Abiraterone Acetate for Cushing Syndrome: Study in a Canine Primary Adrenocortical Cell Culture Model

Karin Sanders,1 Wesley L. de Wit,1 Jan A. Mol,1 Max Kuribaum,2,3 Sabine Kendl,2,3 Matthias Kroiss,2,3,4 Hans S. Kooistra,1 and Sara Galac1

1Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, 3584 CM Utrecht, Netherlands; 2University Hospital Würzburg, Core Unit Clinical Mass Spectrometry, 97080 Würzburg, Germany; 3University Hospital Würzburg, Department of Internal Medicine I, Division of Endocrinology and Diabetology, 97080 Würzburg, Germany; and 4Comprehensive Cancer Center Mainfranken, University of Würzburg, 97080 Würzburg, Germany

ORCID numbers: 0000-0001-9634-9853 (K. Sanders); 0000-0002-4831-4995 (S. Galac).

Abiraterone acetate (AA) is a potent inhibitor of steroidogenic enzyme 17α-hydroxylase/17,20-lyase (CYP17A1). AA is approved for the treatment of prostate cancer but could also be used to treat patients with Cushing syndrome (CS). Similar to humans, canine glucocorticoid synthesis requires CYP17A1, providing a useful animal model. The objective of this study was to preclinically investigate the effect of AA on adrenocortical hormone production, cell viability, and mRNA expression of steroidogenic enzymes in canine primary adrenocortical cell cultures (n = 9) from the adrenal glands of nine healthy dogs. The cells were incubated with AA (0.125 nM to 10 μM) for 72 hours under basal conditions and with 100 nM ACTH(1-24). Adrenocortical hormone concentrations were measured in culture medium using liquid chromatography–mass spectrometry, RNA was isolated from cells for subsequent real-time quantitative PCR analysis, and cell viability was assessed with an alamarBlue™ assay. AA reduced cortisol (IC50, 21.4 ± 4.6 nM) without affecting aldosterone under basal and ACTH-stimulated conditions. AA increased progesterone under basal and ACTH-stimulated conditions but reduced corticosterone under basal conditions, suggesting concurrent inhibition of 21-hydroxylation. AA did not affect the mRNA expression of steroidogenic enzymes and did not inhibit cell viability. In summary, primary canine adrenocortical cell culture is a useful model system for drug testing. For the treatment of CS, AA may to be superior to other steroidogenesis inhibitors due to its low toxicity. For future in vivo studies, dogs with endogenous CS may provide a useful animal model. (Endocrinology 159: 3689–3698, 2018)

Cushing syndrome (CS) is a serious endocrine disorder that results from chronic exposure to excessive glucocorticoids. Endogenous CS is most commonly (~70%) caused by an ACTH-producing pituitary adenoma (Cushing disease) and second most commonly (~20%) by a primary functional adrenocortical tumor (1, 2). When not properly treated, CS is associated with increased morbidity and mortality rates (3).

Medical treatment options target the pituitary (caber-goline, pasireotide), the adrenal glands (ketoconazole, metyrapone, mitotane), or the glucocorticoid receptor (mifepristone). Response to medical treatment targeting the adrenal gland is highly variable (4). All currently available medical treatments have serious shortcomings (e.g., ketoconazole may lead to serious hepatitis, metyrapone may lead to disabling androgen and mineralocorticoid synthesis and requires frequent dosing, and mitotane has serious side effects and requires diligent monitoring) (5, 6). To expand the range of future treatment options, new medical agents with high selectivity and tolerability are needed.

ISSN Online 1945-7170
Copyright © 2018 Endocrine Society
Received 15 June 2018. Accepted 7 September 2018.
First Published Online 13 September 2018

Abbreviations: AA, abiraterone acetate; CS, Cushing syndrome; CYP11A1, cytochrome P450 side chain cleavage; CYP11B1, 11b-hydroxylase; CYP17A1, 17a-hydroxylase/17,20-lyase; D4A, Δ4-abiraterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DMSO, dimethyl sulfoxide; HSD3B2, 3b-hydroxysteroid dehydrogenase 2; LC-MS/MS, liquid chromatography–tandem mass spectrometry; mCRPC, metastatic castration-resistant prostate cancer; PR, progesterone receptor; RT-qPCR, quantitative RT-PCR.

doi: 10.1210/en.2018-00588

An interesting target for selective inhibition of cortisol production is the steroidogenic enzyme 17α-hydroxylase/17,20-lyase (CYP17A1), which plays an important role in glucocorticoid synthesis but not in mineralocorticoid synthesis (7). This enzyme is also required for adrenal androgen production, which stimulates tumor growth in patients with metastatic castration-resistant prostate cancer (mCRPC) (8). To inhibit androgen synthesis in these patients, the potent CYP17A1 inhibitor abiraterone acetate (AA) was approved as treatment in 2011 in Europe and the United States (9, 10). Although generally well tolerated, AA induces hypocortisolism with a concomitant rise in circulating ACTH concentration in patients with mCRPC, which necessitates coadministration of glucocorticoids to reduce the associated side effects (11). Although the concurrent inhibition of cortisol production with AA is unintentional in patients with mCRPC, this offers interesting opportunities for the treatment of patients with CS.

Testing new treatment options for use in humans with CS is hampered by the limited presence of suitable cell culture systems and in vivo models. Most in vitro studies in the field have used the human adrenocortical carcinoma (ACC) NCI-H295 cell line. Only recently, three novel ACC cell lines have been described; however, these cell lines are available in only a few laboratories (12, 13). Clinical trials are impeded by the low estimated incidence of 0.5 to 5.0 cases per million people per year (14). In contrast, spontaneous CS is quite common in dogs, with an estimated incidence of 1 to 2.5 cases per 1000 dogs per year (15, 16). Canine CS shows remarkable similarities to human CS, including the etiology, clinical presentation, and treatment options, and can therefore be regarded as a spontaneous animal model for human CS (17, 18). Glucocorticoid synthesis in dogs also requires the AA target CYP17A1, which is not the case in, for example, adult rats, mice, and rabbits (19). To elucidate how AA affects adrenocortical cells, we hypothesized that an in vitro system with cultured canine primary adrenocortical cells may convey information that is useful for subsequent in vivo studies.

In this study, we evaluated whether and how AA affects the hormone production, mRNA expression of steroidogenic enzymes, and cell viability of canine primary adrenocortical cells under basal and ACTH-stimulated conditions.

**Materials and Methods**

**Animals and tissues**

The adrenal glands of 10 healthy dogs (one for a pilot experiment, nine for the following experiments) that were euthanized for reasons unrelated to the current study were used. The current study was approved by the Ethical Committee of Utrecht University. The median age was 17 months (range, 14 to 18 months), and the median body weight was 25 kg (range, 11 to 26 kg). Three dogs were male, and seven were female; all of the dogs were sexually intact. Seven dogs were of mixed breed; three dogs were beagles. The adrenal glands were collected within 10 minutes after euthanasia.

**Primary cell cultures**

Cell suspensions were prepared as described previously (20). In brief, the adrenal cortices were enzymatically digested in a collagenase solution, filtered, washed, and centrifuged. The cells were diluted to 1 × 10⁵ cells/mL and seeded in Multwell-96-well (100 µL per well) or 24-well (1 mL per well) plates (Primaria™; Corning, Amsterdam, Netherlands). The cells were cultured in a mixture of DMEM F-12 (Gibco, Invitrogen, Breda, Netherlands) with 1% Insulin-Transferrin-Selenium (Gibco), 0.125% BSA, 2.5% Nu-Serum (Corning), and 1% penicillin/streptomycin (Gibco). The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and were left to attach for 4 to 6 days prior to compound incubations.

**Cell treatments**

AA (Sigma-Aldrich, Zwijndrecht, Netherlands) was dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany). To determine the optimal incubation time, one adrenocortical cell culture was incubated for 24, 48, 72, and 96 hours in quadruplicate with increasing concentrations of AA (eight concentrations, 0.125 nM to 10 µM in fivefold dilution series) or with DMSO as vehicle control and coincubated with 100 nM synthetic ACTH(1-24) (Synacthen; Novartis Pharma BV, Arnhem, Netherlands) to induce hypercortisolism. The DMSO end concentration was 0.1% in all conditions. Cortisol concentrations were measured in the culture medium with RIA as described previously (21). To determine the IC₅₀ and maximal inhibitory concentration of AA, nine adrenocortical cell cultures were incubated in quadruplicate with AA or with DMSO as vehicle control for 72 hours and coincubated with 100 nM synthetic ACTH(1-24). Cortisol concentrations were measured in the culture medium with RIA as described previously (21). In the following experiments, the cells were treated in duplicate [quantitative RT-PCR (RT-qPCR) and steroidomics] or quadruplicate (cell viability assay) with 16 nM (approximate IC₅₀) or 2 µM (maximal inhibition) of AA or with DMSO as vehicle control. The cells were coincubated with (ACTH-stimulated cell cultures) and without (basal conditions) 100 nM synthetic ACTH(1-24) to induce hypercortisolism.

**Steroidomics**

After 72 hours of incubation, steroid hormones were measured in the culture medium with liquid chromatography–tandem mass spectrometry (LC-MS/MS). The LC-MS/MS instrumentation consisted of a 6500+ QTRAP (SCIEX®, Framingham, MA) MS-system coupled to a 1290 HPLC system (G4226A autosampler, infinityBinPump, G1316C column-oven, G1330B thermostat; Agilent Technologies, Santa Clara, CA). Analysis was performed with a commercial kit (MassChrom-Steroids in Serum/Plasma®; Chromsystems®, Gräfelfing, Germany) for determination of 13 steroid hormones (MRM-Mode, isotope standards) according to the manufacturer’s instruction. After an off-line solid-phase extraction extraction procedure of 500 µL culture medium, 5 µL were used for analysis. Concentrations were calculated with
100 nM ACTH(1-24) for 72 hours. Data are shown as mean ± SEM of nine individual cell cultures unless indicated otherwise.

Results

Optimal incubation time and concentrations

In the pilot experiment AA most notably inhibited the cortisol production after 72 and 96 hours of incubation (Fig. 1A). To determine the IC50 and maximal inhibitory concentrations, nine adrenocortical cell cultures were incubated with increasing concentrations of AA and with ACTH for 72 hours (Fig. 1B). The mean IC50 of AA was 21.4 ± 4.6 nM (range, 3.0 to 39.8 nM). Because the mean IC50 most closely resembled 16 nM in the dilution series and the maximal inhibition of cortisol production was seen at 2000 nM AA, the following experiments were performed using 0, 16, and 2000 nM AA.

Cell viability

Cell viability was assessed after 72 hours of incubation by using the alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, Breda, Netherlands) as described previously (20). In brief, the cells were incubated with the alamarBlue reagent in the dark at 37°C for 4 hours, after which fluorescence was measured at 535 nm excitation and 595 nm emission.

Statistical analysis

The IC50 values of AA on cortisol production measured with RIA were calculated with sigmoidal dose-response curves using SigmaPlot (Systat Software, San Jose, CA). All hormone concentrations that were nondetectable (specified in the text when applicable) were assigned a value of 0.5 times the quantification limit for statistical analysis. The data were tested for normality using the Shapiro-Wilk test. Normally distributed data (viability data) were analyzed with repeated measures ANOVA with a post hoc Bonferroni correction. Sphericity was checked with Mauchly test, which showed no significant variances of differences. Nongaussian data (RT-qPCR and hormone data) were analyzed with the Friedman test. Significant differences were analyzed post hoc with the Wilcoxon signed-rank test with a Bonferroni correction. P values <0.05 were considered significant. Data are reported as mean ± SEM of nine individual cell cultures unless indicated otherwise.
AA inhibits glucocorticoid production

AA at concentrations of 16 and 2000 nM significantly reduced the cortisol concentration in the culture medium of cell cultures under basal conditions (i.e., without ACTH stimulation) and of ACTH-stimulated cell cultures (Fig. 2). Under basal conditions, the cortisol concentration was undetectable in three out of nine cell cultures incubated with 16 nM AA and in four out of nine cell cultures incubated with 2000 nM. The cortisol precursor 11-deoxycorticosterone concentration was dose-dependently reduced by AA under basal conditions and by 2000 nM in the ACTH-stimulated cell cultures (Fig. 2). The concentration of cortisone was reduced by both AA concentrations under basal conditions and dose-dependently in the ACTH-stimulated cell cultures (Fig. 2).

AA inhibits corticosterone production only under basal conditions but does not affect aldosterone production

Although CYP17A1 is not required for corticosterone production, 2000 nM AA significantly reduced the corticosterone concentration in cell cultures under basal conditions (Fig. 2). In contrast, in the ACTH-stimulated cell cultures, AA slightly but significantly increased the corticosterone concentration at 16 nM but did not affect the corticosterone concentration at 2000 nM (Fig. 2). The aldosterone concentration was unaffected by AA under both basal and ACTH-stimulated conditions (Fig. 2).

AA increases progesterone production and inhibits 17-OH–progesterone production

The concentration of progesterone, a substrate of CYP17A1, was dose-dependently increased by AA under both basal and ACTH-stimulated conditions (Fig. 2). Subsequent 17-hydroxylation of progesterone to 17-OH–progesterone was inhibited by AA because AA dose-dependently reduced the 17-OH–progesterone concentration in the cell cultures under basal conditions and at 2000 nM in the ACTH-stimulated cell cultures (Fig. 2).

AA inhibits androstenedione production when stimulated with ACTH

The concentration of androstenedione, an adrenal androgen precursor, was significantly reduced in cell cultures under basal conditions by 16 nM AA but was unaffected by 2000 nM AA (Fig. 2). In the ACTH-stimulated cell cultures, AA dose-dependently reduced the androstenedione concentration (Fig. 2). The androstenedione concentration was undetectable in four out of nine ACTH-stimulated cell cultures incubated with 2000 nM AA.

The concentrations of dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), estradiol, testosterone, and DHT were measured, but their concentrations were either below the detection limit (DHEA, DHEAS, DHT) or similar to the concentration that was measured in cell-free culture medium (estradiol, testosterone) in all or in the majority of the cell cultures and were therefore excluded from further analyses.

AA inhibits 17-hydroxylation, 21-hydroxylation, and 11β-hydroxylation

To determine why AA increased the concentration of progesterone but not of corticosterone, we performed additional measurements of 11-deoxycorticosterone in a small number of key samples (single measurements of 0, 16, and 2000 nM conditions of four adrenocortical cell cultures under basal and ACTH-stimulated conditions). Under basal conditions, AA reduced the mean 11-deoxycorticosterone concentration (0 nM AA, 12.9 ± 5.5 nmol/mg protein; 2000 nM AA, 4.8 ± 0.9 nmol/mg protein), whereas under ACTH-stimulated conditions AA increased the mean 11-deoxycorticosterone concentration (0 nM AA, 16.8 ± 10.9 nmol/mg protein; 2000 nM AA, 33.5 ± 3.1 nmol/mg protein). We calculated the ratios relative to the 0 nM control of 17-OH–progesterone/progesterone (17-hydroxylase), 11-deoxycorticosterone/progesterone (21-hydroxylase), and progesterone/11-deoxycorticosterone (11β-hydroxylase) (Fig. 3). As expected, 2000 nM AA substantially decreased the 17-OH–progesterone/progesterone ratio (17-hydroxylase) for adrenocortical cell cultures under basal and ACTH-stimulated conditions. In addition, to a lesser extent AA decreased the ratios of 11-deoxycorticosterone/ progesterone (21-hydroxylase) and corticosterone/11-deoxycorticosterone (11β-hydroxylase).

The mRNA expression of steroidogenic enzymes

The AA target CYP17A1 was expressed in all cell cultures under basal conditions, and the addition of ACTH significantly increased its expression (Table 1). The same was true for the other key steroidogenic enzymes. The mRNA expression of the steroidogenic enzymes was unaffected by either AA concentration, except for that of CYP11A1 under basal cell culture conditions, which was significantly higher at 2000 nM AA (Fig. 4).

AA does not inhibit cell viability

The cell viability was not inhibited by AA under basal (Fig. 5A) or ACTH-stimulated (Fig. 5B) conditions.

Discussion

To expand the range of treatment options for patients with CS, new drugs that inhibit glucocorticoid production with high selectivity and tolerability are required. This study contributes to the current knowledge on the mechanism of action of AA, and our findings warrant future studies to
determine whether AA is a potential treatment option for both human patients and canines with endogenous CS.

In a previous study, we showed that cultured canine primary adrenocortical cells retain their ability to produce cortisol and to respond to ACTH (20) and are therefore a useful in vitro model to study adrenocortical hormone secretion. In contrast, the commonly used cell line NCI-H295R responds only mildly to ACTH stimulation (29), and these cells are therefore an imperfect model to study adrenocortical hormone secretion or, in

**Figure 2.** The effects of AA on steroid hormone production as measured with LC-MS/MS. Steroid hormone production was measured in canine primary adrenocortical cell cultures (n = 9) under basal and ACTH-stimulated conditions. Data are shown as mean ± SEM of nanomole of hormone per milligram protein. Scale bars are adjusted to each hormone concentration to aid visualization. *P < 0.05.
An advantage of using canine over, for example, murine adrenocortical cells is that they require and express CYP17A1 for glucocorticoid production and therefore produce the same glucocorticoids as humans (19), which is important when testing CYP17A1 inhibitors such as AA. The cultured cells in the current study indeed expressed mRNA of the AA target CYP17A1, which was greatly upregulated by ACTH. The inhibition of this enzyme by AA substantially reduced the cortisol and other glucocorticoid concentrations in this study, which is an important goal of treatment in CS.

Although CYP17A1 is not required for corticosterone production, AA also significantly reduced the corticosterone concentration in the cell cultures under basal conditions, which implies that AA also inhibits 21-hydroxylation or 11β-hydroxylation. Indeed, recent studies have shown that AA inhibits the 21-hydroxylase activity of CYP21A2 in NCI-H295R cells (30, 31). To determine whether this inhibition of 21-hydroxylase occurred in the canine adrenocortical cells, we performed additional measurements in a small number of samples that included 11-deoxycorticosterone measurements. Although the limited number of samples hampers statistical analyses, under basal conditions AA increased progesterone but decreased 11-deoxycorticosterone, suggesting that 21-hydroxylation is indeed inhibited. The calculation of the 11-deoxycorticosterone/progesterone ratios suggests that, in cell cultures under both basal and ACTH-stimulated conditions, 21-hydroxylase and 11β-hydroxylase were inhibited by AA. AA has also been shown to inhibit the human HSD3B2 enzyme (32). In this study, we were not able to measure all required hormones to test whether this was also the case in the canine adrenocortical cells. However, the substantially and significantly increased progesterone concentration, which is a product of HSD3B2 conversion, suggest that if HSD3B2 is indeed inhibited, its capacity remains extensive.

AA is the ester prodrug of abiraterone. AA gets rapidly deacetylated to the active form abiraterone in vivo, and its bioavailability strongly depends on whether it is taken with food (33, 34). Abiraterone can be converted to the active metabolite Δ4-abiraterone (D4A) by HSD3B isoforms (35). In the culture medium of two of the adrenocortical cell cultures described in this study, the percentage of the D4A metabolite that we could detect was 13% to 38% of the added 2000 nM AA [preliminary data, method similar to that described by van Nuland et al. (36)]. AA supposedly is deacetylated to abiraterone in our cell culture system by nonspecific esterases. Because the canine adrenocortical cells highly express HSD3B2 (Table 1), this enzyme presumably converts a

### Table 1. mRNA Expression of Steroidogenic Enzymes in Canine Primary Adrenocortical Cell Cultures (n = 9) Under Basal Conditions in ΔCT and Fold Change After Stimulation With 100 nM ACTH(1-24)

<table>
<thead>
<tr>
<th>Gene</th>
<th>ΔCT Basal</th>
<th>Fold Change + ACTH</th>
<th>P Value + ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAR</td>
<td>1.44 ± 0.37</td>
<td>15.4 ± 3.5</td>
<td>0.012</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>2.88 ± 0.33</td>
<td>10.9 ± 2.5</td>
<td>0.012</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>6.87 ± 0.44</td>
<td>106.7 ± 29.3</td>
<td>0.008</td>
</tr>
<tr>
<td>HSD3B2</td>
<td>1.37 ± 0.47</td>
<td>23.0 ± 7.1</td>
<td>0.008</td>
</tr>
<tr>
<td>CYP21A2</td>
<td>5.70 ± 0.43</td>
<td>33.9 ± 9.2</td>
<td>0.012</td>
</tr>
<tr>
<td>CYP11B1</td>
<td>10.62 ± 0.58</td>
<td>27.0 ± 8.3</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM relative to the geometric mean CT value of the reference genes (ΔCT basal) or as fold change relative to the expression under basal conditions (fold change + ACTH).

Abbreviations: CT, cycle threshold; STAR, steroidogenic acute regulatory protein.

Figure 3. The effects of AA on steroid hormone ratios. Steroid hormone production was measured with LC-MS/MS in canine primary adrenocortical cell cultures (n = 4) under basal and ACTH-stimulated conditions. Ratios were calculated between the indicated enzymes in Canine Primary Adrenocortical Cell Cultures (n = 9) Under Basal Conditions in ΔCT Basal and ACTH-stimulated Conditions in Fold Change + ACTH.
large portion of the released abiraterone to D4A. This D4A metabolite has been shown to inhibit CYP21A2 (37), which might contribute to the inhibition of 21-hydroxylase.

When AA was administered without concurrent glucocorticoids to patients with advanced castration-resistant prostate cancer, the decreased glucocorticoid production resulted in substantially increased plasma ACTH concentrations (11). This in turn markedly increased steroids upstream of CYP17A1 that can exert mineralocorticoid activity (e.g., 11-deoxycorticosterone), which can lead to the mineralocorticoid excess syndrome. Because of subsequent suppression of the renin-angiotensin-aldosterone system, aldosterone itself did not increase. Coadministration of glucocorticoids inhibited the increased ACTH secretion and was thereby able to overcome the adverse effects caused by mineralocorticoid excess syndrome in most patients (11). Although 2000 nM AA decreased the mean 11-deoxycorticosterone concentration in our culture system under basal conditions, it increased the mean 11-deoxycorticosterone concentration when stimulated with ACTH (n = 4). This effect might be greater in vivo when the ACTH secretion increases due to a low cortisol concentration. However, whereas the goal of treatment in patients with castration-resistant prostate cancer is to completely block androgen production, the goal of treatment in patients with CS would be to achieve normocortisolism. Nevertheless, the potential increase in ACTH secretion in vivo warrants caution and possibly requires coadministration of other drugs, such as aldosterone receptor antagonists (e.g., spironolactone, eplerenone) or epithelial sodium channel inhibitors (e.g., amiloride) (38, 39).

Recently, Fiorentini et al. (40) reported that AA inhibited the cell viability of the human ACC cell line NCI-H295R and of primary cell cultures derived from ACCs that were hormonally active but not from those that were hormonally inactive or of the hormonally inactive SW13 cell line. When they cultured the cells in the presence of the progesterone receptor (PR) antagonist mifepristone or when they silenced the PR gene, the inhibition of cell viability was prevented, indicating that the increased progesterone concentration has a role in the inhibition of cell viability through the PR (40). In contrast, in our current study, cell viability was not inhibited but slightly increased in the cell cultures under basal conditions, possible due to a compensatory increased cellular mitochondrial activity. The lack of inhibition of

![Figure 4](https://academic.oup.com/endo/article/159/11/3689/5095428)

**Figure 4.** The effects of AA on the mRNA expression of steroidogenic enzymes. Gene expression was measured in canine primary adrenocortical cell cultures (n = 9) under (A) basal and (B) ACTH-stimulated conditions. Data are shown as mean ± SEM fold change relative to the corresponding DMSO vehicle control. The dotted line represents control. The circles above and below the box plots indicate outliers. *P < 0.05. StAR, steroidogenic acute regulatory protein.

![Figure 5](https://academic.oup.com/endo/article/159/11/3689/5095428)

**Figure 5.** The effects of AA on cell viability. Cell viability was measured with alamarBlue™ assay in canine primary adrenocortical cell cultures (n = 9) under (A) basal and (B) ACTH-stimulated conditions. Data are shown as mean ± SEM of the percentage of detected fluorescence with each DMSO vehicle control (0 nM AA) set to 100%. *P < 0.05; **P < 0.01.
cell viability was not caused by a lack of hormone production because these cells were hormonally active with also dramatically increased progesterone concentrations after AA incubation. Earlier work by our group showed that the canine adrenal cortex does express the PR (41). There are two PR isoforms that are synthesized from the PR gene in both humans and dogs: PRα and PRβ. In NCI-H295R cells, the predominant isoform of the PR is PRβ (40), whereas in dogs PRB has been shown to have very limited transactivation potential (42). This difference in isoform activity between the two species could explain the contradictory results. An alternative explanation for the contradictory effects of AA on cell viability is that healthy adrenocortical cells possibly respond differently to a high progesterone concentration.

In the cell cultures under basal conditions, 2000 nM AA significantly increased the mRNA expression of CYP11A1. Because this condition contained the lowest cortisol concentration in the culture medium, we hypothesize that this increase in CYP11A1 expression might be related to an intra-adrenal feedback regulation of cortisol production. Although relatively little is known about the intra-adrenal feedback mechanism, a recent study provided evidence that cortisol can inhibit its own production (43). In the cell cultures under basal conditions, the cortisol concentrations inhibited by AA were in every case lower than in the ACTH-stimulated cell cultures under basal conditions, which was discussed in the current study, so we were not able to test this hypothesis. In the ACTH-stimulated cell cultures, androstenedione was inhibited substantially by AA, as also reported in vivo in women with 21-hydroxylase deficiency treated with AA (46). An advantage of concurrent inhibition of androgen production is that an ACC often co-secretes glucocorticoids and androgens (47), in which case administration of AA could inhibit the virilization symptoms (48).

Although many similarities exist between humans and dogs in terms of CS and adrenal physiology, there are some differences. For example, whereas humans have two different isoforms of the cytochrome P450 11B enzyme to catalyze the last steps in cortisol (CYP11B1) and aldosterone (aldosterone synthase; CYP11B2) synthesis, dogs appear to have only one functional isoform of this enzyme (19). It is therefore prudent to keep these species-related differences in mind when interpreting the results of this study.

In summary, the results of this study show that AA effectively inhibits cortisol production but does not affect aldosterone production. Future studies are warranted to determine whether AA can be a valuable addition to the current treatment options for both human and canine CS. To determine the in vivo efficacy, tolerability, and optimal dosing of AA, dogs with spontaneous CS may provide a useful animal model.

**Acknowledgments**

The authors thank Merel van Nuland and Jos H. Beijnen (Antoni van Leeuwenhoekziekenhuis, Amsterdam, Netherlands) for the D4A measurements and Adri Slob (Utrecht University, Utrecht, Netherlands) for RIA cortisol measurements and technical assistance.

**Financial Support:** This study was supported by the Deutsche Forschungsgemeinschaft (DFG) within the CRC/Transregio 205/1 “The Adrenal: Central Relay in Health and Disease” (Project B16) to M. Kroiss and an individual research grant from the DFG to M. Kroiss (KR 4371/1-2).

**Correspondence:** Sara Galac, DVM, PhD, Yalelaan 108, 3584 CM Utrecht, Netherlands. E-mail: s.galac@uu.nl.

**Disclosure Summary:** The authors have nothing to disclose.

**References**


