  $IC_{50}$ . A direct comparison is difficult, because this was partly based on other primary cultures. To date, no research has been performed yet focusing on determinants of sensitivity to stereoisomers of ketoconazole on cellular level. Direct effects might be stronger in ACC and hyperplasia compared to ACA, implying tissue entity specific effects. However, these differences have to be interpreted with caution, considering the relatively low number of cultures. Previously, a single-nucleotide polymorphism in the CYP17A1 gene has been shown to be associated with the response to ketoconazole and metyrapone in CS patients (15). Considering the small sample size and individual dose titration schemes, these results have to be confirmed in larger populations. Additional underlying hypothetical explanations of variable sensitivity include differences in basal enzyme levels between specimens and tissues, other genetic abnormalities, differences in breakdown of levoketoconazole in the cell or cell-dependent differences in uptake. Further research could focus on elucidating this issue in an attempt to make the first step toward selecting patients in whom ketoconazole enantiomers are most effective.

In a pharmacokinetic study investigating administration of a relatively low concentration of 200 mg ketoconazole in healthy volunteers, plasma concentrations up to 11  $\mu$ M could be reached (16). At therapeutic concentrations, plasma levels can thus be expected even higher. Furthermore, plasma levels of levoketoconazole are expected to be higher compared to racemic ketoconazole, since it has been suggested that liver extraction of this enantiomer is lower (6). Effects as observed in the present study were found at even lower concentrations, which implies that these effect can be observed *in vivo* as well based on the concentrations.

To obtain insights into the mechanism of action of levoketoconazole on adrenal steroidogenesis, multisteroid analysis by LC-MS/MS was used. For reliable measurements,

it seems essential to select primary adrenocortical cultures with no molecular alterations. For example, in 35% to 65% of the cortisol-producing ACA, recurrent activating mutations in protein kinase 3',5'-cyclic adenosine 5'-monophosphate-activated catalytic subunit alpha, encoding the catalytic subunit  $\alpha$  of protein kinase A, have recently been identified (17). This suggests that ACTH-dependent adrenal hyperplasias include the most suitable candidate specimens. From measurement of the steroid profile, it appears that differences between levoketoconazole and racemic ketoconazole are most pronounced at concentrations approximating the  $IC_{50}$  value for cortisol inhibition. Maximum inhibitory effects seem to be highly comparable. We show that effects of levoketoconazole on the steroid profile may be variable dependent on the adrenocortical culture and condition; in some cases the production of all steroids is inhibited, whereas in other conditions there is accumulation of progesterone, corticosterone, 17-OHP, and 11-DOC. These differences might be related to the relative amounts of the various steroidogenic enzymes in the tissue samples. The changes in the steroid profiles suggest that levoketoconazole inhibits several steroidogenic enzymes. Furthermore, the effects of levoketoconazole and racemic ketoconazole seem overall relatively comparable. Differences in percentage change are subtle, although consistently favoring a more potent effect of levoketoconazole compared to racemic ketoconazole. In HAC15 cells, adrenal androgens are inhibited more strongly by levoketoconazole, and this was confirmed only in ACTH-dependent adrenal hyperplasia no. 3 and cortisol-producing ACA no. 2 for androstenedione. In male patients, inhibition of adrenal or testicular androgen production by ketoconazole can result in hypogonadism and gynecomastia (18,19). Exact percentages are however unknown. Long-term treatment with ketoconazole only minimally affects testosterone levels, potentially explaining the few androgen-related reported side effects (19).

The absence of strong accumulation equal to the total sum of inhibition of steroids, suggests an inhibition of the proximal steps of the steroid biosynthetic pathway, like cholesterol side chain cleavage enzyme or steroidogenic acute regulatory protein. We hypothesize that the extent of this proximal inhibition might be higher for levoketoconazole compared to racemic ketoconazole, as demonstrated by a greater negative balance for levoketoconazole in the majority of adrenocortical cultures. We do have to acknowledge that we did not measure all steroids of the profile, which can influence the balance. Thereby, a direct comparison between the 2 compounds was only possible in a subset of primary cultures due to limited yield of cells at isolation.

In both ACTH-dependent adrenal hyperplasias in which the steroid hormone profile was measured, there is a trend toward accumulation of corticosterone at lower concentrations and a decrease at higher concentrations of levoketoconazole and racemic ketoconazole. This implies that specificity of both compounds for inhibition of steroidogenic enzymes is concentration-dependent. In a study in which human adrenal tissue slices were incubated with ketoconazole, it has been shown that CYP17A1 lyase is inhibited at the lowest concentration (IC<sub>50</sub> 2 μM), followed by CYP17A1 hydroxylase (IC<sub>50</sub> 18 μM), CYP11B2 (18-hydroxylase, IC<sub>50</sub> 28 μM), and CYP11B1 (IC<sub>50</sub> 35 μM) (20). The relatively potent inhibition of CYP17A1 might explain the difference in effect between corticosterone and cortisol and, furthermore, the accumulation of corticosterone at lower concentrations.

We also demonstrated that both levoketoconazole and racemic ketoconazole affect corticotroph ACTH-secreting cells. The inhibitory effect of ketoconazole on ACTH secretion by pituitary adenomas has been described before, showing decreased ACTH secretion in 2 primary human corticotroph pituitary adenoma cultures (21). This might be mediated via inhibition of 3',5'-cyclic adenosine 5'-monophosphate formation, as was demonstrated to be the case in rat pituitary cells (22). The inhibitory effect of ketoconazole on corticotroph ACTH secretion could be one of the explanations of the unexpected absence of increased ACTH in a subset of patients with a corticotroph pituitary adenoma treated with ketoconazole for a longer period (18). In our study, levoketoconazole and racemic ketoconazole inhibited cell growth and ACTH production corrected for cell amount in a dose- and time-dependent manner in AtT20 cells. Furthermore, in 1 of the 2 human corticotroph pituitary adenoma cultures, levoketoconazole inhibited cell growth, whereas this effect was not observed after treatment with racemic ketoconazole. No effect was observed on ACTH secretion in these 2 corticotroph pituitary adenoma cultures, which might be due to the applied correction for cell amount.

In conclusion, we show that levoketoconazole is a potent inhibitor of cortisol secretion in primary human adrenocortical cells, and that levoketoconazole might inhibit steroidogenesis more potently compared to racemic ketoconazole. In addition, levoketoconazole may have pituitary-directed effects. Together with the previously reported potential advantages of increased efficacy in vivo, a favorable safety profile, and increased therapeutic index, this makes levoketoconazole a very promising novel treatment option for CS.

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## Additional Information

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**Data Availability:** Some or all data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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