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ANTISECRETIVE AND ANTITUMOR ACTIVITY OF ABIRATERONE ACETATE IN HUMAN ADRENOCORTICAL CANCER: A PRECLINICAL STUDY.

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Abstract

Context: Patients with adrenocortical carcinoma (ACC) frequently suffer from cortisol excess, which portends a negative prognosis. Rapid control of cortisol hypersecretion and tumor growth are the main goals of ACC therapy. Abiraterone acetate (AA) is a potent inhibitor of 17alpha-hydroxylase/17,20-lyase, a key enzyme of adrenal steroidogenesis.

Objective: To investigate the therapeutic use of AA in preclinical models of ACC.

Design: AA antisecretive and antiproliferative effects were investigated *in vitro* using NCI-H295R and SW13 ACC cell lines and human primary ACC cell cultures, as well as *in vivo* using immunodeficient mice.

Methods: Steroid secretion, cell viability and proliferation were analyzed in untreated and AA-treated ACC cells. The ability of AA to affect the Wnt/beta-catenin pathway in NCI-H295R cells was also analyzed. Progesterone receptor (PgR) gene was silenced by the RNA interference approach. The antitumor efficacy of AA was confirmed *in vivo* in NCI-H295R cells xenografted in immunodeficient mice.

Results: AA reduced the secretion of both cortisol and androgens, increased production of progesterone and induced a concentration-dependent decrease of cell viability in the NCI-H295R cells and primary secreting ACC cultures. AA also reduced beta-catenin nuclear accumulation in NCI-H295R cells. AA administration to NCI-H295R-bearing mice enhanced progesterone levels and inhibited tumor growth. The cytotoxic effect of AA was prevented by either blocking PgR or by gene silencing.

Conclusion: AA is able to inhibit hormone secretion and growth of ACC both *in vitro* and *in vivo.* It also reduces beta-catenin nuclear accumulation. The cytotoxic effect of AA appears to require PgR.

Introduction

Adrenocortical carcinoma (ACC), an extremely rare cancer with an incidence of 0.5–2 cases per million population per year [1], is highly malignant and resistant to treatment. A key molecular event that contributes to ACC formation and aggressiveness is the constitutive activation of the Wnt/β-catenin signaling pathway due to mutation of the *CTNNB1* gene [2, 3]. Approximately 50% of ACC in adults are hormone-secreting tumors that produce cortisol (Cushing's syndrome) or androgen or multiple steroids [4]. Cortisol excess negatively influences the outcome of ACC patients with early [5] and advanced stages [6, 7] of disease. The adverse prognosis associated with secreting tumors is due to cortisol-related comorbidities, including hypertension, hypokaliemia, hyperglycemia, and infections. Moreover, the immunosuppressive effects of cortisol excess may promote tumor progression [6, 7]. Hypertension and hypokaliemia are worsened by co-secretion of steroids with mineralcorticoid activity. Rapid control of hypersecretion is therefore crucial for effective treatment. Surgery with radical intent is the treatment of choice, whereas for patients with inoperable metastatic disease, mitotane alone or in combination with chemotherapy is the reference therapy [1,8]. However, because the efficacy of current medical treatment of advanced ACC is limited, there is an urgent need for new drugs.

Abiraterone acetate (AA) is a potent inhibitor of 17alpha-hydroxylase/17,20-lyase (CYP17A1), a key enzyme for steroid hormone synthesis [9]. Due to its ability to inhibit androgen synthesis by the adrenal gland, AA is effective in metastatic castration-resistant prostate cancer (CRPC), as demonstrated by two large randomized clinical studies [10, 11]. Besides reducing androgen levels, the drug rapidly impairs cortisol synthesis [12]; therefore, it could be potentially used in the management of Cushing's syndrome. AA efficacy in controlling Cushing's syndrome was recently observed by our group in a ACC patient [13]. As compared with other steroid synthesis inhibitors currently used in the treatment of Cushing's syndrome, AA is less hepatotoxic than ketoconazole, and the concomitant inhibitory effect of cortisol and androgen synthesis is a potential advantage of AA over metyrapone [14], since ACC often co-secretes both hormones.

In the present study, we investigated the effects of AA on hormone secretion and tumor viability *in vitro* in two established ACC cell lines (NCI-H295R and SW13 [15]) and in ACC primary cultures, and *in vivo*, in NCI-H295R mouse xenografts.

Materials and Methods

Chemical and reagents. Chemical and reagents are reported in the Supplementary Materials and Methods.

Cell lines. NCI-H295R and SW13 ACC cell lines were obtained from the American Type Culture Collection and cultured as suggested by ATCC. Cells were authenticated by the AmpFISTR Identifiler PCR amplification kit (Applied Biosystems, Foster City, CA, USA).

Primary cell cultures. Human ACC primary cells were derived from two female patients bearing cortisol-secreting tumors (ACC01 and ACC02) and from three patients with non–secreting tumors (ACC03, male; ACC06 and ACC08, females) (Supplementary Table 1). The project was approved by the local Ethical Committee and written informed consent was obtained from all patients. After surgical removal, cells were enzymatically digested with collagenase and cultured in the same medium of NCI-H295R cells.

Cell treatments. Cells were treated with increasing concentrations of AA (10-400 nM) for different times in charcoal-dextran-treated Nu-Serum (cNS)-medium. For the liquid cromatography-tandem mass spectrometry (LC-MS/MS) experiments, conditioned media were centrifuged at 1500 x *g* for 5 min at 4°C and stored at -20°C until use. NCI-H295R cells were also exposed to mifepristone (0.1-100 nM), with or without AA.

Quantitative RT-PCR (qRT-PCR). Gene expression was evaluated by qRT-PCR (iCycler iQ real-time PCR detection system, BioRad Laboratories, Milan, Italy), using SYBR Green as fluorochrome, as described elsewhere [16]. The sequences of sense and antisense oligonucleotide primers of genes are listed in the supplementary data (Supplemental Table 2).

Cell viability assays. Cell viability was evaluated by cell counting, as previously described [17]. Cell viability was also determined by 3-(4,5-Dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay [18]. Briefly, untreated and AA-treated ACC primary cultures and cell lines were incubated with MTT dye (5 mg/mL) and solubilized with DMSO. Absorbance was measured by a spectrophotometer at 540/620 nm (GDV, Rome, Italy).

Immunofluorescence. Cells were permeabilized with a solution containing 0.2% Triton X-100 for 60 min at room temperature and then incubated overnight at 4°C with anti-beta catenin primary antibody (1:400, Cell Signaling Technologies, Milan, Italy). After extensive washes, the Alexa Fluor488 anti-rabbit secondary antibody (Life Technologies, Milan, Italy) was applied for 60 min at room temperature. For nuclear counterstaining, cells were incubated with Hoechst (Sigma Aldrich, Milan, Italy) for 5 minutes. Coverslips

were mounted using FluorPreserve^{TM} Reagent and cell staining was detected using a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

PgR gene silencing by RNA interference. Two small interfering RNA (siRNA) duplexes targeting regions of two isoforms of the PgR (PgRA and PgRB) gene (si-PgR) and a non-targeting negative control siRNA duplex were used (Silencer® Select Pre-Designed siRNAs, LifeTechnologies). The si-PgR sequences were 5'-GGUUUUCGAAACUUACAUATT-3' (si-PgR 10416), and, 5'-GACAAGUCUUAAUCAACUATT-3' (si-PgR 10417). NCI-H295R cells were transfected with si-PgR or si-control using Lipofectamine RNAiMAX (LifeTechnologies). Preliminary experiments showed that transfection with 10 nM si-PgR 10416 and 30 nM si-PgR 10417 efficiently down-regulated PgRA and PgRB mRNA already after 24 h of exposure.

Liquid Cromatography-Tandem Mass Spectrometry. Steroid extraction was performed as described in [19] and is reported in the Supplementary Materials and Methods. Analysis was carried out using a high-performance liquid chromatography (HPLC) system (1260 Series Agilent Technologies; Santa Clara, CA, USA) equipped with a reversed-phase Hypersil Gold C8 (100 x 3mm 3um) analytical column (Thermo Finnigan, San Jose, CA, USA) connected to a detection system (Agilent 6460 triple quadrupole mass spectrometer) with a Jet Stream Electrospray ion source operating in positive-ion mode (ESI+). Data were acquired in MRM (multiple reaction monitoring) mode. Transitions, cone voltage, and collision energy were optimized using the MassHunter Optimization program (Mass Hunter Optimizer Triple Quad B04.01 Agilent Technologies). Data acquisition was controlled using Mass Hunter software and processed with QuanL0 software (Agilent Technologies). For calibration, blank medium (0.5 ml) was spiked with all solutions of the steroids in methanol, resulting in calibrator concentrations of 1.0- 50.0 ng/ml and 0.1-5.0 ng/ml for Pg.

Antitumor activity of AA in NCI-H295R ACC xenograft mice model. Experiments were carried out using 8-10 week-old female SCID mice (Charles River, Calco, Italy), maintained in laminar flow rooms at constant temperature and humidity, with free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale dei Tumori, Milan, and communicated to the Ministry of Health, in compliance with Italian law. Exponentially growing tumor cells (10^7 cells/mouse) were resuspended in medium and Matrigel Basement Matrix (BD Biosciences, Milan, Italy) (total volume 20 μL, ratio 1:1), and subcutaneously injected into the right flank of groups of 12 mice. AA was administered i.p (196 mg/Kg) daily for 16 days (qdx16); control mice received the vehicle (2.5% benzyl alcohl, 97.5% safflower oil). Toxicity of the drug treatment was monitored as body weight loss. To evaluate plasma and tumor steroid content, at the end of treatment, 6 mice/group were bled under

anaesthesia and then sacrificed by cervical dislocation. Plasma was prepared using standard protocols. Tumors were removed, weighted, and frozen in liquid nitrogen.

Statistical Analysis. Statistical analysis was carried out using GraphPad Prism software (version 5.02, GraphPad Software, La Jolla, CA, USA). One-way ANOVA with Bonferroni's correction was used for multiple comparisons. Unpaired two-tailed Student's *t* test was used for statistical comparison of mean tumor volume (TV) in mice and of steroid levels in tumors and plasma. P values < 0.05 were considered statistically significant. Unless otherwise specified, data are expressed as mean ± SEM of at least three experiments run in triplicate.

Results

AA inhibits cortisol synthesis in NCI-H295R cells

The human NCI-H295R and SW13 cell lines were analyzed for CYP17A1 gene expression by qRT-PCR. CYP17A1 mRNA was highly expressed in NCI-H295R cells (ΔCt 4.41±0.10) and undetectable in SW13 cells, which were then used as negative control. The mRNAs of other key enzymes involved in the steroid hormones biosynthesis was also expressed in NCI-H295R cells: CYP11A1 (ΔCt 5.14±0.11), CYP21A2 (ΔCt 8.86±0.12), CYP11B1 (ΔCt 11.94 ±0.22), CYP11B2 (ΔCt 12.53 ±0.16), 3βHSD2B (ΔCt 10.35±0.11), SULT2A1 (ΔCt 7.69±0.08) and CYP19A1 (ΔCt 7.17±0.08).

Cortisol concentration was measured in cell culture supernatants following treatment of NCI-H295R and SW13 cells with increasing concentrations of AA at different times (2-6 days). As shown in Fig. 1, starting from 2 days of treatment, AA significantly and steadily reduced cortisol secretion at all concentrations in the supernatants of NCI-H295R cells but not of the SW13 cells (data not shown). Cortisol was almost undetectable in the supernatants of cells treated for 2 days with 100-400 nM AA. Cortisol concentration in the supernatants of control cells was slightly increased after 2 days (1.01 ± 0.16 ng/ml) and then stabilized around the starting values (0.86 ± 0.01 ng/ml) after 6 days.

CYP17A1 inhibition by AA inhibits androgen synthesis and increases progesterone production

NCI-H295R cells were treated with AA and the supernatants analyzed for hormone production. As shown in Table 1, AA-induced inhibition of CYP17A1 (50 nM; 2 days) led to a consistent decrease of testosterone and its precursors (dehydroepiandrosterone [DHEA] and androstenedione [ASD]) in comparison wiht untreated cells, whereas the CYP17A1 substrate progesterone increased by about 13 times. The progesterone-down-stream hormones corticosterone and aldosterone decreased after AA treatment.

AA induces apoptosis-mediated cytotoxic effects in NCI-H295R cells

Besides revealing an antisecretive effect, microscope analyses showed a concentration- and time-dependent reduction in vital NCI-H295R cells after AA exposure. After 2 days of treatment, 200 nM AA significantly reduced cell number (% of reduction: 22±1; $P < 0.01$ vs. untreated cells), an effect that was drastically augmented after 4 and 6 days of treatment (% of reduction: 47±6; $P < 0.05$ vs. untreated cells and 62±3; $P < 0.001$ vs. untreated cells, respectively). AA up to 400 nM did not further affect NCI-H295R cell number. SW13 cell viability was completely unaffected by AA (Supplemental Fig. 1), suggesting that the antineoplastic effect of AA was related to the inhibition of CYP17A1 activity.

The cytotoxic effects in NCI-H295R cells were further investigated by MTT assay. As shown in Fig. 2A, AA reduced cell viability in a concentration-dependent manner, with an IC_{50} value of 62.9 nM (95% confidence interval [CI] 54.14 to 73.06). The maximum effect was observed after 4 days of treatment, with no significant change up to 6 days (IC_{50} value of 70.1 nM; 95% CI 48.02 to 87.29). All subsequent experiments were then performed by treating NCI-H295R cells with the IC_{50} value for 4 days. Apoptosis was the main mechanism involved in the observed cytotoxicity (Supplemental Fig. 2).

AA inhibits tumor growth in the NCI-H295R xenograft model and interferes with steroid production

NCI-H295R cells were subcutaneously inoculated into immune-deficient mice. AA inhibited tumor growth when administered daily for 16 days to mice bearing established tumors. AA treatment, which was well tolerated, started to reduce tumor growth at day 36 (tumor volume inhibition [TVI] 38%) ($P = 0.07$ for AA-treated vs. control) - i.e., 8 days from the start of treatment - and this trend was maintained ($P = 0.0707$ and $P = 0.0646$ for AA-treated vs. control at day 40 and 43, respectively) until day 61, when significant tumor growth inhibition was observed ($P = 0.009$ for AA-treated vs. control, TVI 34%) (Fig. 2B). Analysis of the steroid levels in plasma and tumor samples showed that progesterone levels were increased in both plasma and tumor specimens (Fig. 2C, panel a and b), whereas cortisol levels were not significantly modified (Supplemental Table 3). Testosterone levels were significantly decreased in AA-treated tumors (Fig. 2C, panel c), but no significant differences were observed between the plasma levels in the treated versus the untreated animals (Supplemental Table 3). Higher levels of the mineralcorticoid hormones, corticosterone and aldosterone, were found in the plasma and tumor tissues of the AA-treated mice than the controls.

AA affects beta-catenin translocation into the nucleus in NCI-H295R cells

The pathogenic p.S45P mutation of *CTNNB1* gene, which results in abnormal beta-catenin nuclear accumulation [20, 21], is characteristic of NCI-H295R cells. The effect of AA on beta-catenin sub-cellular localization was investigated. Immunofluorescence analyses showed high expression of beta-catenin in the nucleus of cells under basal condition (Fig. 3A), whereas beta-catenin was mainly retained in cytoplasmic sites following AA treatment (63 nM) (Fig. 3B).

AA induces cytotoxic effects in primary human ACC cells

The effect of AA on cell viability was tested on five ACC primary cultures derived from the ACC patients. Primary cells were treated with AA for 4 days. In cortisol-secreting ACC tumors (ACC01 and ACC02), AA exerted a concentration-dependent inhibition of cell viability, as shown by MTT assay, with an IC_{50} values of 127.8 nM (95% CI 60.9 to 178.4) and 96 nM (95% CI 90.4 to 101.9) for ACC01 and ACC02, respectively

(Fig. 4). In these cells, AA-induced CYP17A inhibition resulted in a marked increase in progesterone levels (from 1.9±0.1 to 22±1.4 ng/ml/10^5 cells in ACC01 and from 1.4±0.1 to 43.7±3.4 ng/ml/10^5 cells in ACC02), whereas cortisol secretion was drastically reduced (from 0.2±0.001 to 0.06±0.002 ng/ml/10^5 cells in ACC01 and from 5.7±0.5 to 1.2±0.2 ng/ml/10^5 cells in ACC02).

In the three primary cultures from non-secreting ACC tumors (ACC03, ACC06, ACC08), steroid hormones were almost undetectable in cell supernatants (data not shown) and AA treatment, up to 4 days, did not affect cell viability (Fig. 4).

AA-mediated increase of progesterone secretion in NCI-H295R cells as a mechanism of drug-induced decrease in cell viability

This preclinical study was conducted in only one cell model and few primary cultures; nonetheless, the inability of AA to affect the viability of primary cells from non-secreting ACC suggests a hormone-related mechanism for the decrease in cell viability. MTT assay showed that hydrocortisone (0.1-50 μM) did not significantly affect NCI-H295R cell viability (OD 540/620 nm values: ctrl: 0.53±0.03; hydrocortisone 50 μM: 0.51±0.028), whereas cell exposure to increasing concentrations of dihydrotestosterone ([DHT], 0.01 pM-1 nM) had a significant inhibitory effect (IC_{50} value of 3.15 pM; 95% CI 1.5 to 6.6). The role of progesterone was next assessed by qRT-PCR and western blot assay of progesterone receptor (PgR) expression. PgR mRNA was detected, the ΔCt value being 10.97±0.13 (ΔCt value of the positive control - MCF7 - was 10.14±0.16). In addition, a specific primary antibody recognizing both the PgRA and PgRB isoforms (MW: 94 and 114 kDa, respectively) revealed that PgRB was the predominant isoform expressed in NCI-H295R cells (Supplemental Fig. 3).

NCI-H295R cells were then treated with AA in the presence of increasing concentrations of the PgR antagonist mifepristone (0.1 pM-100 pM) and analyzed for cell viability. As shown in Fig. 5A, the cytotoxic effect of AA was prevented in a concentration-dependent manner by mifepristone, whereas exposure to mifepristone alone did not affect NCI-H295R cell viability (Supplemental Fig. 4). The role of Pg was highlighted using an RNA interference approach and by silencing the PgR gene (Supplemental Fig. 2). The effect of PgR knock-down on AA cell viability was then investigated in NCI-H295R cells exposed to either si-PgR 10416 (10 nM), si-PgR 10417 (30 nM) or si-control for 2 days, and then treated with AA IC_{50} for 4 days. Silencing NCI-H295R cells by both siRNAs significantly prevented AA-induced cell death (Fig. 5B).

Discussion

New drugs for the rapid control of cortisol and androgen hypersecretion are need in the management of ACC. Since AA is able to selectively and irreversibly inhibit the CYP17A1 related enzymes 17α-hydroxylase and c17, 20-lyase, which are critical for cortisol and androgen biosynthesis, the drug holds promise as a potential component in the treatment algorithm of patients with ACC.

This study was designed to evaluate the antisecretive properties of AA in ACC models, including the human NCI-H295R cell line, a widely accepted model of hormonally active ACC [15], which expresses high amounts of CYP17A1 mRNA and other key enzymes of steroidogenesis. We found that AA significantly and steadily inhibited cortisol synthesis in NCI-H295R cells, but not in SW13 cells, an *in vitro* model of hormonally inactive ACC, which had undetectable CYP17A1 expression. AA-induced CYP17A1 inhibition in NCI-H295R cells also led to a marked reduction in androgen synthesis in cell medium, whereas progesterone concentration consistently increased. Of note is that the IC_{50} value of 63 nM fell within the range of the reported Cmax plasma drug concentrations at steady-state when AA was administered to CRPC patients at a daily dose of 1000 mg (226 ± 178 ng/ml; corresponding to a range from 19 to 157 nM) (Drugdex-Micromedex 2.0). Interestingly, corticosterone and aldosterone levels decreased when the cells were exposed to AA. These data are shared by those reported in a recent study performed on NCI-H295R cells: *in vitro* AA was found to inhibit the 21-hydroxylase (CYP21A2) enzyme that converts progesterone to hydroxy-corticosterone, a precursor of both corticosterone and aldosterone [22].

Intriguingly, we also found that AA significantly reduced cell viability in a concentration- and time-dependent manner in NCI-H295R cells, with the maximum effect occurring after exposure for 4 days. The reduction in cell viability was mainly due to an increased rate of apoptosis.

The animal models using NCI-H295 cells xenografted into immunodeficient mice indicated that AA is able to significantly inhibit tumor growth, which was associated with the concomitant increase in progesterone levels. While cortisol levels were not affected, corticosterone and aldosterone levels were increased in the plasma and tumors from the AA-treated mice. The data on plasma hormone levels can be explained by the fact that, unlike humans, the adrenals of rodents do not express the CYP17A1 gene and therefore have no P450C17 enzyme for cortisol production, but produce corticosterone [23]. However, we find no plausible explanation for why the cortisol concentration in the tumor tissues of the AA-treated mice did not differ from that of the control mice. It should be noted that, because this is a cross-sectional comparison, we did not have data on cortisol concentration in tumor tissue in the AA-treated mice at baseline condition before AA

administration. Another possible explanation is that while this mouse model is a workable experimental model to measure antineoplastic AA activity on ACC, it is not the appropriate model to measure changes in steroidal hormone secretion.

The antitumor properties of AA were also evident in the two primary cell cultures obtained from the patients bearing cortisol-secreting ACC. Similar to what we observed in the NCI-H295R cells, AA exerted concentration-dependent cytotoxic activity in these cultures, with marked reduction in cortisol and androgen concentrations in the medium, whereas the progesterone levels increased significantly. By contrast, AA was ineffective in affecting cell viability in the three primary cell cultures derived from the non-secreting ACC tumors, and these findings were consisted with those observed in SW13 cells. Taken together, our results indicate that the antineoplastic activity of AA is limited to hormone-secreting ACC. We then wanted to determine whether the AA-induced changes in hormonal levels were responsible for the decrease in cell viability. In agreement with previous reports, we observed that cortisol exposure did not significantly affect NCI-H295R cell viability [24], whereas DHT exerted a significant concentration-dependent cytotoxic effect [25]. These data suggest that the reduction in both cortisol and androgen levels induced by AA was unlikely to be related to the observed drug-induced decrease of cell viability.

We therefore focused our attention on the AA-induced increase in progesterone, as already observed in CRPC patients [9]. The biological functions of progesterone are mediated by nuclear PgRs, which are expressed in two different isoforms, the full length PgRB and the short form PgRA. In humans, the ratio of the two isoforms is critical for regulating biological responses of PgR in target tissues [26]. The two PgR isoforms are present in normal adrenal tissue and in ACC and have a similar expression rate [27], whereas NCI-H295R cells display a higher expression of PgRB than PgRA [27]. We found that PgRB is predominantly expressed in NCI-H295R cells and that the PgR antagonist mifepristone was able to antagonize AA-induced NCI-H295R cell toxicity in a concentration-dependent manner. In addition, the cytotoxic effect induced by AA was completely reverted by silencing PgR. These data provide indirect evidence for a role of PgR in mediating the AA-induced decrease in cell viability. Pertinently, estradiol was previously found to induce NCI-H295R cell proliferation by a non-genomic signaling stimulation of ERK1/2, and AKT phosphorylation [28], and drugs targeting estrogen receptor α were found to effectively control the proliferation of ACC *in vitro* [28].

The Wnt/beta-catenin pathway plays a central role in ACC pathophysiology and is a potential therapeutic target for drug development [3]. Interestingly, progesterone contributes to the dynamic modification of the

endometrial epithelium during the menstrual cycle by inhibiting Wnt/beta-catenin signaling, which is also required for maintaining endometrial homeostasis and for contrasting progression toward hyperplasia and carcinogenesis [29, 30]. We found that AA induced an increase in progesterone levels and prevented the nuclear accumulation of beta-catenin in NCI-H295R cells. Collectively, these results suggest that PgR signaling, via negative modulation of the Wnt/beta-catenin pathway, could contribute to AA inhibition of cell viability in ACC. We are now studying whether progesterone is able to modify Wnt/beta-catenin signaling and the molecular mechanism underlying this phenomenon.

In conclusion, our preclinical data suggest that AA may be of value in the management of secreting ACC. AA may possess not only antisecretive but also antitumor activity perhaps mediated through inhibition of the Wnt/beta-catenin pathway. The inhibition of AA antineoplastic activity by silencing PgR is a relevant finding that suggests that PgR may be a new target of antineoplastic therapy for ACC. On the basis of these results, we are conducting a prospective clinical trial to test AA activity in the management of Cushing's syndrome induced by ACC (Eudract: n. 2016-000945-29).

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Legends

Figure 1. Effect of AA on cortisol secretion in NCI-H295R cells. Cells were treated with increasing concentrations of AA (50-400 mM) for up to 6 days. Steroid analyses was carried out in the conditioned media by the HPLC. Limits of detection (LOD) and limit of quantification (LOQ) were measured by evaluating the signal/noise (S/N) ratio of three replicates for each compound at proper concentrations. LOD was fixed at the concentration with a S/N > 3 while concentrations of analyses with a S/N > 10 were chosen as LOQ. * P <0.01 versus untreated cells; ** P < 0.001 versus untreated cells.

Figure 2. AA-induced effects on NCI-H295R cells and NCI-H295R ACC xenograft model. A) Cells were treated with increasing concentrations of AA (10-200 nM) for 4 days. Cell viability was measured by MTT assay. Results are expressed as percent of viable cells versus control (ctrl) cells. Data are the mean ± SEM of three independent assays performed in triplicate. *P < 0.01 versus ctrl cells. B) AA was administered i.p. daily for 16 days in female SCID mice with a mean tumor volume (TV) of approximately 200 mm^3. Control (ctrl) mice received vehicle. Tumor growth was recorded by biweekly measurements of tumor diameter using a Vernier caliper. TV was calculated as described in Methods. The efficacy of drug treatment was assessed as TV inhibition percentage (TVI %) in treated versus control mice, calculated as follows: TVI%=[100-(mean TV treated/mean TV control x 100)]. *P = 0.009 for AA-treated versus control mice. C) Control and AA-treated mice (6 mice/group) were sacrificed by cervical dislocation and analyzed for plasma and tumor steroid content by HPLC as described in Methods. Panel a: Pg plasma, *P < 0.05 versus control mice; panel b: Pg tumor, *P < 0.05 versus control mice; panel c: testosterone tumor, *P < 0.05 versus control mice.

Figure 3. **AA treatment alters the subcellular localization of beta-catenin in NCI-H295R cells.** Cells were treated with AA (63 nM) for 3 days. Untreated (A) and AA-treated (B) cells were then analysed for beta-catenin following by incubation with Hoechst for nuclear staining. Panels a, d: Hoechst; panels b, e: beta-catenin; panels c, f: co-staining (Magnification 40×).

Figure 4. Cytotoxic effect of AA in primary cultures derived from ACC tumors. Cortisol-secreting ACC01 (■) and ACC02 (▲) and non–secreting ACC03 (Δ), ACC06 (◊) and ACC08 (○) primary cultures of human ACC cells were treated with increasing concentrations of AA (10-300 mM) for 4 days. Cell viability was measured by using MTT. Results are expressed as percent of viable cells versus control (ctrl) cells. Data

are the mean ± SEM of three independent MTT assays performed in triplicate. *$P < 0.01$ versus ctrl cells; **$P < 0.001$ versus ctrl cells.

Figure 5. AA reduction of cell viability in NCI-H295R cells requires PgR. A) NCI-H295R cells were treated with AA (63 nM) in combination with increasing concentration of the PgR antagonist mifepristone (0.1-100 pM) for 4 days. Cell viability was measured by MTT. Results were expressed as percent of viable cells versus untreated cells. Data are the mean ± SEM of three independent assays performed in triplicate. * $P < 0.05$ vs control (ctrl) cells; ** $P < 0.001$ vs ctrl cells. B) Untreated (ctrl), si-PgR 10416 (10 nM) and si-PgR 10417 (30 nM)- treated NCI-H295R cells were incubated with AA (63 nM) for 4 days. Cell viability was measured by MTT. Results are expressed as percent of viable cells versus ctrl cells. Data are the mean ± SEM of three independent MTT assays performed in triplicate. *$P < 0.01$ versus ctrl cells; ** $P < 0.001$ versus ctrl cells; ## $P < 0.001$ versus AA-treated cells.

Table 1. NCI-H295R cells were treated with AA (50 nM) for 2 days and the supernatants analyzed for hormone concentrations by using the Liquid Cromatography-Tandem Mass Spectrometry.