Domestic Animal Endocrinology 63 (2018) 23–30

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: https://www.journals.elsevier.com/domestic-animal-endocrinology

Steroidogenic factor-1 inverse agonists as a treatment option for canine hypercortisolism: in vitro study

K. Sanders, J.A. Mol, A. Slob, H.S. Kooistra, S. Galac*

Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, 3584 CM Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 21 September 2017
Received in revised form 7 November 2017
Accepted 10 November 2017

Keywords:
Cushing's syndrome
NR5A1
Ad4BP
ACTH
Pituitary
Adrenal

ABSTRACT

Hypercortisolism is one of the most commonly diagnosed endocrinopathies in dogs, and new targeted medical treatment options are desirable. Steroidogenic factor-1 (SF-1), an orphan nuclear hormone receptor, is a key regulator of adrenal steroidogenesis, development, and growth. In pituitary-dependent hypercortisolism (PDH), high plasma ACTH concentrations increase the transcriptional activity of SF-1. In adrenal-dependent hypercortisolism, SF-1 expression is significantly greater in dogs with recurrence after adrenalectomy than in those without recurrence. Inhibition of SF-1 could therefore be an interesting treatment option in canine spontaneous hypercortisolism. We determined the effects of 3 SF-1 inverse agonists, compounds IsoQ A, #31, and #32, on cortisol production, on the messenger RNA (mRNA) expression of steroidogenic enzymes and SFs, and on cell viability, in primary adrenocortical cell cultures of 8 normal adrenal glands and of 3 cortisol-secreting adrenocortical tumors (ATs). To mimic PDH, the normal adrenocortical cell cultures were stimulated with ACTH. The results show that only compound #31 inhibited cortisol production and SF-1 target gene expression in non–ACTH-stimulated and ACTH-stimulated normal adrenocortical cells but did not affect cell viability. In the AT cell cultures, the effects of #31 on cortisol production and target gene expression were variable, possibly caused by a difference in the SF-1 mRNA expressions of the primary tumors. In conclusion, inhibition of SF-1 activity shows much promise as a future treatment for canine hypercortisolism.

1. Introduction

In dogs, one of the most frequently diagnosed endocrine disorders is hypercortisolism (Cushing's syndrome). Spontaneous hypercortisolism is caused by an ACTH-secreting pituitary adenoma (pituitary-dependent hypercortisolism [PDH]) in 80% to 85% of cases and by a primary functional cortisol-secreting adrenocortical tumor (AT) in 15% to 20% of cases, of which the majority is an adrenocortical carcinoma [1,2].

For medical treatment of spontaneous canine hypercortisolism, the adrenocorticolytic drug mitotane (o,p'-DDD) and the adrenal enzyme inhibitor trilostane are used most often. Mitotane destroys adrenocortical cells, but because it can cause serious side effects [3–5], its use has been largely replaced by trilostane. Trilostane competitively inhibits the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase 2 (HSD3B2) and thereby inhibits cortisol production. The drawbacks of treatment with trilostane include disturbance of the renin-angiotensin-aldosterone axis, and possibly occurrence of apoptosis and necrosis in the adrenal cortex [6–8].

* Corresponding author. Tel.: +31 30 253 9683; fax: +31 30 2518126.
E-mail address: s.galac@uu.nl (S. Galac).

0739-7240/$ – see front matter © 2017 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.domaniend.2017.11.001

Moreover, trilostane does not affect the growth of an AT or its metastases [5,9].

To improve the medical treatment of dogs with spontaneous hypercortisolism, new targeted medical treatment is desirable. For this new medical treatment, an interesting target could be steroidogenic factor-1 (SF-1/Ad4BP; NR5A1), which is an orphan nuclear receptor that regulates adrenal steroidogenesis, development, and growth [10]. Steroidogenic factor-1 was initially discovered as a transcription factor for genes encoding steroidogenic enzymes [11,12]. This transcriptional activity of SF-1 can be stimulated by the binding of ACTH to the melanocortin 2 receptor (MC2R) in the adrenal cortex [13]; therefore, increased SF-1 activity is an important characteristic of PDH. Steroidogenic factor-1 was later discovered to be essential also in adrenal development. Mice with targeted disruption of the *SF-1* gene (SF-$1^{-/-}$ mice) were born without adrenal glands and gonads and died shortly after birth [14,15]. Not only the presence or absence but also the dose of SF-1 is important [16–18]. Greater SF-1 dosages increased proliferation in the human adrenocortical carcinoma cell line NCI-H295R and induced adrenocortical neoplasia in mice [19]. Furthermore, SF-1 messenger RNA (mRNA) expression was significantly greater in dogs with ATs that had recurrence of hypercortisolism within 2.5 yr after adrenalectomy than in dogs that had no recurrence for at least 2.5 yr after adrenalectomy [20]. Taken together, these data suggest that inhibition of SF-1 activity might lead to inhibition of steroidogenesis and inhibition of AT growth.

Previous studies have focused on identifying compounds that can inhibit human SF-1 activity. One such study, using ultra-high-throughput screening, identified 2 isoquinolinone analogs, SID7969543 (IsoQ A) and SID7970631 (IsoQ B), as selective SF-1 inverse agonists [21]. Identification of these compounds led to the development of analogs with improved SF-1 inhibitor potency, lower cellular toxicity, and improved selectivity: compounds #31 and #32 [22]. Doghman et al [23] demonstrated that, in conditions of increased SF-1 expression, compounds IsoQ A, #31, and #32 selectively decreased forskolin-stimulated steroid hormone production and inhibited NCI-H295 R cell proliferation.

Based on the importance of SF-1 in adrenal steroidogenesis and proliferation, inhibition of SF-1 activity could be an interesting treatment option in canine hypercortisolism. This study is the first to investigate the effects of SF-1 inverse agonists in canine primary adrenocortical cell culture of normal adrenals, stimulated with synthetic ACTH to mimic PDH, and of ATs.

2. Materials and methods

2.1. Animals and tissues

For primary cell cultures, the adrenal glands of 8 healthy dogs were used. These dogs were euthanized for reasons unrelated to the present study, which was approved by the Ethical Committee of Utrecht University. The dogs aged between 14 and 62 mo (median 35 mo) and weighed between 10 and 29 kg (median 16 kg). Four dogs were of mixed breed and 4 dogs were Beagles. Two dogs were female and 6 were male, all dogs were intact.

The cortisol-producing ATs were retrieved after unilateral adrenalectomy of 3 dogs. The dogs aged between 98 and 134 mo old (median 118 mo) and weighed between 10 and 18 kg (median 13 kg). One dog was an intact male Fox Terrier, one dog was an intact female Västgötaspets, and one dog was male, castrated, and of mixed breed. The suspicion of hypercortisolism was based on the medical history and the findings on physical examination. The diagnosis of a cortisol-producing AT was confirmed by the finding of nonsuppressible hypercortisolism with endocrine testing, combined with the demonstration of an AT with an atrophic contralateral adrenal gland by ultrasonography or computed tomography [24]. The dogs did not receive any drugs to inhibit cortisol production prior to the adrenalectomy. Histopathological evaluation confirmed the diagnosis of an AT in all dogs. The ATs were classified by a single pathologist based on the criteria described by Labelle et al [2], which classified 2 ATs as carcinomas and one AT as an adenoma.

For reverse transcriptase quantitative PCR (RT-qPCR) analysis of primary tissues, the aforementioned ATs and the adrenal glands of 4 healthy dogs were used. The healthy dogs aged between 14 and 58 mo (median 24 mo) and weighed between 10 and 26 kg (median 23 kg). Three dogs were of mixed breed, and one dog was a Beagle. Three dogs were female and one was male, all dogs were intact.

2.2. Cell culture

The adrenal glands were collected in ice-cold Hanks Balanced Salt Solution (Gibco, Invitrogen, Merelbeke, Belgium) and cleared of surrounding tissues. Normal adrenals were cut in half length-wise to scrape out the medulla. In the case of the ATs, only apparent tumorous tissue was used.

The adrenal tissues were cut into pieces and digested for 60 to 75 min at 37°C in a mixture of Leibowitz L15 (Gibco, Invitrogen, Breda, the Netherlands), 3 mg/mL collagenase 1A (Sigma-Aldrich, Zwijndrecht, the Netherlands), 0.05 mg/mL DNAse (Sigma-Aldrich), 20 mM D-(+)-Glucose monohydrate (Sigma-Aldrich), 0.2% bovine serum albumin (BSA; Sigma-Aldrich) ,and 1% penicillin/streptomycin (Gibco). The digested tissue solutions were filtered through 100 and 70 μm EASYstrainer filters (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) and subsequently mixed in a 1:1 ratio with Leibowitz L15 containing 20% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin. A 3% BSA cushion was pipetted at the bottom of the tube to remove cell debris, after which the tissue solutions were centrifuged at 190 × *g* for 10 min at 4°C. The pellets were washed in Leibowitz L15 containing 10% FBS and 1% penicillin/streptomycin and again centrifuged.

The cells were counted with a Bürker Türk counting chamber and diluted to 1×10^5 cells/mL with Dulbecco's Modified Eagle Medium F-12 (Gibco) containing 1% Insulin-Transferrin-Selenium (Gibco), 0.125% BSA, 2.5% Nu-Serum (Corning, Amsterdam, the Netherlands), and 1% penicillin/streptomycin. The cells were seeded in Multiwell 96 well plates (1×10^4 cells per well, Primaria, Corning) to measure cortisol and DNA concentrations and cell viability, and in

Multiwell 24 well plates (1×10^5 cells per well) to isolate RNA for RT-qPCR analysis.

The cells were left to attach for 4 to 7 d at 37°C in a humidified atmosphere of 95% air and 5% CO_2; then, the culture medium was refreshed before the cells were incubated with the compounds.

2.3. Chemicals

Three compounds were used: compound IsoQ A (SID7969543, Sigma-Aldrich, Zwijndrecht, the Netherlands), compound #31 (F1808-0154, Life Chemicals, Kiev, Ukraine), and compound #32 (F1808-0165, Life Chemicals). The compounds were dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany) at 20 mM, then aliquoted and stored at −20°C.

The cells were incubated with 10 nM, 100 nM, 1 μM, and 10 μM of the compounds, and with (normal adrenocortical cells) and without (normal adrenocortical and AT cells) 1 μM ACTH [1–24] (Synacthen, Novartis Pharma BV, Arnhem, the Netherlands). The DMSO end concentration was 0.05% in all conditions. Incubations were performed in quadruplicate in the 96-well plates and in duplicate in the 24-well plates.

After 72 h of incubation, the culture medium was removed and used to measure cortisol concentrations by RIA as described previously [25].

2.4. DNA measurements

After the culture medium was removed, the culture plates underwent 3 freeze/thaw cycles. Thereafter, 50-μL Tris/EDTA (10-mM Tris, 1-mM EDTA, pH 8.0) was added to each well, after which the Qubit dsDNA HS Assay Kit (Fisher Scientific) was used according to the manufacturer's instructions. Deoxyribonucleic acid concentrations were measured with a Qubit 2.0 Fluorometer (Fischer Scientific).

2.5. Viability

After 72 h of incubation, 10 μL of alamarBlue reagent (Thermo Fisher) was added to each well in 96-well plates. The cells were incubated with the alamarBlue reagent for 4 h at 37°C in the dark, after which fluorescence was measured on a DTX 880 Multimode Detector (Beckman Coulter, Woerden, the Netherlands) at 535 nm excitation and 595 nm emission.

2.6. Reverse transcriptase quantitative PCR

For RT-qPCR analysis of the primary ATs, tissue fragments were snap frozen in liquid nitrogen within 10 to 20 min after surgical removal and RNA was isolated with the RNeasy Mini Kit (QIAGEN, Venlo, the Netherlands) according to the manufacturer's instructions.

For RT-qPCR analysis of cultured cells, the culture medium was removed from the 24-well plates after 72 h of incubation with compounds and RNA was isolated from the cells with the RNeasy Micro Kit (QIAGEN), including a DNAse (QIAGEN) step to avoid genomic contamination, according to the manufacturer's instructions.

The RNA concentrations were measured with NanoDrop (ND-1000, Isogen Life Science, Utrecht, the Netherlands). The iScript cDNA Synthesis Kit (Bio-Rad) was used according to the manufacturer's instructions to synthesize complementary DNA (cDNA), which was subsequently diluted to 1 ng/μL.

Reverse transcriptase quantitative PCR analysis was used to determine the mRNA expression of 3 steroidogenic enzymes and 3 SFs. The steroidogenic enzymes that were analyzed were 17α-hydroxylase/17,20-lyase (CYP17), HSD3B2, and 21-hydroxylase (CYP21). The SFs that were analyzed were SF-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1 (DAX-1), and MC2R. Primers used for CYP17, HSD3B2, CYP21, MC2R, and SF-1 have been described previously [26]. New primers were designed for DAX-1 (forward 5′-CTTTAACCCGGACC TGC-3′, reverse 5′-GCCTGAAGAATAGCTCCAC-3′). The primer products were sequenced using an ABI3130XL Genetic analyzer to confirm the correct identity of the transcript.

To correct for differences in cDNA concentrations, the reference genes that were analyzed were ribosomal protein S5, ribosomal protein S19, signal recognition particle receptor, and hypoxanthine-guanine phosphoribosyltransferase [27,28].

The RT-qPCR reactions were performed using SYBR-green supermix (Bio-Rad) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) instrument. To analyze the expression levels of the reference genes, GeNorm software [29] was used, which justified their use. To calculate the normalized relative mRNA expression of each target gene, the $2^{-\Delta\Delta Ct}$ method [30] was used.

2.7. Statistical analysis

Normally distributed data (cortisol and DNA data) were analyzed with repeated measures analysis of variance with a post hoc Bonferroni correction. Sphericity was checked using Mauchly's test, and the Greenhouse-Geisser correction was used when the assumption of sphericity was violated. Nonnormally distributed data (alamarBlue and RT-qPCR data) were analyzed with the Friedman test. Significant outcomes were analyzed post hoc with the Wilcoxon signed-rank test with a Bonferroni correction. P values of <0.05 were considered significant.

Cortisol data are reported relative to the DMSO vehicle control in mean ± standard error of the mean.

3. Results

3.1. Cortisol production, DNA content, and cell viability

3.1.1. Normal adrenocortical cell cultures ($n = 8$)

In the normal adrenocortical cell cultures, 10 μM of #31 significantly inhibited the cortisol production of the non–ACTH-stimulated cells by 55 ± 10% ($P = 0.026$) (Fig. 1A) and of the ACTH-stimulated cells by 57 ± 5% ($P = 0.002$) (Fig. 1B). Compounds IsoQ A and #32 did not significantly affect the cortisol production of either non–ACTH-stimulated cells (Fig. 1A) or ACTH-stimulated cells (Fig. 1B).

None of the compounds affected either the DNA content per well or the resazurin reduction in the alamarBlue

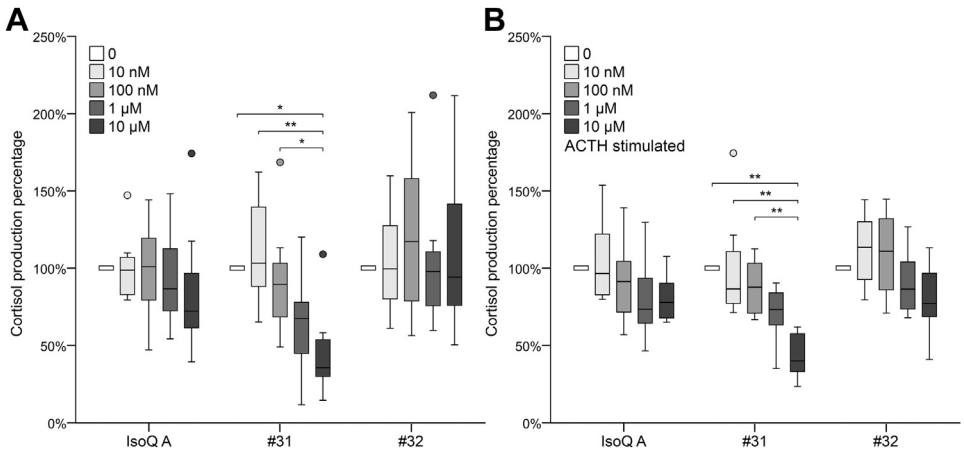

Fig. 1. The effects of compounds IsoQ A, #31, and #32 on the cortisol production of non–ACTH-stimulated (A) and ACTH-stimulated (B) canine primary normal adrenocortical cell cultures (n = 8). Cortisol concentrations are shown in percentages, normalized to the control. Asterisks represent significant differences compared to the control: $^{*}P < 0.05$ and $^{**}P < 0.01$.

viability assay of either non–ACTH-stimulated or ACTH-stimulated normal adrenocortical cells (data not shown).

3.1.2. Adrenocortical tumor cell cultures (n = 3)

In the AT cell cultures, 10 μM of #31 inhibited the cortisol production of 2 ATs but did not inhibit cortisol production of one AT (Table 1). Compound IsoQ A slightly inhibited the cortisol production of only one AT, and #32 did not inhibit the cortisol production of any AT (Table 1).

None of the compounds affected either the DNA content per well or the resazurin reduction in the alamarBlue viability assay of the AT cells (data not shown).

3.2. Reverse transcriptase quantitative PCR

3.2.1. Normal adrenocortical cell cultures (n = 8)

In the normal adrenocortical cell cultures, addition of 1 μM ACTH (1–24) significantly upregulated the mRNA expression of CYP17 ($P = 0.012$), HSD3B2 ($P = 0.012$), CYP21 ($P = 0.012$), SF-1 ($P = 0.017$) and MC2R ($P = 0.012$), whereas it significantly downregulated the mRNA expression of DAX1 ($P = 0.012$) (Fig. 2).

In the non–ACTH-stimulated normal adrenocortical cell cultures, #31 significantly downregulated the mRNA expression of CYP17 ($P = 0.035$), HSD3B2 ($P = 0.035$), CYP21 ($P = 0.035$) (Fig. 3A), and MC2R ($P = 0.035$) (Fig. 3B).

Table 1
The cortisol production of AT cell cultures compared to the control and the SF-1 mRNA expression of the primary AT cells.[a]

AT	% Cortisol production + IsoQ A	% Cortisol production + #31	% Cortisol production + #32	SF-1 fold change
1	84	106	179	0.8
2	107	85	144	1.7
3	112	66	108	4.1

AT, cortisol-secreting adrenocortical tumor; SF-1, steroidogenic factor-1.
[a] The percentage of cortisol production compared to the DMSO vehicle control after incubation with 10 μM of compounds IsoQ A, #31, or #32 in each individual canine cortisol-secreting AT cell culture and the fold change of SF-1 in the primary AT, relative to 4 normal adrenal glands.

In the ACTH-stimulated normal adrenocortical cell cultures, #31 significantly downregulated the expression of HSD3B2 ($P = 0.035$), CYP21 ($P = 0.035$) (Fig. 3C), and MC2R ($P = 0.035$) (Fig. 3D). Compounds IsoQ A and #32 did not affect the mRNA expression of the genes analyzed in this study in either non–ACTH-stimulated or ACTH-stimulated normal adrenocortical cell cultures (Fig. 3).

3.2.2. Adrenocortical tumor cell cultures (n = 3)

Based on the results from the normal adrenocortical cell cultures, we determined whether #31 had similar effects on the mRNA expressions of CYP17, HSD3B2, CYP21, and MC2R in the AT cell cultures. In all the AT cell cultures, #31 inhibited the expression of CYP17 (fold changes 0.4, 0.7, and 0.7), HSD3B2 (fold changes 0.3, 05, and 0.9), CYP21 (fold changes 0.3, 0.7, and 0.8) and MC2R (0.4, 0.4, 0.5). Due to the low number of AT cell cultures, no statistical analyses were performed on this data.

The fold changes of SF-1 in the primary ATs relative to 4 normal adrenal glands are shown in Table 1, combined with the percentages of cortisol production compared to the DMSO vehicle control when incubated with 10-μM of compounds IsoQ A, #31 and #32.

4. Discussion

This study demonstrates that canine primary adrenocortical cell culture is a valuable in vitro system that can be used to test potential future treatment options for canine hypercortisolism. We have shown that, with our culture protocol, canine primary adrenocortical cells retained their hormone-producing abilities and their responsiveness to ACTH in vitro. This makes this in vitro model superior to the most commonly used human AT cell line NCI-H295R, which responds only mildly or not at all to ACTH stimulation [31] and is therefore not a suitable model to study PDH. Furthermore, the 3 AT cell cultures provide a proof of concept that this primary culture system is not only possible for normal canine adrenocortical cells but also for canine AT cells. Because of the heterogeneous nature of

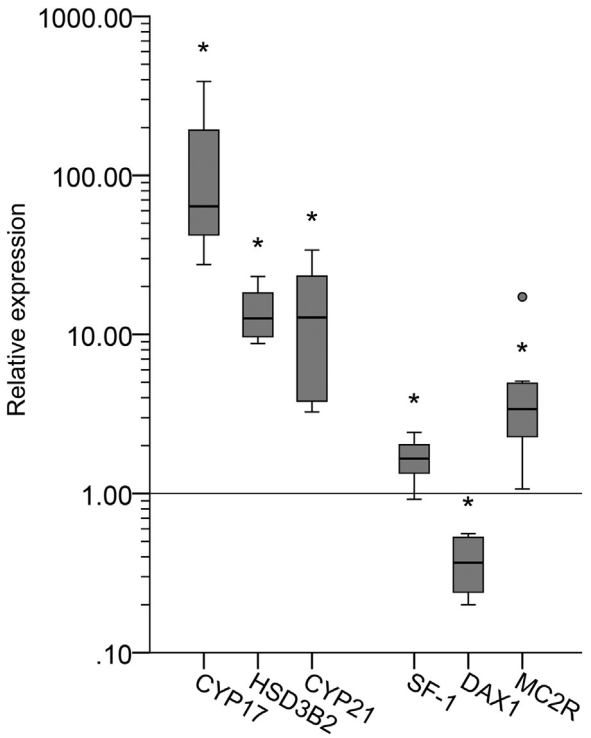

Fig. 2. The effects of addition of 1-μM ACTH [1–24] on the relative mRNA expression of steroidogenic enzymes (CYP17, HSD3B2, and CYP21) and steroidogenic factors (SF-1, DAX1, and MC2R) in canine normal adrenocortical cell cultures (n = 8). Fold changes are normalized to the non–ACTH-stimulated control. Asterisks represent significant differences compared to the non–ACTH-stimulated controls: $^{*}P < 0.05$. CYP17, 17α-hydroxylase/17,20-lyase; CYP21, 21-hydroxylase; DAX-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1; HSD3B2, 3β-hydroxysteroid dehydrogenase 2; MC2R, melanocortin 2 receptor; SF-1, steroidogenic factor-1.

cells in primary cell culture, this in vitro system more closely resembles the heterogeneous in vivo conditions than a single cell line does and could also be used as an animal in vitro model for human hypercortisolism.

This study shows that compound #31 is an effective inhibitor of cortisol production and SF-1 target gene expression in primary cell culture of normal canine adrenocortical cells, both with and without ACTH stimulation. The inhibition of cortisol production does not seem to be caused by a decrease in the number or the viability of the cells, because #31 did not decrease the DNA content or inhibit the cell viability. Compound #31 was more potent than IsoQ A or #32, both in terms of inhibition of mRNA expression levels of SF-1 target genes and of inhibition of cortisol production. This difference in potency makes #31 an interesting candidate for further development into a new drug to treat canine hypercortisolism.

The objective of using SF-1 inverse agonists is to inhibit the transcriptional activity of SF-1. The activity of SF-1 is affected by posttranslational modifications such as phosphorylation, acetylation, and sumoylation [13,32–34]. Although the term "orphan nuclear receptor" implies that it is unknown whether there are physiological ligands that regulate the transcriptional activity of SF-1, more recent structural analyses showed that bacterially expressed SF-1 protein contains a large hydrophobic ligand-binding pocket filled with phospholipids, which could function as regulatory ligands [35–37]. Depending on fatty acid tail length, effects of the phospholipids on bacterially expressed SF-1 activity are either inhibitory or stimulatory [36].

The transcriptional activity of SF-1 can be stimulated by ACTH. Binding of ACTH to the MC2R activates a cyclic AMP (cAMP)-dependent signal transduction cascade that eventually results in increased transcription of steroidogenic enzymes [13,38], as also shown in Figure 2. This increase in transcription is caused, at least partly, by increased SF-1 activity. In NCI-H295R cells, cAMP signaling activates SF-1 by decreasing the amount of inhibitory ligands bound to SF-1 (such as sphingosine) [39] and increasing the amount of stimulatory ligands bound to SF-1 (such as phosphatidic acid) [40]. However, the regulation of nuclear receptors by phospholipids is a relatively young field of research [41], in which there is still much to be explored.

In addition to increasing SF-1 activity, cAMP signaling also increases SF-1 protein expression [42]. Some studies report that this increased protein expression is not caused by increased mRNA expression of SF-1 but by increased stability of the SF-1 protein [42–46], although other studies report that cAMP signaling does increase SF-1 mRNA expression [47–49]. In the present study, we did not determine whether ACTH affected the SF-1 protein expression, but addition of ACTH did significantly increase the SF-1 mRNA expression in the normal canine primary adrenocortical cells. This finding supports the concept that the cAMP-induced increased SF-1 protein expression is at least partly caused by increased SF-1 mRNA expression.

Another important regulator of the transcriptional activity of SF-1 is DAX1. DAX1 inhibits SF-1 activity both by binding to gene promoters that are regulated by SF-1, as well as by directly interacting with SF-1 [50]. Similar to previously reported results [47], our results show that ACTH inhibits DAX1 mRNA expression, which shows another way by which ACTH increases SF-1 activity.

How the compounds used in the present study affect the transcriptional activity of SF-1 is still unknown. Possible mechanisms of action include binding inside the ligand binding pocket of SF-1 or interfering with the interaction of transcriptional cofactors with SF-1 [23]. In the present study, the compounds did not affect the mRNA expression of SF-1, but we did not determine whether the compounds affect the protein expression of SF-1. However, since the compounds did not affect SF-1 protein expression in NCI-H295R cells [23], it seems unlikely that the effects of the compounds are caused by inhibition of the SF-1 protein expression.

In the present study, #31 resulted in different degrees of cortisol inhibition in the AT cell cultures, which could be explained by the different SF-1 mRNA expressions of these tumors. This hypothesis is supported by Table 1, where we see that the adrenocortical cells from the AT with the greatest SF-1 mRNA expression show most cortisol inhibition by #31, whereas the adrenocortical cells from the AT with the lowest SF-1 mRNA expression show least cortisol inhibition by #31. This is similar to the findings by Doghman et al, who showed that the effect of the compounds is

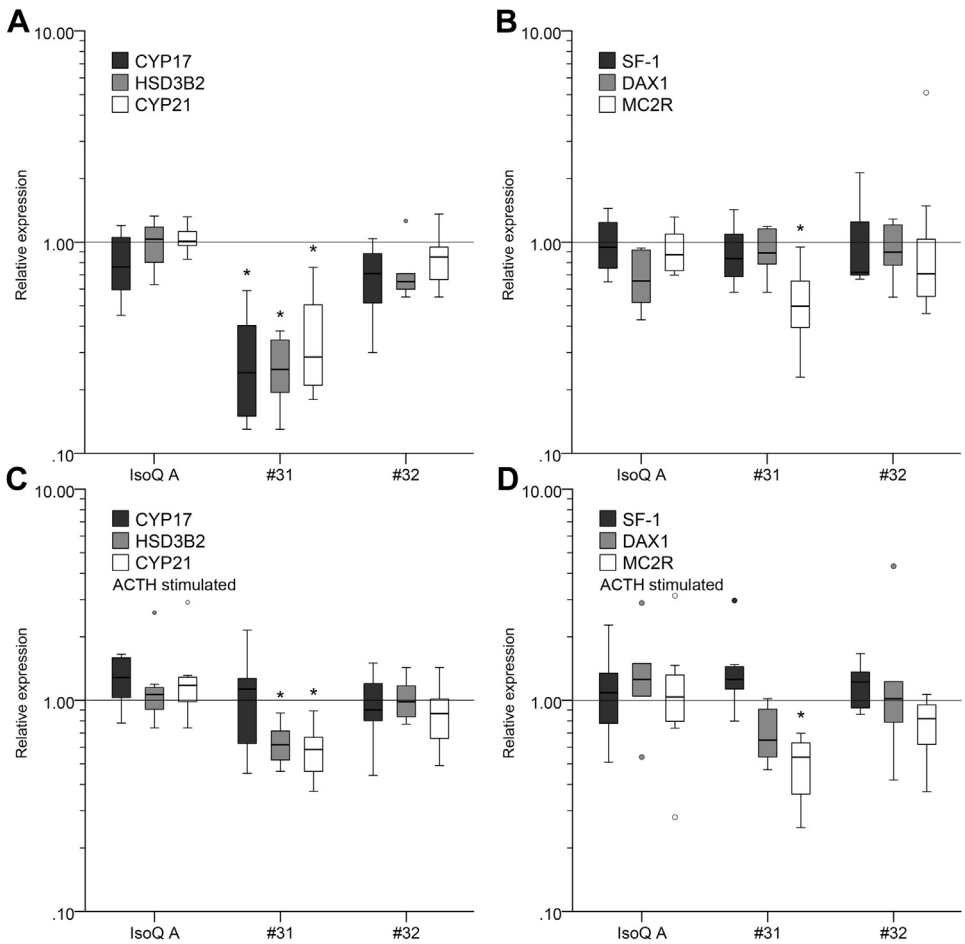

Fig. 3. The effects of compounds IsoQ A, #31, and #32 on the relative messenger RNA expression of steroidogenic enzymes (A and C) and SFs (B and D) in non–ACTH-stimulated (A and B) and ACTH-stimulated (C and D) canine primary normal adrenocortical cell cultures (n = 8). Fold changes are normalized to the controls. Asterisks represent significant differences compared to the ACTH-stimulated controls: $^{*}P < 0.05$. CYP17, 17α-hydroxylase/17,20-lyase; CYP21, 21-hydroxylase; DAX-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1; HSD3B23β-hydroxysteroid dehydrogenase 2; MC2R, melanocortin 2 receptor; SF, steroidogenic factor.

SF-1-dosage dependent [23], which indicates that these compounds specifically target SF-1 activity. However, the low number of ATs used in the present study makes it difficult to draw any reliable conclusions. Nevertheless, we included the 3 ATs to show that #31 may not only have potential in dogs with PDH but also in dogs with ATs that have high SF-1 expression.

Since ACTH increases SF-1 activity and the effects of the compounds are SF-1-dosage dependent, we expected that the effects of the compounds would be more evident in the ACTH-stimulated cells. However, the inhibition of cortisol production by #31 seems to be comparable between non–ACTH-stimulated and ACTH-stimulated normal adrenocortical cells. In fact, the inhibition of the mRNA expression of the steroidogenic enzymes by #31 was even greater in the non–ACTH-stimulated cells than in the ACTH-stimulated cells. Possibly, the metabolic stability of the ACTH was greater than that of the compounds, resulting in an underestimation of the effects of the compounds after 72 h of incubation. To confirm this, repeat experiments could include different incubation times, measuring the compound concentrations in the culture medium at different time points or reincubating the cells with fresh compounds during the course of the experiment.

In ATs, a big advantage of inhibiting SF-1 activity would be to also inhibit AT cell proliferation. In the study by Doghman et al (2009), compounds IsoQ A, #31, and #32 all significantly inhibited the SF-1 overexpression–induced cell proliferation in NCI-H295 R cells, which they determined by counting the cells [23]. In the present study, the compounds did not inhibit the DNA content or cell viability of normal adrenocortical or AT cells after 72 h of incubation. However, the primary adrenocortical cells in our current culture system have a low proliferation rate, and longer incubation times could therefore be necessary to detect an inhibitory effect on cell proliferation. Since our results show that #31 is an effective inhibitor of SF-1–dependent steroidogenesis, the hypothesis that #31 could also inhibit SF-1–dependent proliferation seems likely. To determine whether using SF-1 inverse agonists could be useful to inhibit proliferation of ATs, further research is necessary on a larger AT sample size and in vivo.

Since ACTH increases SF-1 activity, the use of SF-1 inverse agonists could be beneficial in all patients with PDH, ie, the majority of dogs with spontaneous hypercortisolism. As previously mentioned, in ATs, SF-1 inverse agonists will only have additional value in patients of which the ATs have normal to high SF-1 expression. Dogs with ATs with relatively high SF-1 mRNA expression have a greater chance of recurrence of hypercortisolism compared to dogs with relatively low SF-1 mRNA expression [20], similar to humans with ATs in which SF-1 protein expression is an important prognostic factor [51,52]. This group with high SF-1 expression therefore has the most need for additional treatment, and SF-1 inverse agonists could be a promising future treatment in these patients. The greatest chance of therapeutic success would therefore be when SF-1 inverse agonists are used in personalized medicine, where the SF-1 expression of an individual AT is determined prior to initiation of therapy. Since this can only be determined in tumors after a patient has undergone adrenalectomy, this could be feasible as postoperative adjuvant therapy. In addition, further research could focus on whether the SF-1 expression of an AT can reliably be determined from fine needle biopsies taken from canine ATs in situ. Alternatively, it would be very interesting to study whether there are peripheral biomarkers that correlate with a high SF-1 expression in the tumor, for example, circulating cell-free microRNAs [53].

5. Conclusion

Canine primary adrenocortical cell culture is a valuable in vitro culture system that can be used to test potential future treatment options for hypercortisolism. This study shows that #31 is an effective inhibitor of cortisol production and SF-1 target gene expression in vitro. Its effects in vivo and its effects on AT cells are worthwhile to investigate further. In conclusion, inhibition of SF-1 activity shows much promise as a potential future treatment option in canine hypercortisolism.

Acknowledgments

The authors would like to thank Drs. Bart Sjollema, Elaine Naan, and Rob Gerritsen for supplying the patient materials (ATs). This work was supported by Morris Animal Foundation (D15CA-052) and AKC Canine Health Foundation (Grant 02011).

References

[1] Galac S, Reusch CE, Kooistra HS, Rijnberk A. Adrenals. In: Rijnberk A, Kooistra HS, editors. Clinical endocrinology of dogs and cats. Hannover, Germany: Schlütersche; 2010. p. 93–154.
[2] Labelle P, Kyles AE, Farver TB, De Cock HE. Indicators of malignancy of canine adrenocortical tumors: histopathology and proliferation index. Vet Pathol 2004;41:490–7.
[3] Arenas C, Melián C, Pérez Alenza MD. Long-term survival of dogs with adrenal-dependent hyperadrenocorticism: a comparison between mitotane and twice daily trilostane treatment. J Vet Intern Med 2014;28:473–80.
[4] Peterson ME, Kintzer PP. Medical treatment of pituitary-dependent hyperadrenocorticism. Mitotane. Vet Clin North Am Small Anim Pract 1997;27:255–72.
[5] Feldman E. Evaluation of twice-daily lower-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. J Am Vet Med Assoc 2011;238:1441–51.
[6] Reusch CE, Sieber Ruckstuhl N, Wenger M, Lutz H, Perren A, Pospischil A. Histological evaluation of the adrenal glands of seven dogs with hyperadrenocorticism treated with trilostane. Vet Rec 2007;160:219–24.
[7] Galac S, Buijtels JJ, Mol JA, Kooistra HS. Effects of trilostane on the pituitary-adrenocortical and renin-aldosterone axis in dogs with pituitary-dependent hypercortisolism. Vet J 2010;183:75–80.
[8] Chapman PS, Kelly DF, Archer J, Brockman DJ, Neiger R. Adrenal necrosis in a dog receiving trilostane for the treatment of hyperadrenocorticism. J Small Anim Pract 2004;45:307–10.
[9] Benchekroun G, de Fornel-Thibaud P, Lafarge S, Gomez E, Begon D, Delisle F, et al. Trilostane therapy for hyperadrenocorticism in three dogs with adrenocortical metastasis. Vet Rec 2008;163:190–2.
[10] Schimmer BP, White PC. Minireview: steroidogenic factor 1: its roles in differentiation, development, and disease. Mol Endocrinol 2010;24:1322–37.
[11] Lala DS, Rice DA, Parker KL. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. Mol Endocrinol 1992;6:1249–58.
[12] Morohashi K, Honda S, Inomata Y, Handa H, Omura T. A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J Biol Chem 1992;267:17913–9.
[13] Chen W, Juan L, Chung B. SF-1 (nuclear receptor 5A1) activity is activated by cyclic AMP via p300-mediated recruitment to active foci, acetylation, and increased DNA binding. Mol Cell Biol 2005;25:10442–53.
[14] Luo X, Ikeda Y, Parker KL. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 1994;77:481–90.
[15] Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, et al. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proc Natl Acad Sci U S A 1995;92:10939–43.
[16] Beuschlein F, Mutch C, Bayers DL, Ulrich-Lai YM, Engeland WC, Keegan C, et al. Steroidogenic factor-1 is essential for compensatory adrenal growth following unilateral adrenalectomy. Endocrinology 2002;143:3122–35.
[17] Val P, Lefrançois-Martinez AM, Veyssière G, Martinez A. SF-1 a key player in the development and differentiation of steroidogenic tissues. Nucl Recept 2003;1:8.
[18] Bland ML, Jamieson C, Akana S, Dallman M, Ingraham HA. Gene dosage effects of steroidogenic factor 1 (SF-1) in adrenal development and the stress. Endocr Res 2000;26:515–6.
[19] Doghman M, Karpova T, Rodrigues G, Arhatte M, De Moura J, Cavalli L, et al. Increased steroidogenic factor-1 dosage triggers adrenocortical cell proliferation and cancer. Mol Endocrinol 2007;21:2968–87.
[20] Galac S, Kool MM, van den Berg MF, Mol JA, Kooistra HS. Expression of steroidogenic factor 1 in canine cortisol-secreting adrenocortical tumors and normal adrenals. Domest Anim Endocrinol 2014;49:1–5.
[21] Madoux F, Li X, Chase P, Zastrow G, Cameron M, Conkright J, et al. Potent, selective and cell penetrant inhibitors of SF-1 by functional ultra-high-throughput screening. Mol Pharmacol 2008;73:1776–84.
[22] Roth J, Madoux F, Hodder P, Roush W. Synthesis of small molecule inhibitors of the orphan nuclear receptor steroidogenic factor-1 (NR5A1) based on isoquinolinone scaffolds. Bioorg Med Chem Lett 2008;18:2628–32.
[23] Doghman M, Cazareth J, Douguet D, Madoux F, Hodder P, Lalli E. Inhibition of adrenocortical carcinoma cell proliferation by steroidogenic factor-1 inverse agonists. J Clin Endocrinol Metab 2009;94:2178–83.
[24] Galac S, Kool MM, Naan EC, Daminet S, Mol JA, Kooistra HS. Expression of the ACTH receptor, steroidogenic acute regulatory protein, and steroidogenic enzymes in canine cortisol-secreting adrenocortical tumors. Domest Anim Endocrinol 2010;39:259–67.
[25] Meijer JC, de Bruijne JJ, Rijnberk A, Croughs RJ. Biochemical characterization of pituitary-dependent hyperadrenocorticism in the dog. J Endocrinol 1978;77:111–8.
[26] Sanders K, Mol JA, Kooistra HS, Slob A, Galac S. New insights in the functional zonation of the canine adrenal cortex. J Vet Intern Med 2016;30:741–50.
[27] Brinkhof B, Spee B, Rothuizen J, Penning L. Development and evaluation of canine reference genes for accurate quantification of gene expression. Anal Biochem 2006;356:36–43.

[28] Stassen QE, Riemers FM, Reijmerink H, Leegwater PA, Penning LC. Reference genes for reverse transcription quantitative PCR in canine brain tissue. BMC Res Notes 2015;8:761.
[29] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:34.
[30] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
[31] Rainey WE, Saner K, Schimmer BP. Adrenocortical cell lines. Mol Cell Endocrinol 2004;228:23–38.
[32] Lewis A, Rusten M, Hoivik E, Vikse E, Hansson M, Wallberg A, et al. Phosphorylation of steroidogenic factor 1 is mediated by cyclin-dependent kinase 7. Mol Endocrinol 2008;22:91–104.
[33] Winnay J, Hammer G. Adrenocorticotropic hormone-mediated signaling cascades coordinate a cyclic pattern of steroidogenic factor 1-dependent transcriptional activation. Mol Endocrinol 2006;20:147–66.
[34] Jacob AL, Lund J, Martinez P, Hedin L. Acetylation of steroidogenic factor 1 protein regulates its transcriptional activity and recruits the coactivator GCN5. J Biol Chem 2001;276:37659–64.
[35] Krylova I, Sablin E, Moore J, Xu R, Waitt G, MacKay JA, et al. Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. Cell 2005;120:343–55.
[36] Li Y, Choi M, Cavey G, Daugherty J, Suino K, Kovach A, et al. Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1. Mol Cell 2005;17:491–502.
[37] Sablin E, Blind R, Krylova I, Ingraham J, Cai F, Williams J, et al. Structure of SF-1 bound by different phospholipids: evidence for regulatory ligands. Mol Endocrinol 2009;23:25–34.
[38] Sewer M, Nguyen V, Huang C, Tucker P, Kagawa N, Waterman M. Transcriptional activation of human CYP17 in H295R adrenocortical cells depends on complex formation among p54(nrb)/NonO, protein-associated splicing factor, and SF-1, a complex that also participates in repression of transcription. Endocrinology 2002;143:1280–90.
[39] Urs A, Dammer E, Sewer M. Sphingosine regulates the transcription of CYP17 by binding to steroidogenic factor-1. Endocrinology 2006;147:5249–58.
[40] Li D, Urs A, Allegood J, Leon A, Merrill A, Sewer M. Cyclic AMP-stimulated interaction between steroidogenic factor 1 and diacylglycerol kinase theta facilitates induction of CYP17. Mol Cell Biol 2007;27:6669–85.
[41] Crowder M, Seacrist C, Blind R. Phospholipid regulation of the nuclear receptor superfamily. Adv Biol Regul 2017;63:6–14.
[42] Aesøy R, Mellgren G, Morohashi K, Lund J. Activation of cAMP-dependent protein kinase increases the protein level of steroidogenic factor-1. Endocrinology 2002;143:295–303.
[43] Zhang P, Mellon SH. The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3′,5′-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17 alpha-hydroxylase/c17-20 lyase). Mol Endocrinol 1996;10:147–58.
[44] Chau YM, Crawford PA, Woodson KG, Polish JA, Olson LM, Sadovsky Y. Role of steroidogenic-factor 1 in basal and 3′,5′-cyclic adenosine monophosphate-mediated regulation of cytochrome P450 side-chain cleavage enzyme in the mouse. Biol Reprod 1997;57:765–71.
[45] Mamluk R, Greber Y, Meidan R. Hormonal regulation of messenger ribonucleic acid expression for steroidogenic factor-1, steroidogenic acute regulatory protein, and cytochrome P450 side-chain cleavage in bovine luteal cells. Biol Reprod 1999;60:628–34.
[46] Simmonds PJ, Phillips ID, Poore KR, Coghill ID, Young IR, Canny BJ. The role of the pituitary gland and ACTH in the regulation of mRNAs encoding proteins essential for adrenal steroidogenesis in the late-gestation ovine fetus. J Endocrinol 2001;168:475–85.
[47] Osman H, Murigande C, Nadakal A, Capponi A. Repression of DAX-1 and induction of SF-1 expression. Two mechanisms contributing to the activation of aldosterone biosynthesis in adrenal glomerulosa cells. J Biol Chem 2002;277:41259–67.
[48] Kulcenty K, Holysz M, Trzeciak WH. SF-1 (NR5A1) expression is stimulated by the PKA pathway and is essential for the PKA-induced activation of LIPE expression in Y-1 cells. Mol Cell Biochem 2015;408:139–45.
[49] Enyeart JJ, Boyd RT. ACTH and AII differentially stimulate steroid hormone orphan receptor mRNAs in adrenal cortical cells. Mol Cell Endocrinol 1996;124:97–110.
[50] Lalli E, Sassone Corsi P. DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. Mol Endocrinol 2003;17:1445–53.
[51] Sbiera S, Schmull S, Assie G, Voelker H, Kraus L, Beyer M, et al. High diagnostic and prognostic value of steroidogenic factor-1 expression in adrenal tumors. J Clin Endocrinol Metab 2010;95:161–71.
[52] Duregon E, Volante M, Giorcelli J, Terzolo M, Lalli E, Papotti M. Diagnostic and prognostic role of steroidogenic factor 1 in adrenocortical carcinoma: a validation study focusing on clinical and pathologic correlates. Hum Pathol 2013;44:822–8.
[53] Armand Labit V, Pradines A. Circulating cell-free microRNAs as clinical cancer biomarkers. Biomol Concepts 2017;8:61–81.