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Research Article

Ribociclib Cytotoxicity Alone or Combined With Progesterone and/or Mitotane in in Vitro Adrenocortical Carcinoma Cells

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Abstract

Mitotane is the only approved drug for treating adrenocortical carcinoma (ACC). The regimen added to mitotane is chemotherapy with etoposide, doxorubicin, and cisplatin. This pharmacological approach, however, has a limited efficacy and significant toxicity. Target-therapy agents represent a new promising approach to cancer therapy. Among these, a preeminent role is played by agents that interfere with cell-cycle progression, such as CDK4/6-inhibitors. Here, we investigate whether ribociclib could induce a cytotoxic effect both in ACC cell line and patient-derived primary cell cultures, alone or in combined settings. Cell viability was determined by 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide assay, whereas cell proliferation was evaluated by direct count. Binary combination experiments were performed using Chou and Talalay method. Gene expression was analyzed by quantitative RT-PCR, whereas protein expression was evaluated by immunofluorescence. A double staining assay revealed that ribociclib induced a prevalent apoptotic cell death. Cell-cycle analysis was performed to evaluate the effect of ribociclib treatment on cell-cycle progression in ACC cell models. Our results indicate that ribociclib was cytotoxic and reduced the cell proliferation rate. The effect on cell viability was enhanced when ribociclib was combined with progesterone and/or mitotane. The effect of ribociclib on cell-cycle progression revealed a drug-induced cell accumulation in G2 phase. The positive relationship underlined by our results between ribociclib, progesterone, and mitotane strengthen the clinical potential of this combination.

Key Words: ribociclib, progesterone, mitotane, adrenocortical carcinoma, CDK4/6-inhibitor

Abbreviations: ACC, adrenocortical carcinoma; CSS, charcoal-stripped serum; DMSO, dimethyl sulfoxide; EDP-M, mitotane, etoposide, doxorubicin, and cisplatin; Fa, fraction affected; IC_{50}, half maximal inhibitory concentration; MTT, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide

Adrenocortical carcinoma (ACC) is a rare endocrine neoplasia characterized by a poor prognosis, with clinical manifestations that are the consequence of either steroid excess or the tumor mass growing. Surgery is the mainstay of treatment; unfortunately, about 50% of ACC patients are diagnosed at an advanced stage, and a consistent proportion of patients undergoing radical surgery are destined to develop metastases during follow-up. The 5-year survival of patients with metastatic ACC is less than 15%. Mitotane is the only drug approved for the treatment of advanced ACC until now. It is recommended either in an adjuvant setting or when managing advanced/metastatic disease (for extensive review, see (1, 2)). The standard systemic treatment for advanced/metastatic ACC patients not eligible for surgery is the combination of mitotane with etoposide, doxorubicin, and cisplatin (EDP-M regimen) (2, 3). Although some pathological responses have been observed (4), the efficacy of EDP-M is limited, and most initially responding patients are destined to relapse and die of disease (5). Other cytotoxic therapies, administered to patients with disease progression to EDP-M, did not show a remarkable activity (6-8). Moreover, despite intensive efforts, no effective targeted treatment is available for patients with advanced ACC (9), whereas immunotherapy with modern immune check point inhibitors has shown some promising results (10); however, strategies to overcome mechanisms of primary immune resistance of ACC should be implemented (11, 12). New treatment strategies are therefore needed. Molecular alteration that directly or indirectly

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abnormally induces G1-S phase cell-cycle progression, such as proteins involved in the cell cycle or tyrosine kinase receptors cyclin-dependent kinases, represent one of the most promising drug targets in the management of ACC (13-15). Our group showed that the CDK4/6 inhibitor palbociclib significantly affects cell viability of both ACC cell lines and patient-derived primary cell cultures, providing preclinical evidence that CDK4/6 targeting agents could be effective in the ACC treatment (16). These results were confirmed by another recently published in vitro study (15) When translated to clinic, however, CDK4-CDK6 inhibitors have limited value as antineoplastic agents in monotherapy (17, 18). Indeed, they offer greater promise when combined with other targeted therapies (19). The third-generation CDK4/6 inhibitors palbociclib, ribociclib, and abemaciclib are approved in for managing advanced HR+, HER2- breast cancer in association with endocrine therapies such as aromatase inhibitors or fulvestrant (20). Interestingly, we demonstrated that progesterone possesses both antisecretive and cytotoxic activity in ACC cells (21, 22).

Using 3 experimental cell models, in line with the ACC heterogeneity observed in clinic, we investigated the in vitro effects of the CDK4/6 inhibitor on ACC cells, focusing our attention on the combination setting. In particular, we investigated whether ribociclib exerted in vitro antineoplastic activity, either alone or combined with progesterone, using 2 different approaches for combination experiments. We also evaluated whether mitotane could be added to the combined therapy, performing ternary combinations. Finally, we evaluated the effect of ribociclib on cell-cycle progression in our cell models.

Materials and Methods

ACC Cell Lines

Human ACC cell lines, namely NCI-H295R and MUC-1, were used. NCI-H295R cells were established from a secreting human ACC and represents the most widely used experimental cell model to study ACC in vitro (23), whereas MUC-1 cells were derived from a neck metastasis of an EDP-M-treated patient (24). NCI-H295R cells were purchased from American Type Culture Collection (ATCC) and maintained in culture according to ATCC instructions. The MUC-1 cell line was kindly provided by Dr. Hantel and cultured as indicated in Hantel et al (24). Media and supplements were purchased from Euroclone (Milano, Italy). Cell lines was periodically tested for mycoplasma and authenticated short tandem repeats profile by BMR Genomics srl (Padova, Italy).

Patient-derived Primary Culture

ACC115m primary cell culture was derived from a patient diagnosed with ACC who underwent surgical resection. In particular, cells were established from a lymph-node localization in a patient who experienced disease progression after the EDP-M. The project was approved by the Ethics Committee, and informed consent was received from each patient enrolled in the study. The primary ACC culture was obtained following the method described previously (25). To assess the adrenal origin, primary cell cultures were then characterized, evaluating the steroidogenic factor 1 expression by quantitative RT-PCR, as described previously [26], and by immunofluorescence. The primary culture was also analyzed by BMR Genomics srl, and the stability of the short tandem repeats profile was confirmed every 5 passages.

Cell Viability and Cell Proliferation Assay

Cell viability was determined by 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay, as previously described (25).

Drugs

Ribociclib succinate (ribociclib) was kindly given by Novartis Pharma S.p.A., dissolved in dimethyl sulfoxide (DMSO), and a stock solution of 18 mM was prepared, aliquoted, and stored at -80°C. Progesterone (Merk, Milano, Italy) was dissolved in DMSO in a stock solution of 100 mM, aliquoted, and stored at -20°C. Mitotane (Selleckchem Chemicals, DBA Italia, Segrate, Milano, Italy) was dissolved in DMSO in a stock solution of 180 mM, aliquoted, and stored at -80°C.

Single-drug Cell Treatment

NCI-H295R cells (30 000 cells/well), MUC-1, and ACC115m cells (20 000 cells/well) were seeded in 24-well plates and treated with increasing concentrations of ribociclib. The length of treatment was chosen relating to the doubling time calculation for each cell line, according to the ATCC method.

NCI-H295R cell lines and ACC115m primary culture were exposed to ribociclib (2.5-100 μM and 2.5-75 μM respectively) for 4 days, whereas MUC-1 cells were exposed to ribociclib (2.5-100 μM) for 5 days. After the end of the treatment, cells were analyzed for cell viability and cell proliferation.

Binary and Ternary Drug Cell Treatment

Ribociclib and progesterone combination experiments were performed to evaluate their potential interaction on the viability of NCI-H295R and MUC-1 cell lines and on ACC115m

Table 1. Oligonucleotide sequences

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)
CDK4	GCCTCGAGATGTATCCTGC	AGTCAGCATTTCCAGCAGCA
CDK6	ATCTCTGGAGTGTTGGCTGC	GGCAACATCTCTAGGCCAGTC
P107	ACGATTCTGCACTGTGGGAG	GTCCCTGCACATTTCCTCCA
P130	CACCCCTCAGATCCAGCAG	CGTGTAGCTTTCGCTCATGC
PgR	CGCGCTCTACCCTGCACTC	TGAATCCGGCCTCAGGTAGTT
SF1	CAGCCTGGATTTGAAGTTCC	TTCGATGAGCAGGTTGTTGC
Rb	CATCGAATCATGGAATCCCT	GGAAGATTAAGAGGACAAGC
β-actin	TCTTCCAGCCTTCCTTCCTG	CAATGCCAGGGTACATGGTG

primary cells. Before treatment, for steroid hormonal depletion, the entire medium was switched to dextran-treated charcoal-stripped serum (CSS) containing medium (CSS medium). Cells were seeded as previously described. NCI-H295R, MUC-1, and ACC115m cells were treated for 4, 5, and 4 days, respectively, with ribociclib (2.5-100 μM) and progesterone (3.75-150 μM) alone or in combination with a fixed ratio (ribociclib:progesterone = 1:1.5), as recommended for the most efficient data analysis according to the Chou and Talalay method (27). Cells were analyzed for cell viability using MTT. Data were then converted to fraction affected (Fa [range, 0-1], where Fa = 0, indicating 100% cell viability and Fa = 1, indicating 0% cell viability) and analyzed using the CompuSyn software (ComboSyn inc. Paramus, NJ, USA) to calculate the combination index. A combination index level < 0.9 is an indication of synergism, 09 to 1.1 an indication of additive effect, and > 1.1 is an indication of antagonism. A different approach to the binary combination experiment was also used in NCI-H295R and MUC-1 cells. Cells were treated with increasing concentrations of progesterone or ribociclib alone or combined with a fixed concentration of the other drug. Cells were analyzed for cell viability. For the ternary treatments, NCI-H295R cells were seeded as previously described and treated with 3 different concentrations (corresponding to 0.5 × half maximal inhibitory concentration [IC_{50}], IC_{50}, and 2 × IC_{50} values) of ribociclib, progesterone, and mitotane alone or in combination for 96 hours. Before treatment, the complete medium was switched to CSS medium. Cells were analyzed for cell viability.

Quantitative RT-PCR

Gene expression was evaluated by quantitative RT-PCR (ViiA7 Real-Time PCR System, Thermo-Fisher Scientific, Milan, Italy), using SYBR Green as fluorochrome, as described elsewhere (25). The sequences of sense and antisense oligonucleotide primers are listed in Table 1. Differences in the cycle threshold values between the β-actin housekeeping gene and the studied genes were then calculated as an indicator of the amount of mRNA expressed. The Livak method was applied to analyze the relative changes in gene expression (28).

Measurement of Cell Apoptosis

The Pacific Blue Annexin V/ SYOX AADVanced apoptosis kit (Invitrogen) was applied to investigate ribociclib induced cell death. Cells (3 × 10^5 cells/well) were seeded in 6-well plates in complete medium; 24 hours later, cells were treated with ribociclib concentration corresponding to IC_{50}-calculated values for 3 or 4 days. Cells were collected, washed with ice-cold PBS, resuspended in the binding buffer, and stained with Pacific Blue Annexin V/ SYOX AADVanced, according to the manufacturer instructions. Cells were then analyzed using MACSQuant10 cytometer (Miltenyi) using unlabeled cells as negative control. Quantification of apoptosis was determined by FlowJo v10.6.2 software. Annexin V+/SYTOX- and Annexin V+/SYTOX+ cells were considered early- and late-phase apoptotic cells, according to the manufacturer instructions.

Cell-cycle Analysis

Flow cytometric cell-cycle analysis was performed as described (29), with minor modifications. Briefly, untreated and ribociclib-treated NCI-H295R and MUC-1 cells were fixed, treated with Rnase A (12.5 μg/mL), stained with propidium iodide (40 μg/mL) (Sigma Aldrich Italia), and analyzed by flow cytometry using a MACS Quant Analyzer (Miltenyi Biotec GmbH) for cell-cycle status. Data were analyzed using FlowJo (TreeStar).

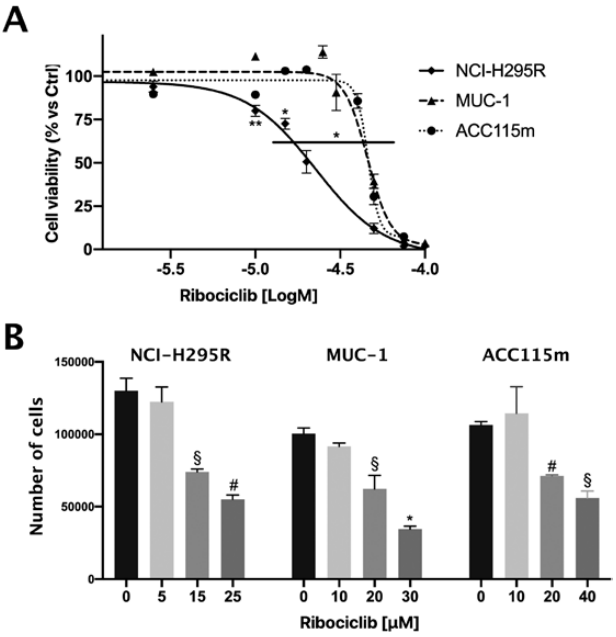

Figure 1. Cytotoxic effect of ribociclib in ACC cell models. (A) Concentration-response curve ribociclib-induced inhibition of cell viability of ACC cell models. NCI-H295R and MUC-1cells were treated with increasing concentrations of ribociclib (2.5-100 μM) for 4 and 5 days, respectively. ACC115m cells were treated with increasing concentrations of ribociclib (2.5-75 μM) for 4 days. (B) Effect of ribociclib on cell proliferation. NCI-H295R, MUC-1, and ACC115m cells were treated for 4, 5, and 4 days, respectively, with 3 concentrations of ribociclib. Cell viability was evaluated by MTT assay, whereas cell proliferation was assessed after cell count with trypan blue exclusion. Results are expressed as percent of viable cells vs untreated cells (Ctrl) ± SEM. *$P < 0.0001$; **$P < 0.001$; #$P < 0.01$; §$P < 0.05$.

Table 2. mRNA coding for CDK4, CDK6, Rb, P130, and P107 expression in ACC cells

Cells	CDK4	CDK6	Rb	P130	P107
NCI-H295R	4.30 ± 0.21	6.98 ± 0.04	ND	11.07 ± 0.11	7.86 ± 0.20
MUC-1	6.45 ± 0.12	7.89 ± 0.02	7.89 ± 0.12	9.78 ± 0.03	8.66 ± 0.11
ACC115m	3.50 ± 0.09	6.48 ± 0.05	8.69 ± 0.24	13.11 ± 0.20	12.07 ± 0.10

Results are presented as ΔCt (Ct of β-actin – Ct gene of interest) ± SD.
Abbreviations: ACC, adrenocortical carcinoma; Ct, cycle threshold; ND, no data.

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 correction was used for multiple comparisons. Where appropriate, the unpaired *t* test was used. Unless otherwise specified, data are expressed as mean ± SEM of at least 3 experiments run in triplicate. *P* values < 0.05 were considered statistically significant. Cytotoxicity experiments were carried out at least 3 times, each point run in triplicate.

Results

Ribociclib Target Expression

The genes expression of the ribociclib molecular targets CDK4 and CDK6 were evaluated in the ACC cell lines and in the primary cell culture. CDK genes were abundantly expressed in each cell model; this finding is consistent with our previous published results (16), which are listed in Table 2.

Ribociclib-induced Cytotoxicity in ACC Cell Models

Exposure of NCI-H295R cells to increasing concentrations of ribociclib (2.5-100 μM) for 4 days led to a

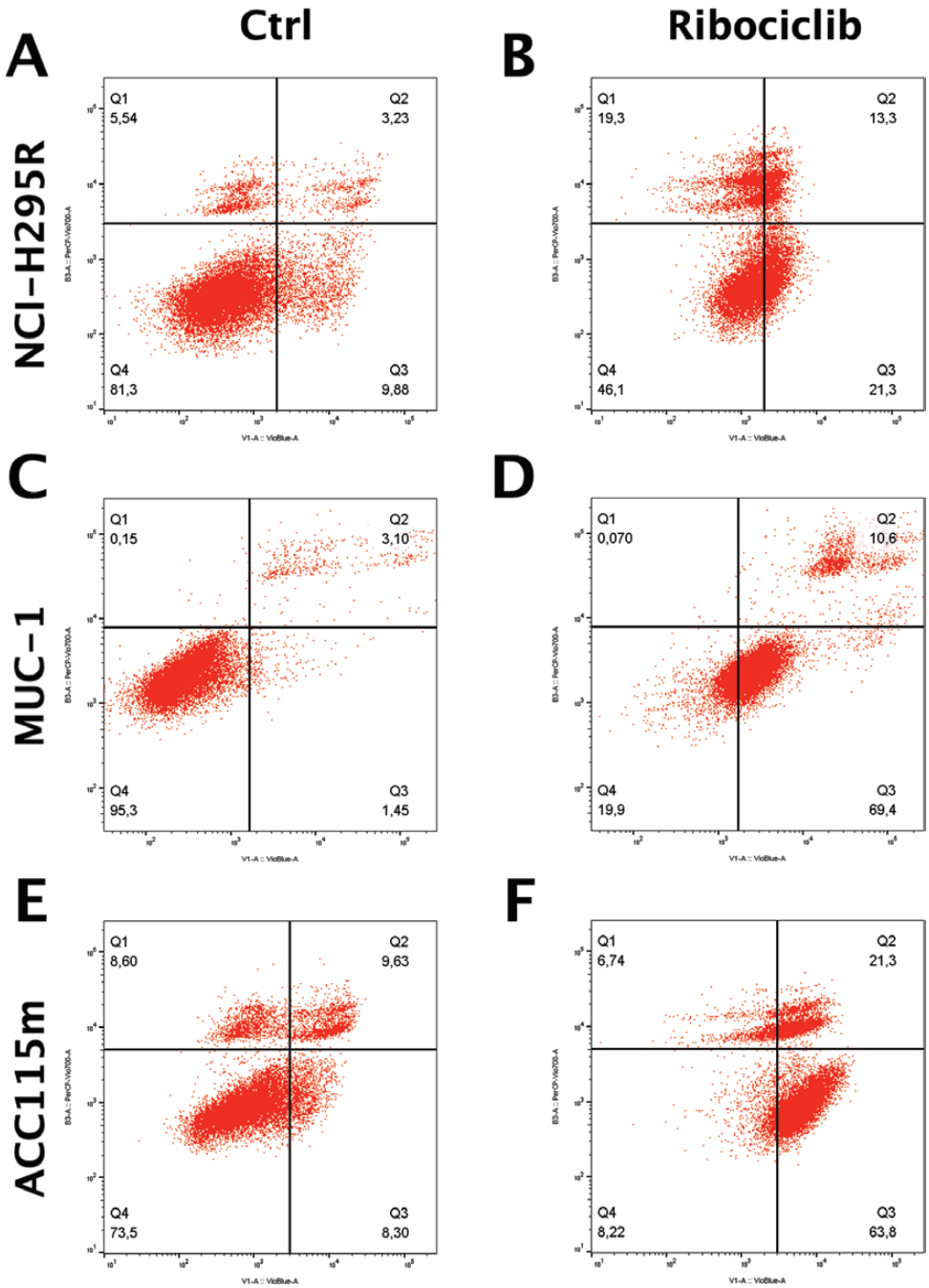

Figure 2. Representative images of cell apoptosis in ACC cells after 72 hours' treatment with ribociclib. Alive cells are shown in the lower left part of the panel (Q4); early apoptotic cells are shown in the lower right part of the panel (Q3); late apoptotic cells are shown in the upper right part of the panel (Q2); necrotic cells are shown in the upper left part of the panel (Q1).

concentration-dependent reduction of cell viability. Sigmoidal concentration-response function was applied to calculate the IC_{50} value of ribociclib, which was 21.60 μM (95% CI, 18.63-25.04). The highest concentration tested of 100 μM left about 2% of viable cells, indicating the high ribociclib efficacy in this cell line (Fig. 1A). Ribociclib also reduced the cell proliferation rate of NCI-H295R cells, as shown in Fig. 1B. The metastasis-derived cell line MUC-1 was found to be responsive to ribociclib (2.5-100 μM); indeed, after 5 days' exposure, a concentration-dependent decrease of cell viability was observed. The calculated IC_{50} value was 48.16 μM (95% CI, 39.64-50.13). In this cell line, ribociclib showed a decreased potency compared with NCI-H295R, with a similar efficacy (Fig. 1A). The effect on the cell proliferation rate was also evaluated and results reported in Fig. 1B demonstrate a ribociclib concentration-dependent reduction of cell number. We then investigated the effect of ribociclib in a patient-derived primary cell culture, namely the ACC115m cells. The adrenal origin of these cells was assessed both at mRNA and protein levels (see Figure S1 (30)). When ACC115m cells were exposed to increasing concentration of ribociclib (2.5-75 μM) for 4 days, a concentration-dependent decrease of cell viability was observed. The sigmoidal concentration-response function was applied to calculate the IC_{50} value, which was 46.1 μM (95% CI, 44.56-47.64). In these primary cells, ribociclib induced an effect on cell viability similar to that observed in MUC-1 (Fig. 1A), according to the origin of both ACC cells from patients underwent progression after EDP-M. The effect on cell proliferation was evaluated as well and demonstrated that ribociclib reduced the rate of ACC115m cell proliferation (Fig. 1B).

Ribociclib-induced Increase of Apoptotic Cells

To investigate the mechanism of cell death induced by ribociclib in our experimental models, we performed a double-staining assay as described in Methods in untreated and ribociclib-treated cells. Results are reported in Fig. 2. The exposure of both cell lines and primary cells to a concentration of ribociclib corresponding to the calculated IC_{50} values for 3 days induced an increase in apoptotic cells (NCI-H295R apoptotic cells: untreated cells, 12.0% ± 1.0%; ribociclib-treated cells, 31.0% ± 4.0%; $P < 0.05$. MUC-1 apoptotic cells: untreated cells, 4.5% ± 0.5%; ribociclib-treated cells, 87.5% ± 7.5%; $P < 0.01$. ACC115m apoptotic cells: untreated cells, 19.0% ± 1.0%; ribociclib-treated cells, 83.5% ± 1.5%; $P < 0.001$). The analysis was performed after 4 days of treatment, and results confirm the observed trend (not shown).

Effect of Ribociclib on Cell Cycle

Ribociclib is known to interfere with the cell-cycle progression (31). The gene expression of proteins belonging to the pRb protein family was first evaluated in our ACC experimental cell models. Results reported in Table 2 are consistent with our previous published results (16). To evaluate the effect of ribociclib treatment on the distribution of NCI-H295R and MUC-1 cells in the cell cycle, we performed preliminary time-course evaluation of cell-cycle distribution to define the best treatment time for subsequent experiments (data not shown). Then, cells were treated with a concentration corresponding to the IC_{50} value of ribociclib for 3 days; the results reported in Fig. 3 indicate an increase of the percentage of cells in G1 phase in ACC115m primary cells (cells in G1 phase: untreated cells, 60.8% ± 3.0%; ribociclib-treated cells, 75.5% ± 1.9%; $P < 0.01$), whereas an increase in G2 phase was observed in both NCI-H295R (cells in G2 phase: untreated cells, 26.0% ± 2.0%; ribociclib-treated cells, 35.1% ± 0.9%; $P < 0.05$) and MUC-1 (cells in G2 phase: untreated cells, 23.6% ± 1.4%; ribociclib-treated cells, 44.3% ± 3.0%; $P < 0.01$) cell lines.

Progesterone Enhanced the Ribociclib Effect in ACC Cell Models

We have already demonstrated the cytotoxic effect of increasing concentrations of progesterone in NCI-H295R cells (22) and in MUC-1 cells (32), with differences in potency and efficacy. Binary combination experiments were then performed, drawing the concentration-response curves according to the Chou-Talalay method: NCI-H295R and MUC-1 were treated with ribociclib and progesterone alone or in combination. Cells were exposed to a drug fixed ratio (ribociclib: progesterone = 1:1.5). Concentration-response curves of combination experiments are reported in Fig. 4A and in Fig. 4C. Data on cell viability were converted in FA and the combination index was calculated with the CompuSyn Software. Figure 4B and in Fig. 4D show the semilogarithmic-combination index plots. When values of Log(combination index) > 0, the effect is antagonistic; when Log(combination index) = 0 and < 0, the effect is additive and synergic, respectively. The results indicate that, on average, ribociclib and progesterone have an additive relationship in both ACC cell lines. Finally, we evaluated the ribociclib/progesterone combination cytotoxic effect also in the primary cell culture, namely the ACC115m cells, which showed a response to the cytotoxic effect of progesterone superimposable to that observed in MUC-1 cells (32). Cells were indeed treated with ribociclib and progesterone alone or in combination with a fixed ratio of concentrations (ribociclib:progesterone = 1:1.5). Concentration-response curves of combination experiments are reported in Fig. 4E.

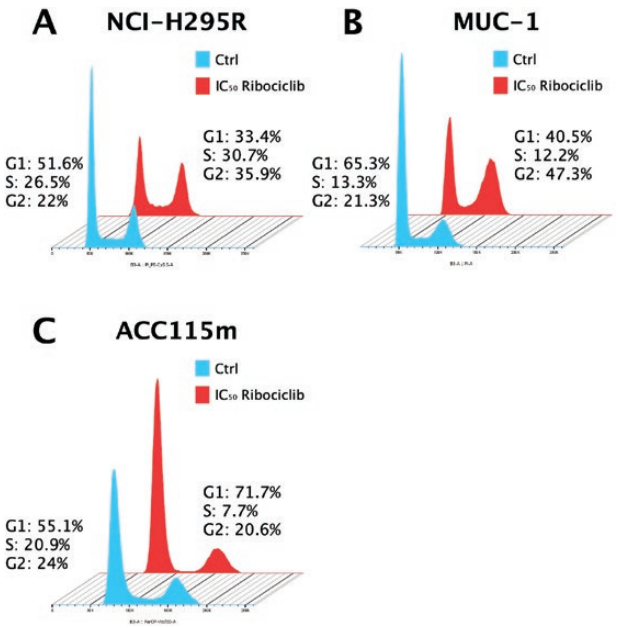

Figure 3. Cell-cycle analysis after ribociclib treatment. DNA histograms. (A) NCI-H295R untreated cells and 3-day ribociclib-treated cells. (B) MUC-1 untreated cells and 3-day ribociclib-treated cells. (C) ACC115m untreated cells and 3-day ribociclib-treated cells.

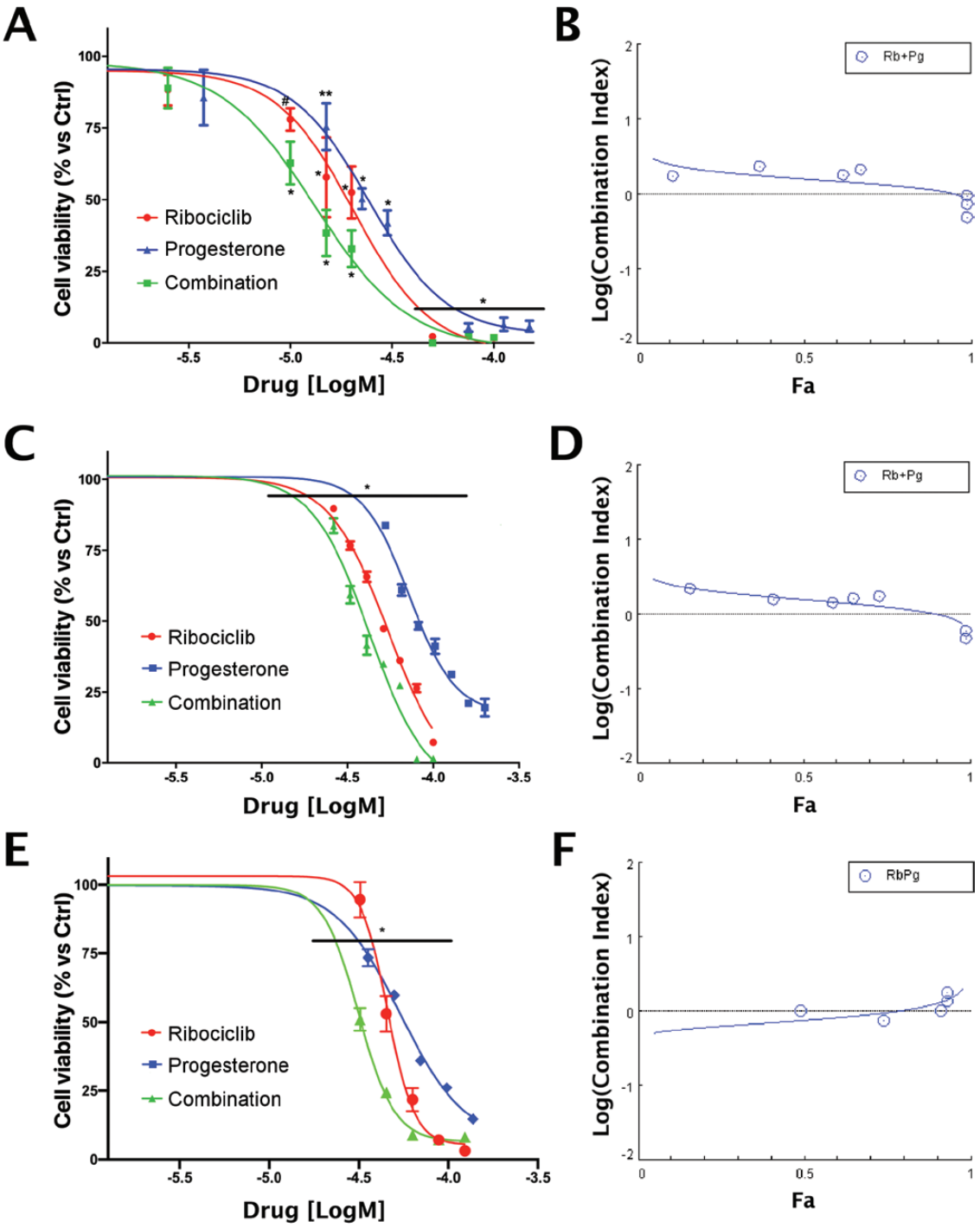

Figure 4. Effect of the ribociclib/progesterone combination on ACC cell models viability. Cells were exposed to increasing concentrations of ribociclib and progesterone alone or in combination at fixed concentration ribociclib:progesterone = 1:1.5 molar ratio. Cell viability was measured by MTT. Concentration-response curves in (A) NCI-H295R cells, (C) MUC-1 cells, and (E) ACC115m cells. Results are expressed as percent of viable cells vs untreated cells (Ctrl) ± SEM. (B, D, F) Combination index plot in NCI-H295R, MUC-1, and ACC115m cells, respectively. Dose and effect data obtained were converted to Fa values and analyzed with CompuSyn software. $*P < 0.0001$; $**P < 0.001$; $\#P < 0.01$.

As reported in Fig. 4F, the results of the combination index plot indicate in these cells a moderate additive effect also. To investigate whether low concentrations of ribociclib could still positively influence the response of these cells to progesterone, our ACC cell models were treated with increasing concentrations of progesterone alone or combined with a fixed dose of ribociclib. The ribociclib cytotoxic IC_{15} was chosen for each cell lines (NCI-H295R = 10 µM; MUC-1 = 30 µM; ACC115m = 40 µM). Results are reported in Fig. 5. The effect in NCI-H295R cells did not differ between the single drug or the combination (Fig. 5A), whereas, in MUC-1 cells and in ACC115m cells, the combination of increasing concentrations of progesterone with a low concentration of ribociclib induced an increase in the potency of progesterone (Fig. 5B and Fig. 5C). Indeed, the IC_{50} values of the combination were 45.77 µM (95% CI, 34.99-59.87 µM) and 29.60 µM (95%

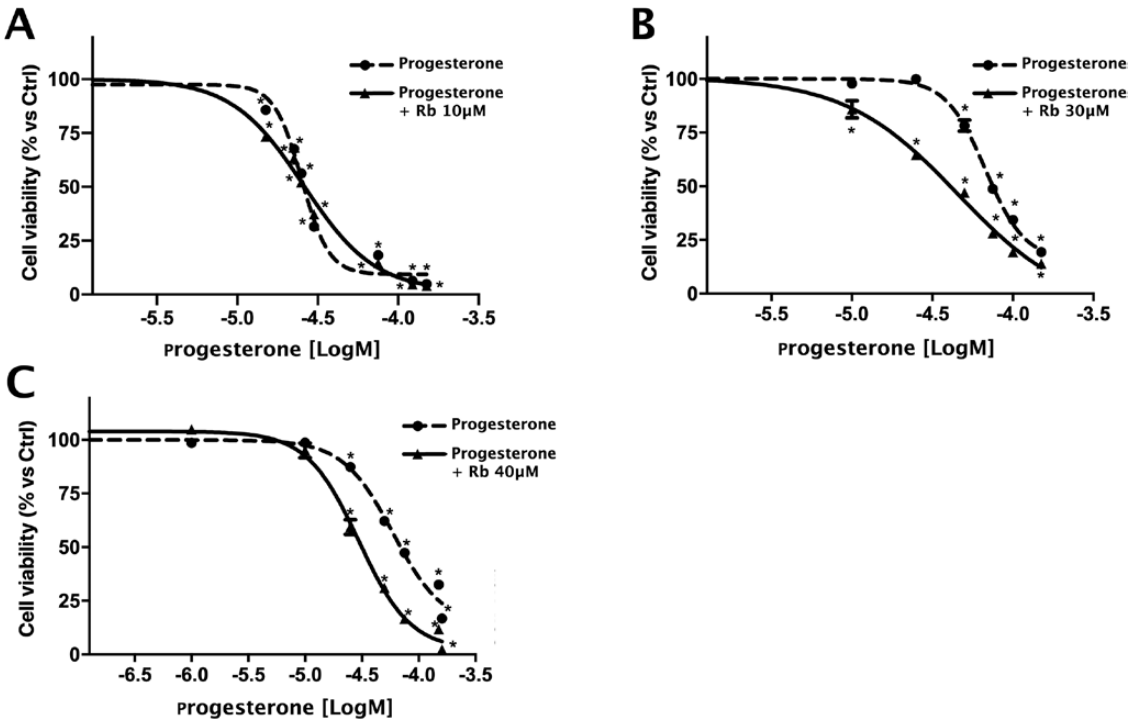

Figure 5. Progesterone alone or combined with a fixed dose of ribociclib ACC cell models. (A) Concentration-response curve in NCI-H295R cells. Cells were treated with increasing concentrations of progesterone alone or combined with 10 μM of ribociclib for 4 days. (B) Concentration-response curve in MUC-1 cells. Cells were treated with increasing concentrations of progesterone alone or combined with 30 μM of ribociclib for 5 days. (C) Concentration-response curve in ACC115m cells. Cells were treated with increasing concentration of progesterone alone or combined with 40 μM of ribociclib for 4 days. Cell viability was evaluated by MTT. Results are expressed as percent of viable cells vs untreated cells (Ctrl) ± SEM. *$P < 0.0001$.

CI, 26.67-32.73) in MUC-1 and ACC115m, respectively. They were significantly ($P < 0.01$ for both cell models) reduced compared with the IC_{50} values of progesterone alone, which were 67.58 μM (95% CI, 63.44-71.98) and 59.52 μM (95% CI, 51.06-69.39) for MUC-1 and ACC115m, respectively, in line with our previous results (31).

Effect of Ternary Combination of Ribociclib, Progesterone, and Mitotane on NCI-H295R Cell Viability

The effect of mitotane on NCI-H295R cells is well documented (22, 33). Here, the response of MUC-1 cells and of ACC115m cells to mitotane treatment (see Figure S2 (30)) was evaluated. MUC-1 cells were responding to mitotane in a high range of concentrations, and our results are in line with published literature (34). According to the origin of this primary culture, derived from a patient who underwent progression on mitotane, ACC115m cells were responsive to high concentrations of mitotane. Considering the different sensitivity of the ACC cell models to the drugs, all of them were used to evaluate the effect on the viability of the ternary treatment. The reduction of cell viability is reported in Fig. 6. In each ACC cell model, the effect of the combination was more evident for values corresponding to $0.5 \times IC_{50}$ and $1 \times IC_{50}$, where a greater and statistically significant reduction in the cell viability in the combined treatment compared with individual treatments was observed.

Discussion

In this study, we evaluated the effect of the CDK4/6 inhibitor ribociclib in preclinical models of ACC, with a particular focus on a combined setting. The pharmacokinetic properties of ribociclib indicated a long half-life and a rapid absorption not influenced by fed state (35). To date, 3 CDK4/6 inhibitors are available for clinical use: palbociclib, ribociclib, and abemaciclib. Although all of them target CDK4 and CDK6, differences have been reported in terms of pharmacodynamic, pharmacokinetic, and toxicological characteristics (20, 35). Ribociclib induced a cytotoxic effect and a reduction in cell proliferation in all our ACC cell models. NCI-H295R cells, which derived from a primitive tumor (23) seems to be more sensitive to ribociclib effect compared with the metastatic models, MUC-1 cell line, and ACC115m primary cell that are responsive both in terms of cytotoxicity and cell proliferation, however. In particular, results reported here demonstrate that ribociclib was cytotoxic in in vitro experimental cell models of ACC, with an IC_{50} within the micromolar concentrations. Plasma concentrations of ribociclib measured in clinical studies were generally lower than the concentrations used in the present work (36, 37), but based on LogP (38), volume of distribution (39), and results of ribociclib measurement in the tumor mass (37), it could be hypothesized that the in vitro concentrations used for cell treatments could be consistent with amount of drug expected in the adrenal gland. Unfortunately, no data are available in adrenal glands; however, according to the lipophilic characteristic of the adrenal tissue, an accumulation in this region could occur. We are aware that this point needs to be evaluated in a clinical study that includes pharmacokinetics. Ribociclib interferes with the cell-cycle progression, causing its arrest. According to this mechanism of action, ribociclib is known to induce apoptosis in different cellular models (40, 41). Our results

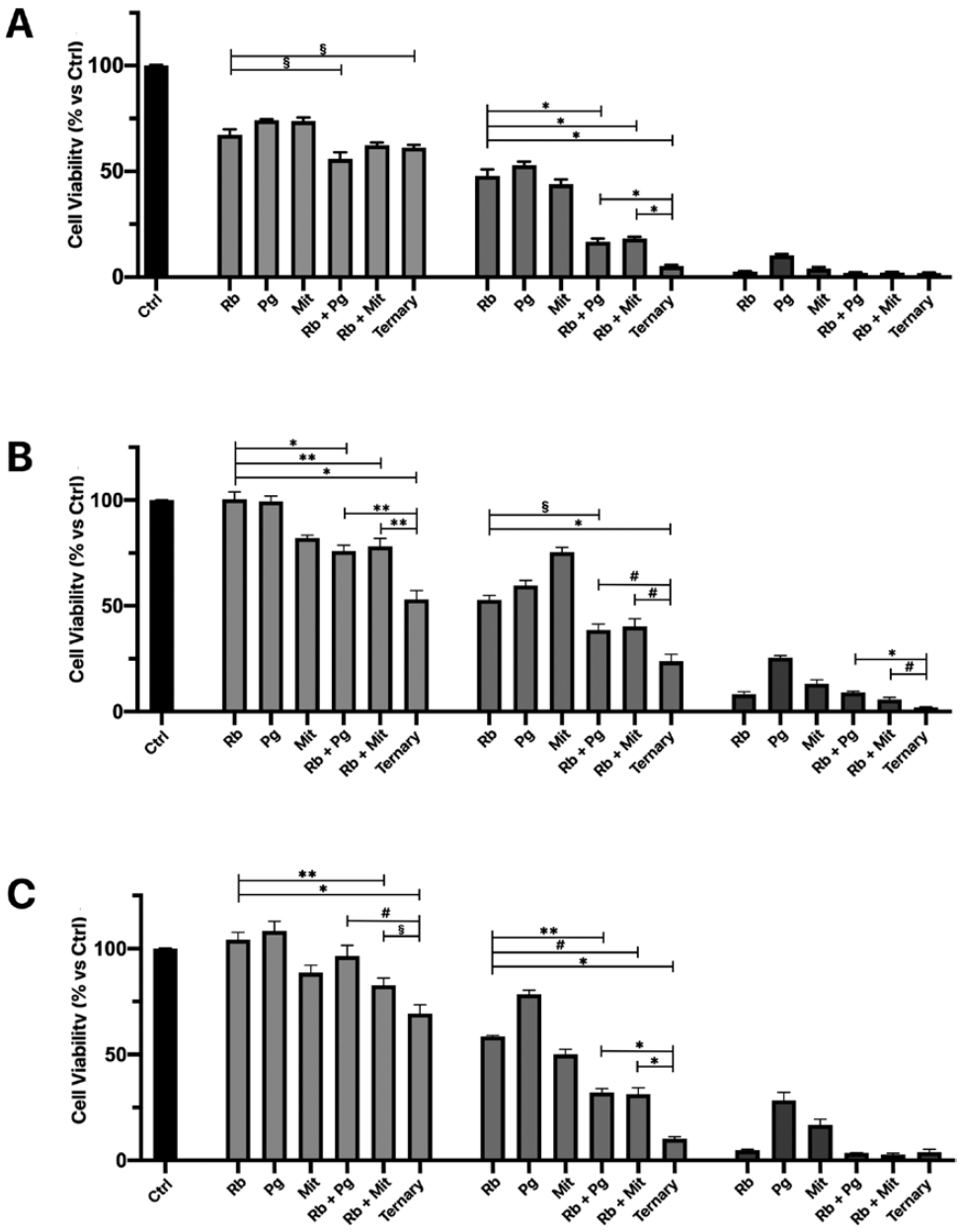

Figure 6. Effect of ribociclib, progesterone and mitotane on cell viability in ACC cell models. (A) NCI-H295R, (B) MUC-1, and (C) ACC115m cells were exposed to 3 concentrations of each drug, corresponding to 0.5×, 1×, and 2× IC_{50} values, alone or in ternary combination for 4, 5, and 4 days, respectively. Results are expressed as percent of viable cells vs untreated cells (Ctrl) ± SEM. $*P < 0.0001$; $**P < 0.001$; $\#P < 0.01$; $\S P < 0.05$.

indicate that ribociclib treatment increases the number of apoptotic cells relative to control cells. Furthermore, we also observed an increase in necrotic cells in NCI-H295R after treatment. Taken together, these results suggest that ribociclib causes cell death, mainly with an apoptotic mechanism in our cells, and reflect the relevant effect induced by ribociclib on ACC cells viability and proliferation.

Subsequently, to evaluate the effect of ribociclib treatment on the cell-cycle progression in ACC cells, we analyzed the cell-cycle distribution. We observed an accumulation of cells in the G2 phase both in NCI-H295R and MUC-1 cells, and an accumulation of cells in the G1 phase was reported in ACC115m primary cells. Although ribociclib is a CDK4/6 inhibitor and its role in the G1 phase arrest is well described (42), our observations on the 2 cell lines are in line with published literature describing a G2 arrest resulting from ribociclib treatment in germinal cell tumor cell lines, where it is shown that in these cell models, the predicted G1 arrest after ribociclib treatment was bypassed and the cell cycle stopped at the G2 or M phases (43). The different effects on cell-cycle progression in our ACC cells induced by ribociclib exposure could be explained by the possible differences in the molecular setting of each cell model, in particular at the level of protein related to cell-cycle progression. The investigation of the exact molecular mechanism by which this occurs is beyond the scope of this work and it is now matter of investigation in our laboratory. Results of different combined treatment schemes with ribociclib plus progesterone revealed a positive interaction between these 2 drugs, particularly in MUC-1 cells and

ACC115m cells. This aspect is even more interesting because MUC-1 cells derived from a heavily treated patient with a metastatic disease, in progression after EDP-M, as well as the ACC115m primary cell culture, gives strength to the possible clinical application of this combination. Interestingly, the advantage of using progesterone in a combination therapy could be of considerable interest for its possible clinical application because progesterone and its derivatives are commonly administered in oncological patients for their anticachectic effect (44). Furthermore, the association of CDK inhibitors and endocrine therapy is a pharmacological approach already in use in the treatment of breast cancer (45, 46). Both CDK inhibitors and progesterone derivatives have been shown to have manageable safety profiles (44, 47). Also, results about the ternary combination demonstrate a positive relation between ribociclib and the reference drug in ACC, mitotane. For this reason, evidence of a positive relationship between mitotane and a drug such as ribociclib could further strengthen the clinical potential of this combination.

Mitotane and ribociclib both interact with CYP3A4, causing opposite effects (48, 49); moreover, ribociclib is a substrate of this enzyme (36). The clinical outcome of this interaction is unknown, requiring a dedicated evaluation in a pharmacokinetic study, as already underlined. Although we are aware that our results on ribociclib and/or progesterone and/or mitotane in in vitro ACC cell models deserve to be deepened at a preclinical level that studies the possible intracellular pathways involved in this effect, we believe that these models gave the bases to explore these different combinations in a clinical setting, with dedicated clinical trials.

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Disclosures

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Data Availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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