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ORIGINAL ARTICLE

# In vitro antitumor activity of progesterone in human adrenocortical carcinoma

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## Abstract

**Purpose** The management of patients with adrenocortical carcinoma (ACC) is challenging. As mitotane and chemotherapy show limited efficacy, there is an urgent need to develop therapeutic approaches. The aim of this study was to investigate the antitumor activity of progesterone and explore the molecular mechanisms underlying its cytotoxic effects in the NCI-H295R cell line and primary cell cultures derived from ACC patients.

**Methods** Cell viability, cell cycle, and apoptosis were analyzed in untreated and progesterone-treated ACC cells. The ability of progesterone to affect the Wnt/β-catenin pathway in NCI-H295R cells was investigated by immunofluorescence. Progesterone and mitotane combination experiments were also performed to evaluate their interaction on NCI-H295R cell viability.

**Results** We demonstrated that progesterone exerted a concentration-dependent inhibition of ACC cell viability. Apoptosis was the main mechanism, as demonstrated by a significant increase of apoptosis and cleaved-Caspase-3 levels. Reduction of β-catenin nuclear translocation may contribute to the progesterone cytotoxic effect. The progesterone antineoplastic activity was synergically increased when mitotane was added to the cell culture medium.

**Conclusions** Our results show that progesterone has antineoplastic activity in ACC cells. The synergistic cytotoxic activity of progesterone with mitotane provides the rationale for testing this combination in a clinical study.

**Keywords** Adrenocortical carcinoma · Progesterone · Progesterone receptor · Cell viability

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s12020-018-1795-x) contains supplementary material, which is available to authorized users.

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**Abbreviations**

| | |
|---|---|
| ACC | adrenocortical carcinoma |
| PgR | progesterone receptor |
| IC | Interval of Confidence |
| MTT | 3-(4,5-Dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide |
| mPR | progesterone membrane receptor |
| PGRMC1 | progesterone receptor membrane component 1 |

## Introduction

Adrenocortical carcinoma (ACC) is a rare aggressive endocrine tumor [1] that in approximately 50% of adults is capable of hormone secretion [2]. Cushing's syndrome is the most commonly associated endocrine disorder [3]. The systemic therapies have a limited efficacy [4–6]; thus, the prognosis of advanced ACC patients, not amenable to radical extirpation, is poor with a 5-year survival rate of 15% [7]; moreover, histological and molecular diagnostic parameters are not still completely shared [8]. Mitotane (o, p′-dichlorodiphe nyldichloroethane, o, p′-DDD) is the reference drug; however, toxicity and narrow therapeutic index limit its efficacy [9]. Therefore, there is an urgent need of new therapeutic approaches.

We have recently observed that abiraterone acetate (abiraterone) has both antisecretive and antitumor activities in ACC cell lines [10]. The antisecretive effect of abiraterone is mediated by the inhibition of 17alpha-hydroxylase/17,20-lyase (CYP17A1), a key enzyme for steroid hormone synthesis [11, 12] leading to a rapid inhibition of cortisol secretion [10, 13]. Abiraterone mechanism of action may involve, at least in part, the Wnt/β-catenin signaling pathway [10] that is constitutively active in approximatively 30% of ACC [14] and is a potential target for new molecular therapies. The cytotoxic effect of abiraterone remains to be fully elucidated, but evidence strongly indicates that it is directly associated with the drug-induced increase of progesterone levels, requiring the activation of the intracellular progesterone receptors (PgRs). Interestingly, in addition to the well-known role of PgRs as nuclear transcription factors, different members of membrane progesterone receptors (mPRs) have been identified and the term "extranuclear" or "nongenomic" effects of progesterone was suggested to specifically defined mPR functions [15]. Another putative membrane-specific progesterone receptor, distinct from known mPRs and nuclear PgR, was isolated from different tissues and called Progesterone Receptor Membrane Component 1 (PGRMC1) [15, 16]. The extranuclear receptor activation leads to a rapid signaling, linked to various second messenger cascades, including extracellular signal-regulated kinases (Erk 1/2, p42/44, p38 MAPKs) [15] and regulation of intracellular calcium mobilization [16].* These effects are progesterone-dependent but independent of PgR transcriptional activity, and are integral part of progesterone cellular effects [17].

In this study, we investigated the cytotoxic effects of progesterone and the molecular mechanisms underlying its antitumor activity in NCI-H295R ACC cell line [18] and in ACC primary cell cultures derived from patients with either cortisol-secreting or nonsecreting ACC.

## Materials and methods

### Cell lines

NCI-H295R ACC cell line was obtained from the American Type Culture Collection (ATCC) and cultured as suggested by the manufacturer. Cells were authenticated by the AmpFISTR Identifiler PCR amplification kit (Applied Biosystems, Foster City, CA, USA). Media and supplements were supplied by Sigma Italia (Milano, Italy). SW13 cell line was obtained from ATCC and cultured as suggested by the manufacturer.

### Primary cell cultures

Human ACC primary cells were derived from three patients with cortisol-secreting tumors (ACC01, ACC02, and ACC16) and from two patients with nonsecreting tumors (ACC03, ACC08). Clinical and histological features are reported in Table 1. After surgical removal, cells were enzymatically digested with (0.1 mg/mL) collagenase (Sigma Italia, Milano, Italy) and cultured in the same medium of NCI-H295R cells. The project was approved by the local Ethical Committee and written informed consent was obtained from all patients.

### Immunohistochemistry

Immunohistochemistry for PgR was performed on 2 µm sections from formalin fixed-paraffin-embedded ACC tissues. Ventana BenchMark Ultra platform was used according to the manufacturer's recommended settings. Ultra Cell Conditioning 1 (CC1) solution was used for heat-induced epitope retrieval (95 °C for 64 min). Slides were incubated (36 °C for 16 min) with the ready-to-use anti-PgR antibody (monoclonal rabbit anti-human PR clone 1E2, Roche) [19] and followed by UltraView Universal DAB Detection Kit. Positive and negative controls from breast cancer tissue microarrays were included in the same slides.

**Table 1** Clinical characteristics of ACC patients

| Primary culture identification | Tumor specimen | Histology | Disease stage | Hormone hypersecretion | PgR expression |
|---|---|---|---|---|---|
| ACC01<br>Female<br>66-year-old | Primary ACC | Weiss score 8<br>Mitotic index: >50/50 HPF Ki67 70% | Stage IV (hepatic metastases) | Cortisol (severe Cushing's syndrome) | 40% |
| ACC02<br>Female<br>63-year-old | Peritoneal metastases | Weiss score not available<br>Mitotic index: >50/50 HPF Ki67 50% | Stage IV (peritoneal dissemination) | Cortisol (mild clinical signs of hypercortisolism) | 70% |
| ACC16<br>Male<br>55-year-old | Primary ACC | Weiss score not available<br>Mitotic index: 10/50 HPF Ki67 50% | Stage IV<br>(bone and multiple abdominal lymphonodal metastases) | Cortisol (severe Cushing's syndrome) | 40% |
| ACC03<br>Male<br>59-year-old | Local relapse of ACC | Weiss score 8<br>Mitotic index: 25/50 HPF Ki67 20% | Stage IV (left hypochondrium soft tissue relapse and peritoneal dissemination) | No secretion | 3–5% |
| ACC08<br>Female<br>50-year-old | Lung metastases | Weiss score 8<br>Oncocytic features<br>Mitotic index: 10/50 HPF Ki67 20% | Stage IV (lung and bone metastases) | No secretion | 1–2% |

## Cell treatments

NCI-H295R cells and ACC primary cultures were seeded in 24-well plates and cultured in complete medium. Before treatment, culture medium was switched into charcoal-dextran-treated Nu-Serum (cNS)-medium with increasing concentrations of progesterone (0.1–160 μM) and/or mitotane (25 nM−40 μM) for 4 days. Both progesterone and mitotane were dissolved in Dimethyl-Sulfoxide (DMSO). NCI-H295R cells were also exposed to mifepristone (0.1 –500 nM) in combination with progesterone (25 μM) for 4 days. Mifepristone and mitotane were supplied by Selleckchem Chemicals (DBA Italia, Milano, Italy) and progesterone was purchased from Sigma Italia (Milano, Italy). SW13 cells were seeded in 24-well plates and cultured in complete medium. Before treatment, culture medium was switched into charcoal-dextran-treated Foetal Bovine Serum (FBS)-medium with increasing concentrations of progesterone (0.1–100 μM) for 3 days. Cell exposure to DMSO alone did not modify cell viability in any of the cell cultures used.

## Cell viability assay

Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay according to the manufacturer's protocol (Sigma Italia, Milan, Italy). Absorbance was measured by a spectrophotometer at 540/620 nm (GDV, Rome, Italy).

## Drug combination experiments

Progesterone and mitotane combination experiments were performed to evaluate their interaction on NCI-H295R cell viability, according to the Chou and Talalay method [20]. Cells were treated for 4 days with progesterone (0.1–160 μM) and mitotane alone (25 nM−40 μM) or with progesterone in combination with mitotane at a fixed ratio (progesterone:mitotane = 4:1), as recommended for the most efficient data analysis [21, 22]. Cells were analyzed for cell viability using MTT. Data were then converted to Fraction affected (Fa, range from 0 to 1 where Fa = 0 indicating 100% of cell viability and Fa = 1 indicating 0% of cell viability) and analyzed using the CompuSyn software (ComboSyn inc. Paramus, NJ, USA) to calculate the combination index (CI), being the CI value < 0.9 an indication of synergism, a CI = 0.9–1.1 an indication of additive effect and CI > 1.1 and indication of antagonism.

## Quantitative RT-PCR (qRT-PCR)

Gene expression was evaluated by qRT-PCR (ViiA7 Real-Time PCR System, ThermoFisher Scientific, Milan, Italy), using SYBR Green as fluorochrome, as described elsewhere [23]. The sequences of sense and antisense oligonucleotide primers are listed in Supplemental Table 1. Differences in the threshold cycle Ct values between the beta-actin housekeeping gene and the studied genes (ΔCt) were then calculated as an indicator of the amount of mRNA expressed.

## Western blot

Whole cell lysates were prepared in ice-cold buffer with protease and phosphatase inhibitor cocktails (Roche, Milano, Italy) [24]. Equal amounts of protein were separated by electrophoresis on a 4−12% NuPAGE Bis-Tris Gel System (Life Technologies, Milan, Italy) and electroblotted to a nitrocellulose membrane. Rabbit monoclonal antibody against human Caspase-3 (Cell Signaling Technology,

Milan, Italy) [25] was used, both at a final concentration of 0.1 µg/mL. The Erk protein was detected using anti-total Erk and anti-phospho-Erk antibodies (Santa Cruz Biotechnologies, Heidelberg, Germany) [26] at a final concentration of 0.7 µg/mL. Primary antibodies anti-mPR (0.5 µg/mL final concentration) and anti-PRGMC1 (1 µg/mL final concentration) were purchased from Abcam (Cambridge, UK) [27] and Santa Cruz Biotechnologies (Heidelberg, Germany) [28]. A mouse monoclonal antibody directed against the N-terminal region of human α-Tubulin (Sigma Italia, Milano, Italy) was used to normalize the values. Secondary HRP-labeled anti-rabbit and anti-mouse antibodies (Santa Cruz Biotechnologies, Heidelberg, Germany) were used and the specific signal was visualized by the ECL-LiteAblot Extend Long (Euroclone, Milano, Italy). Densitometric analysis of the immunoblots was performed using the GelPro-Analyzer v 6.0 (MediaCybernetics, Bethesda, MD, USA).

### Double staining AO/EtBr

NCI-H295R cells were treated with (progesterone (25 µM) for 4 days. A double staining with acridine orange (AO) and ethidium bromide (EtBr) was performed to visualize and quantify the number of viable, apoptotic, and necrotic cells, as previously described [10]. Cells were examined by a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss AG, Germany). Several fields, randomly chosen, were digitalized and scored by using the NIH ImageJ software.

### Cell cycle analyses

Flow cytometric cell cycle analysis was performed as described, with minor modifications [29]. Briefly, untreated and progesterone-treated NCI-H295R cells were fixed, treated with RNase A (12.5 µg/mL), stained with propidium iodide (40 µg/mL) (Sigma Italia, Milan, Italy) and analyzed by flow cytometry using an MACS Quant Analyzer (Miltenyi Biotec GmbH) for cell cycle status. Data were analyzed using FlowJo (TreeStar).

### Immunofluorescence

Cells were grown onto 12 mm poly-L-lysine-coated coverslips and treated with $IC_{50}$ value of progesterone for 3 days, with or without mifepristone (100 nM). Cells were then fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 1 h. Nonspecific binding was blocked by incubation in PBS containing 0.2% Triton X-100 and 10% normal goat serum for 1 h. Cells were then incubated overnight at 4 °C with anti-β catenin primary antibody (14.2 ng/mL, Cell Signaling Technologies, Milan, Italy). After extensive washes, the Alexa Fluor488 anti-rabbit secondary antibody (Life Technologies, Milan, Italy) was applied for 1 h at room temperature, followed by counterstaining with Hoechst (Sigma Aldrich, Milan, Italy) for 5 min. After rinsing in PBS, coverslips were mounted using FluorPreserve™ Reagent and cell staining was detected using a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss AG, Oberkochen, Germany). NIH-ImageJ software was used for image analysis and processing.

### 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. Unless otherwise specified, data are expressed as mean ± SEM of at least three experiments run in triplicate. $P$ values $< 0.05$ were considered statistically significant.

## Results

### Progesterone effects on NCI-H295R cell viability

The predominant expression of the intracellular full-length PgR B isoforms was previously described in NCI-H295R cells [10]. The exposure of NCI-H295R cells to increasing concentrations of progesterone (0.1–160 µM) for 4 days led to a reduction in NCI-H295R cell viability in a concentration-dependent manner (Fig. 1a). Sigmoidal concentration−response function was applied to calculate the $IC_{50}$ value of progesterone in NCI-H295R cells, which was 25.5 µM (95% Interval of Confidence (IC), 19.9−32.9). Time-course experiments in NCI-H295R cells treated with progesterone at the $IC_{50}$ value demonstrated that the reduction of cell viability reached its maximum at 4 days, with no significant change up to 6 days ($IC_{50}$ value of 29.6 µM; 95% CI 23.6−37.0) (data not shown). Pretreatment of NCI-H295R cells with increasing concentration of the PgR antagonist mifepristone (0.1−500 nM) antagonized the cytotoxic effect elicited by progesterone at its $IC_{50}$ for 4 days (Fig. 1b). This provides evidence that the antineoplastic activity of progesterone requires the stimulation of the PgRs. Mifepristone alone (0.1–500 nM; 4 days) did not affect NCI-H295R cells viability (data not shown).

### Progesterone induces NCI-H295R cells apoptosis, without inducing changes in the cell cycle distribution

To provide explanation on the mechanism underlying the progesterone-induced NCI-H295R cell toxicity, cells were

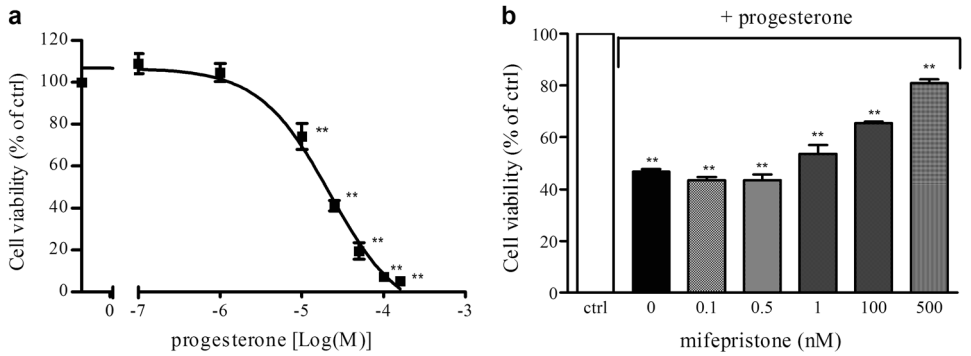


**Fig. 1** Cytotoxic effect of progesterone in NCI-H295R cells. **a** NCI-H295R cells were treated with increasing concentration of progesterone (0.1−160 µM) for 4 days. Cell viability was analyzed by MTT assay. Results are expressed as percent of viable cells vs. control (ctrl) cells. Data are the mean ± SEM of three experiments performed in triplicate. $^{**}P < 0.001$ vs. ctrl. **b** Cells were treated with progesterone (25 µM) for 4 days in the presence of the PgR antagonist mifepristone (0.1–500 nM). Cell viability was analyzed by MTT assay. Results are expressed as percent of viable cells vs. control (ctrl) cells. Data are the mean ± SEM of three experiments performed in triplicate. $^{**}P < 0.001$ vs. ctrl

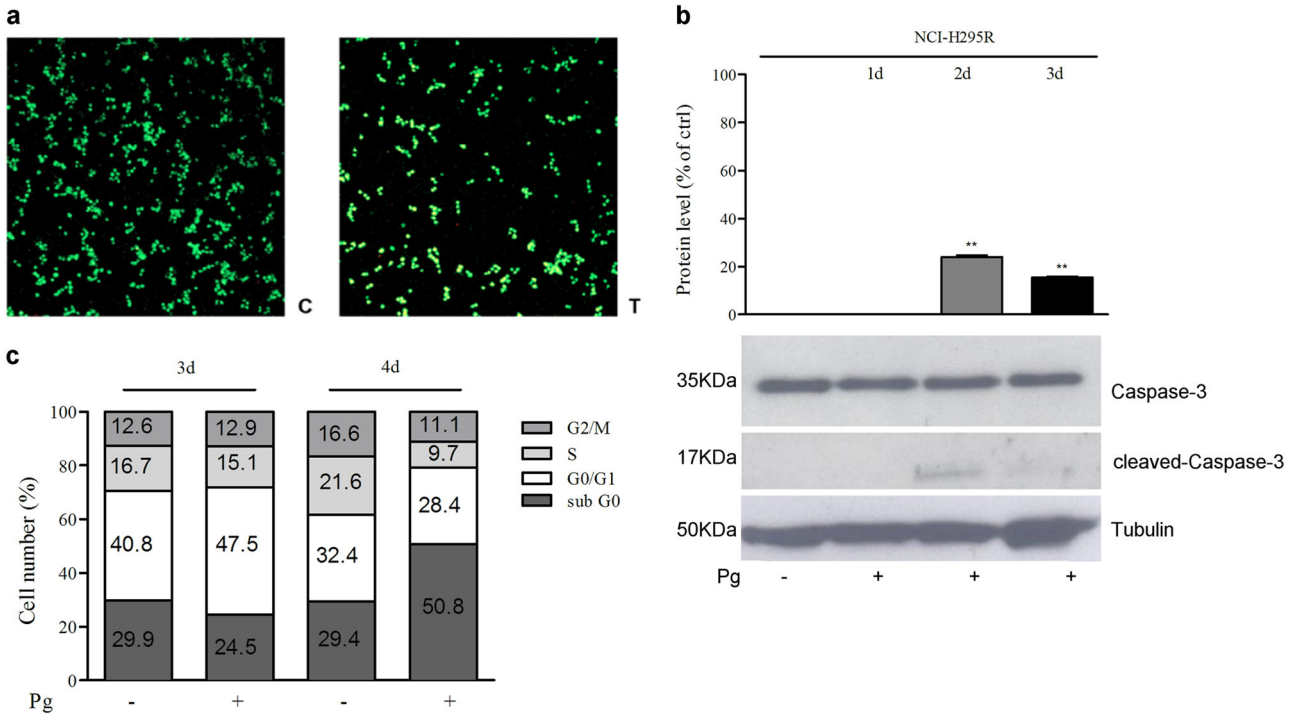


**Fig. 2** Progesterone promotes apoptotic events in NCI-H295R cells, with no influences on cell cycle phases. **a** NCI-H295R cells were treated with progesterone (25 µM) for 4 days. Untreated (C) and progesterone-treated (T) cells were then stained with AO/EtBr. Viable (green), apoptotic (yellow), and necrotic (red) cells were scored under a confocal laser-scanning microscope. Magnification, ×10. The images were representative of at least three independents experiments, with superimposable results. **b** Cells were treated with progesterone (25 µM) for 1−3 days and analyzed for Caspase-3 and cleaved-Caspase-3 expression using western blot (WB). The human α-Tubulin was used as internal control. A representative WB is shown. Densitometric analysis of blots ($n = 3$) with specific levels of cleaved-Caspase-3 normalized to the corresponding tubulin levels. Bars represent the mean ± SEM $^{**}P < 0.01$ vs. untreated cells. **c** NCI-H295R cells were treated with progesterone (25 µM), stained with propidium iodide and analyzed for DNA content by flow cytometry. Histograms representative of one out of three experiments were shown in the figure

treated with progesterone (25 µM; 4 days) and stained with AO/EtBr. Progesterone deeply increased the number of apoptotic cells 39 ± 2%; while necrotic and living cells were 2 ± 2% and 59 ± 3% respectively (apoptotic cells: untreated vs. treated cells: $P < 0.001$ Fig. 2a). Time-course experiments were conducted and our results demonstrated that after 2 days of treatment, the effect is as follows: 93 ± 1% living cells, 7 ± 1% apoptotic cells and no necrotic cells

(apoptotic cells: untreated vs. treated cells: $P < 0.05$; Supplemental Fig. 1a). Progesterone-induced apoptotic cytotoxicity reached its maximum after 4 days of treatment, as indicated above, and it was not modified if cells were exposed to progesterone up to 6 days: 47 ± 1% living cells, 43 ± 3% apoptotic cells, 10 ± 1% necrotic cells (apoptotic cells: untreated vs. treated cells: $P < 0.001$; Supplemental Fig. 1b). We next examined the expression of total Caspase-3 and the cleaved-Caspase-3, that play a central role in the execution phase of cell apoptosis [30], in progesterone-treated NCI-H295R cells in comparison to untreated cells (Fig. 2b). Progesterone exposure for 48 h significantly increased the expression of cleaved-Caspase-3 (% of increase: 23.9 ± 1.6) while total Caspase-3 levels were not affected. The analyses of the cell cycle progression by flow cytometry in untreated and progesterone-treated NCI-H295R cells did not show significant differences in cell distribution up to 4 days of treatment (Fig. 2c). However, we observed that treatment with progesterone for 4 days increased the proportion of cells in the sub-G0 phase: 29.7 ± 4.6% untreated cells, 50.3 ± 5.1% progesterone-treated cells ($P < 0.05$), suggestive of DNA fragmentation. Taken together these observations suggest that apoptosis is the main mechanism mediating the progesterone cytotoxicity.

## Effect of progesterone on β-catenin nuclear translocation in NCI-H295R cells

NCI-H295R cells were treated with progesterone and analyzed for β-catenin localization using immunofluorescence analyses. At baseline, β-catenin was highly expressed in the nucleus (Fig. 3a), whereas the cell exposure to progesterone at its $IC_{50}$ reduced β-catenin nuclear localization and increased its retention into cytoplasm (Fig. 3b). The effect of progesterone in sequestering β-catenin in cytoplasm was counteracted by 100 nM mifepristone (Fig. 3c). Immunofluorescence quantification using ImageJ software, reported in Table 2, demonstrated that progesterone significantly reduced β-catenin nuclear localization. The progesterone-induced reduction of nuclear β-catenin induced the decrease of mRNA expression of some of its target genes, namely MYC and survivin, while the mRNA expression level of another gene, CCND1, resulted unchanged by progesterone treatment (Supplemental Fig. 2).

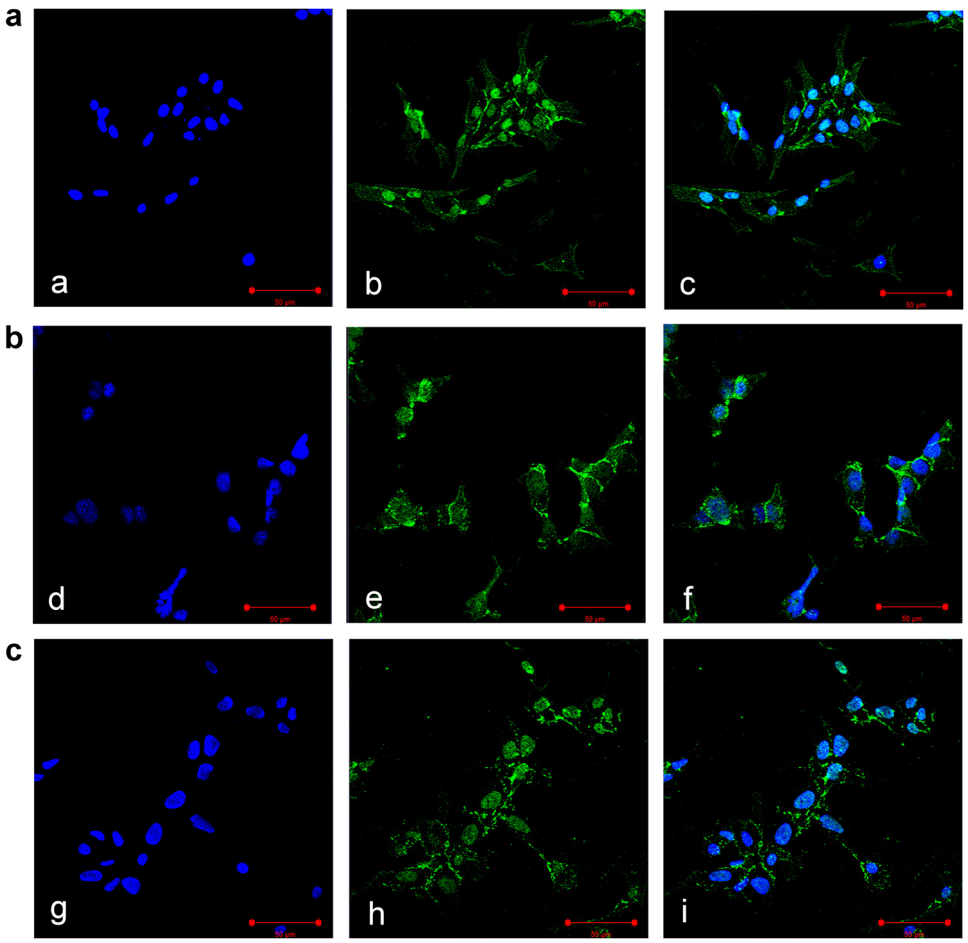


**Fig. 3** Progesterone treatment affects the subcellular localization of β-catenin in NCI-H295R cells. Cells were treated with progesterone (25 μM) alone or in combination with mifepristone (100 nM) for 3 days. Untreated **a**, progesterone-treated **b**, progesterone-mifepristone-treated (**c**) cells were analyzed for β-catenin localization following by incubation with Hoechst for nuclear staining. Panels **a**, **d**, **g**: Hoechst; panels **b**, **e**, **h**: β-catenin; panels **c**, **f**, **i**: merge. The scale bar of 50 μm is automatically inserted by the software ZEN Black

**Table 2** Immunofluorescence quantification of β-catenin expression

| | | % mean ± SEM |
|---|---|---|
| Untreated cells | Nucleus | 51.06 ± 2.3 |
| | Cytoplasm | 48.94 ± 2.3 |
| Progesterone-treated cells | Nucleus | 29.01 ± 3.1 |
| | Cytoplasm | 70.99 ± 3.9* |
| Progesterone-treated cells in the presence of mifepristone | Nucleus | 54.20 ± 2.3 |
| | Cytoplasm | 45.80 ± 2.6 |

Cells were treated with progesterone (25 μM) alone or in combination with mifepristone (100 nM) for 3 days. Quantification was performed using the ImageJ software. Several cells in different fields, randomly chosen, were quantified

*$P < 0.001$ vs. nuclear localization

### mPR and PGRMC1

As underlined in the Introduction, accumulating evidence suggests that rapid progesterone responses are mediated by activation of mPRs [31]. In order to evaluate whether these receptors could contribute to the observed progesterone cytotoxic effect on NCI-H295R, we firstly evaluated their expression. As shown in Supplemental Fig. 3a, NCI-H295R expressed mPRs. These receptors were functionally active, as, when cells were treated with the $IC_{50}$ value of progesterone, we observed a reduction of phospho-Erk protein level (% of decrease: 30.39 ± 1.14) at a very early time, namely 15 min after progesterone exposure (Supplemental Fig. 3b). Finally, we demonstrated that NCI-H295R expressed as well the PGRMC1 (Supplemental Fig. 3a).

### Progesterone enhanced NCI-H295R cytotoxicity induced by mitotane in drug-combination treatments

To evaluate whether progesterone treatment of NCI-H295R cells could enhance the cytotoxicity of mitotane, the combination index (CI) was calculated according to the Chou −Talalay method [21]. NCI-H295R cells were firstly exposed to increasing concentrations of mitotane (25 nM −40 μM) for 4 days and analyzed for cell viability by MTT assay. Sigmoidal concentration−response function was used to calculate the $IC_{50}$ value, which was 3 μM (95% CI, 2.08−4.34) (Fig. 4a). The cytotoxic effect of progesterone in combination with mitotane was evaluated at the 1:4 fixed molar ratio for 4 days (Fig. 4b). We found that in NCI-H295R cells, the combination had a synergistic cytotoxic effect as compared to each single compound at a Fa = 0.09 −0.86 with range of CI, 0.08−0.88 (Fig. 4c).

### Progesterone exerted cytotoxic effect in primary human ACC cells

Primary cultures derived from ACC patients were treated with increasing concentrations of progesterone for 4 days and analyzed for cell viability by MTT assay. In cortisol-secreting ACC cells (ACC01, ACC02, and ACC16), progesterone exerted a concentration-dependent inhibition of cell viability with $IC_{50}$ values of 18 μM (95% CI, 11.4 −31.7), 32.9 μM (95% CI, 26.5−40.9) and 39.2 μM (95% CI, 31.8−48.4) respectively. Immunohistochemical analyses of PgR expression in ACC01, ACC02, and ACC16 tumors showed that at least 40% of neoplastic cells were positive for PgR (Table 1). By contrast, a lesser cytotoxic effect of progesterone was observed in the nonsecreting human ACC cells, ACC03 and ACC08, with $IC_{50}$ values of 73.4 μM (95% CI, 46.1−116.8) and 80.8 μM (95% CI, 50.5 −129.5) respectively (Fig. 5). PgR expression in these cells was detected in less than 5% of ACC cells (Table 1).

### Progesterone effect on SW13 cell line

Finally, as an internal control, we tested the effect of progesterone in the nonsteroidogenic SW13 cell line, which can be found in adrenal small-cell carcinoma and of which the exact histopathological features are still under investigation [32]. We firstly analyzed the mRNA expression of PgRs and the results indicated that SW13 cells were devoid of PgR: indeed, q-RT-PCR analysis revealed a not-detectable PgR mRNA expression in this cell line compared to the ΔCt of 9.01 ± 0.25 in NCI-H295R cells, used as positive control. The western blot analysis of mPR and PGRMC1 expression in SW13 cell line indicated that these receptor proteins were expressed in this cell line (Supplemental Fig. 4a). When exposed to increasing concentrations of progesterone within the same range of concentrations used for NCI-H295R cells, a cytotoxic effect could be observed, although it was non-concentration-dependent (Supplemental Fig. 4b).

## Discussion

In the present study, we demonstrated that progesterone, through its receptors, exerted a concentration-dependent and time-dependent inhibition of ACC cell viability, and this effect was, at least in part, counteracted by the PgR antagonist mifepristone. The role of PgR is further supported by the data published by our group, where the PgR silencing induces the almost complete disappearance of the effect of abiraterone on cell viability [10]. The cytotoxic effect of progesterone was observed in the NCI-H295R cells, an ACC cell line that mainly express the full-length PgR B isoform [10, 33], and

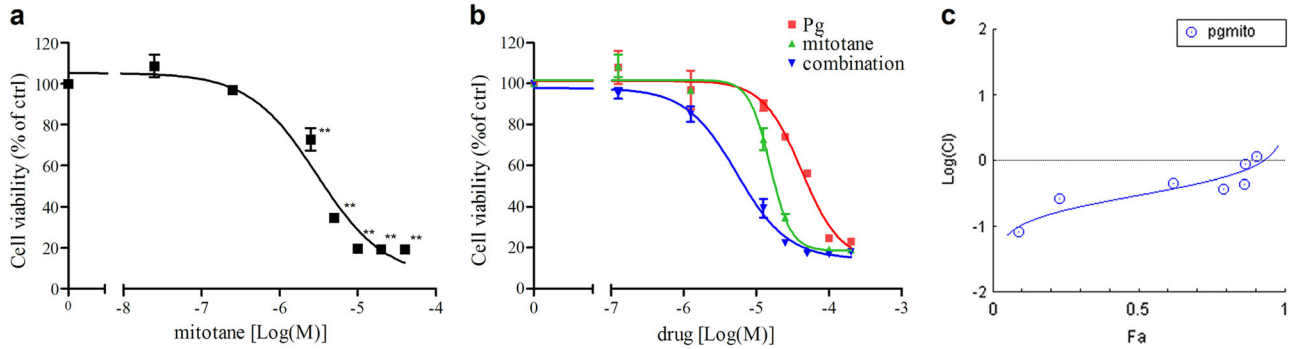


**Fig. 4** Effect of the combination of progesterone with mitotane in NCI-H295R cells. **a** NCI-H295R cells were treated with increasing concentration of mitotane (25 nM−40 μM) for 4 days. Cell viability was analyzed by MTT assay. Results are expressed as percent of viable cells vs. control (ctrl) cells. Data are the mean ± SEM of three experiments performed in triplicate. $^{**}P < 0.001$ vs. ctrl. **b** NCI-H295R cells exposed to increasing concentrations of progesterone and mitotane alone or in combination at fixed concentration at 1:4 molar ratio (progesterone: mitotane) for 4 days. Data are expressed as percent of viable cells vs. control (ctrl) cells. Data are the mean ± SEM of three experiments performed in triplicate. **c** Cell viability from **b** was converted to Fraction affected (Fa) values and resulting data were analyzed with CompuSyn software to obtain combination index (CI) plot. Fa = 0, 100% cell viability; Fa = 1, 0% cell viability; CI value < 0.9, synergism, CI = 0.9–1.1 additive effect and CI > 1.1 antagonism

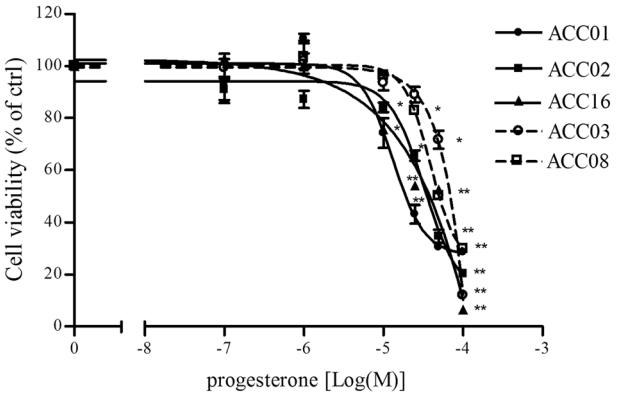


**Fig. 5** Cytotoxic effect of progesterone in human ACC primary cultures. Cortisol-secreting ACC01 (●), ACC02 (■), ACC16 (▲) and nonsecreting ACC03 (○), ACC08 (□) primary cultures of human ACC cells were treated with increasing concentrations of progesterone (0.1–100 μM) for 4 days. Cell viability was evaluated by MTT assay. Results are expressed as percent of viable cells vs. control (ctrl) cells. Data are the mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.01$ vs. ctrl; $^{**}P < 0.001$ vs. ctrl

confirmed in primary cell cultures derived from cortisol-secreting ACC that are characterized by a marked expression of the PgR. Indeed, the cytotoxic effect of progesterone was less evident in nonsecreting ACC tumors in which PgR expression was low. Intriguingly, we demonstrated that NCI-H295R cells expressed also the mPR and PGRMC1 component, suggesting that the progesterone effects that we observed in ACC cells, both in cell line and primary cultures, could be a result of a multifactorial process involving both genomic and nongenomic actions, dependent on both membrane and intracellular progesterone receptor arrangement expressed by each ACC tumor. The scenario is even more complex than expected, in light of the evidence showing that the different effect of progesterone depends on many variables, such as the type of cells, the genomic and/or non-genomic effects linked to the progesterone receptor expression and the concentration of hormone present [34]. As a matter of fact, we observed that the nonsteroidogenic SW13 cell line, established from a small-cell carcinoma of the adrenal [35], was responsive to the cytotoxic effect of progesterone (although without displaying a concentration-dependent curve) despite they expressed only mPR and PGRMC1. On the basis of these preliminary results the characterization of expression and function of all receptor components of progesterone pathway in our experimental models is now undergoing in our lab.

The present results confirm and extend our previous study [10] showing that the in vitro antineoplastic activity of abiraterone is mediated by the drug-induced increase in progesterone levels. PgRs, therefore, could represent a novel promising target in the management of ACC.

In PgR-positive breast cancer cell lines, progestins can induce a growth arrest due to decreased expression and activity of cyclin-dependent kinase (cdk) complexes [36]. In the present study, we showed that the progesterone-induced cytotoxicity of NCI-H295R ACC cells was not cell cycle mediated, but apoptosis represented the main molecular events, with a significant increase of the proapoptotic cleaved-Caspase-3 levels in the initial phase of the treatment. The ability of progesterone in modulating apoptotic events, both in vitro and in vivo, was previously demonstrated in several tumor cells [37–41]. In light of these results, we therefore explored the possible molecular mechanism regulating the progesterone-induced apoptosis in ACC.

The Wnt/β-catenin pathway is frequently altered in ACC, which is characterized by CTNNB1 mutations leading to β-catenin accumulation in the nucleus, where it binds with the T-cell factor (Tcf) and enhances its transcriptional activity.

In the NCI-H295R cell line, harboring the activating CTNNB1 p.S45P mutation, we found that progesterone treatment partially inhibited the β-catenin translocation into the nucleus, thus suggesting the involvement of this pathway in the progesterone antineoplastic activity. These data are in line with our previous in vitro experiments showing that the increased levels of progesterone in NCI-H295R cell culture microenvironment, induced by the block of the CYP17A1 by abiraterone, significantly inhibited the β-catenin migration into the nucleus. Further evidence comes from studies showing that progesterone is able to inhibit the Wnt/β-catenin pathway in endometrial carcinoma [42]. The functional effect of the β-catenin modification of translocation is the downregulation of the expression of some β-catenin target genes, namely MYC and survivin, while CCND1 was not modified.

Taken together these data are suggestive of an involvement of β-catenin inhibition in the progesterone-induced apoptosis of ACC cells. These data, however, are not exhaustive and the full evidence of the inhibitory effect would require the demonstration of a modulation of the expression of other specific β-catenin target genes in NCI-H295R cells by progesterone treatment. These further experiments are outside the scope of the present paper and will be a matter of a future study.

Finally, the in vitro demonstration of the synergistic cytotoxic effect of the combination mitotane + progesterone could be of considerable interest for its possible clinical application, as progesterone and its derivatives are already part of the supportive approach in cancer patients. These preclinical data provide the rationale for a new trial, testing the efficacy of progesterone in association with current systemic therapies in the management of ACC patients.

In conclusion, the present study shows that progesterone exerts a cytotoxic activity in ACC cells, by inducing apoptosis via activation of the progesterone receptors. Both the genomic and nongenomic effects of progesterone seemed to mediate the cytotoxicity, although this point is still under investigation. The synergistic cytotoxic activity of progesterone with mitotane provides the rationale for testing this combination in a prospective clinical study.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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