Domestic Animal Endocrinology 63 (2018) 23–30

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

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