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In vitro cytotoxicity of cabazitaxel in adrenocortical carcinoma cell lines and human adrenocortical carcinoma primary cell cultures

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ABSTRACT

Adrenocortical cancer (ACC) is a rare and aggressive malignancy with a poor prognosis. The overall 5-year survival rate of patients with ENS@T stage IV ACC is less than 15%. Systemic antineoplastic therapies have a limited efficacy and new drugs are urgently needed.

Human ACC primary cultures and cell lines were used to assess the cytotoxic effect of cabazitaxel, and the role of P-glycoprotein in mediating this effect.

Cabazitaxel reduced ACC cell viability, both in ACC cell lines and in ACC primary cell cultures. Molecular and pharmacological targeting of ABCB1/P-gp did not modify its cytotoxic effect in NCI-H295R cells, while it increased the paclitaxel-induced toxicity. Cabazitaxel modified the expression of proteins involved in cellular physiology, such as apoptosis and cell cycle regulation. The drug combination cabazitaxel/mitotane exerted an additive/moderate synergism in different ACC cell experimental models. These results provide a rationale for testing cabazitaxel in a clinical study.

1. Introduction

Adrenocortical cancer (ACC) is a rare disease that is often associated with a severe prognosis, due to its aggressive nature and limited responsiveness to standard systemic therapies (Konda and Kirschner, 2016). Early diagnosis, followed by radical surgical resection and adjuvant mitotane therapy are actually the only valuable options to give to the patients a chance of cure (Terzolo et al., 2014). Unfortunately, one out of four patients have metastatic disease at diagnosis (Puglisi et al., 2018) and most radically resected ACC patients are destined to recur with local or metastatic disease (Libé et al., 2015). In these patients, a large prospective multicenter randomized clinical trial (FIRM- ACT trial) has demonstrated the superiority of etoposide, doxorubicin, cisplatin and mitotane (EDP-M) regimen over the association of streptozotocin and mitotane (Sz-M) in terms of progression free survival and overall survival (Berruti et al., 2008; Fassnacht et al., 2012). The efficacy of EDP-M, however, is modest and almost all treated patients are destined to progress and die of disease. Second line therapies in ACC

Abbreviations: ACC, adrenocortical carcinoma; ABCB1, ATP binding cassette subfamily B member 1; P-gp, P-glycoprotein; MTT, 3-(4,5-Dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide dye; Fa, fraction affected; CI, combination index; AO, acridine orange; EtBr, ethidium bromide; SF1, Steroidogenic Factor 1

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patients with disease progression to EDP-M, such as the combination of gemcitabine plus capecitabine, are poorly efficacious (Henning et al., 2017; Sperone et al., 2010). Taxanes, docetaxel and paclitaxel, are an important class of antineoplastic agents used in the treatment of a wide variety of cancers. The most known mechanism of action of taxanes is as microtubule stabilizer since they shift the equilibrium between soluble tubulin and the microtubule polymer in favor of the latter, and thereby reduce the critical concentration of tubulin required to form a microtubule (reviewed in Rohena and Mooberry, 2014). Other mechanisms of action have been afterward proposed, owing the capability to: 1) block the microtubule disassembly in chromosome separation; 2) modify cell cytoskeleton, with an impact on cellular functions, as many cell components move along

microtubules; 3) directly act on transcriptional activity, inducing apoptosis (von Haefen et al., 2003; Komlodi-Pasztor et al., 2011; Fitzpatrick and de Wit, 2014). Increasing evidence propose that the clinical efficacy of taxanes is mainly due to their inhibition of duplication mechanisms in the cell cycle interphase involving microtubules, rather than their effect on mitosis (Fitzpatrick and de Wit, 2014). Due to these multi-modal mechanisms of action, taxanes can be potentially efficacious in the management of ACC patients. However, published clinical trials did not support their efficacy in this contest since a phase II trial testing the combination of cisplatin plus docetaxel as first line therapy in advanced ACC (Urup et al., 2013) showed an inferior activity of this regimen as compared to EDP-M (Berruti et al., 2005). Furthermore, a phase II trial exploring the association of paclitaxel and sorafenib as second line therapy in ACC patients progressing to EDP-M was interrupted prematurely due to inefficacy (Berruti et al., 2012a). One of the most important mechanism limiting the efficacy of many chemotherapeutic drugs including taxanes in ACC (Lockhart et al., 2003) is the great expression of the ATP binding cassette subfamily B member 1 (ABCB1) (Bates et al., 1991; Villa et al., 1999). The ABCB1 gene encodes the P-glycoprotein (P-gp) that mediates multidrug resistance in cell culture by increasing efflux of chemotherapeutic agents, thus decreasing their intracellular accumulation (Chen and Sikic, 2012).

Cabazitaxel is a new semisynthetic taxane, dimethyl derivate of docetaxel, bearing methoxy groups in place of hydroxyl groups in the side chain at positions C-7 and C-10, that is efficacious in ABCB1 expressing tumors (Kathawala et al., 2015). Resistance to first-generation taxanes is frequently observed in different tumor types, resulting in treatment failure. This finding stimulated large-scale preclinical screening process, in order to identify a taxane-like drug, efficacious also in docetaxel- and paclitaxel-resistant tumors. In both docetaxel-sensitive and docetaxel-resistant cell lines, the chemical structure of cabazitaxel resulted in the greatest increase in *in vitro* potency, without significantly increasing toxicity at the maximum tolerated dose, in contrast to other side chain modifications (Vrignaud et al., 2013). The structure of cabazitaxel confers two advantages over old-taxanes: the poor affinity for P-gp and a marked increase of lipophilicity, that makes this drug able to easily cross the cell membrane and superior in penetration of the blood-brain barrier in preclinical models (Vrignaud et al., 2013; Azarenko et al., 2014). In a large prospective multicenter clinical trial involving metastatic castration-resistant prostate cancer patients who progressed to docetaxel, cabazitaxel treatment was associated with an increased survival over the standard therapy (mitoxantrone plus prednisone) (de Bono et al., 2010). Based on the result of this clinical study, cabazitaxel became a standard therapy in the management of patients with metastatic castration-resistant prostate cancer, previously treated with a docetaxel. The activity of cabazitaxel against several malignancies is currently tested in clinical trials (www.clinicaltrials.gov). The high expression of P-gp in ACC cells and the lyophilic milieu of these tumors, provide a strong rationale for testing this drug in ACC. The present preclinical study was designed to evaluate the *in vitro* preclinical effects of cabazitaxel in ACC cell lines and in human ACC primary cell cultures obtained from patients with ACC who underwent surgery.

Table 1

Clinical and immunohistochemical characteristics of ACC patients.

| Primary culture identification | Tumor specimen | Histology | Disease stage | Hormone hypersecretion | SF-1 expression | P-gp expression |
|-------------------------------------|----------------------|---|-------------------------------------|------------------------|-----------------|-----------------|
| ACC03 Male 59 yr old | Local relapse of ACC | Mitotic index: 25/50 HPF; Ki67: 20% | Local relapse | No secretion | 2+ | 0 |
| ACC06-I Male 42 yr old | Hepatic metastasis | Mitotic index: 18/50 HPF; Ki67: 20% | Stage IV, multiple metastases | No secretion | 2+ | 0 |
| ACC08 Female 50 yr old | Lung metastasis | Oncocytic features, mitotic index: 10/50 HPF; Ki67: 20% | Stage IV, lung and bone metastases | No secretion | 1+ | 0 |
| ACC16 Male 55 yr old | Primary ACC | Mitotic index: 10/50 HPF; Ki67: 50% | Stage IV, Lymphonodal dissemination | Cortisol | 3+ | 2+ |
| ACC29 Female 51 yr old | Primary ACC | Mitotic index: 2/50 HPF; Ki67: < 5% | Stage IV, lung metastases | Cortisol | 2+ | 1+ |
| ACC32 Male 66 yr old | Primary ACC | Mitotic index: > 5/50 HPF; Ki67: 20% | Stage II | No secretion | 1+ | 2+ |

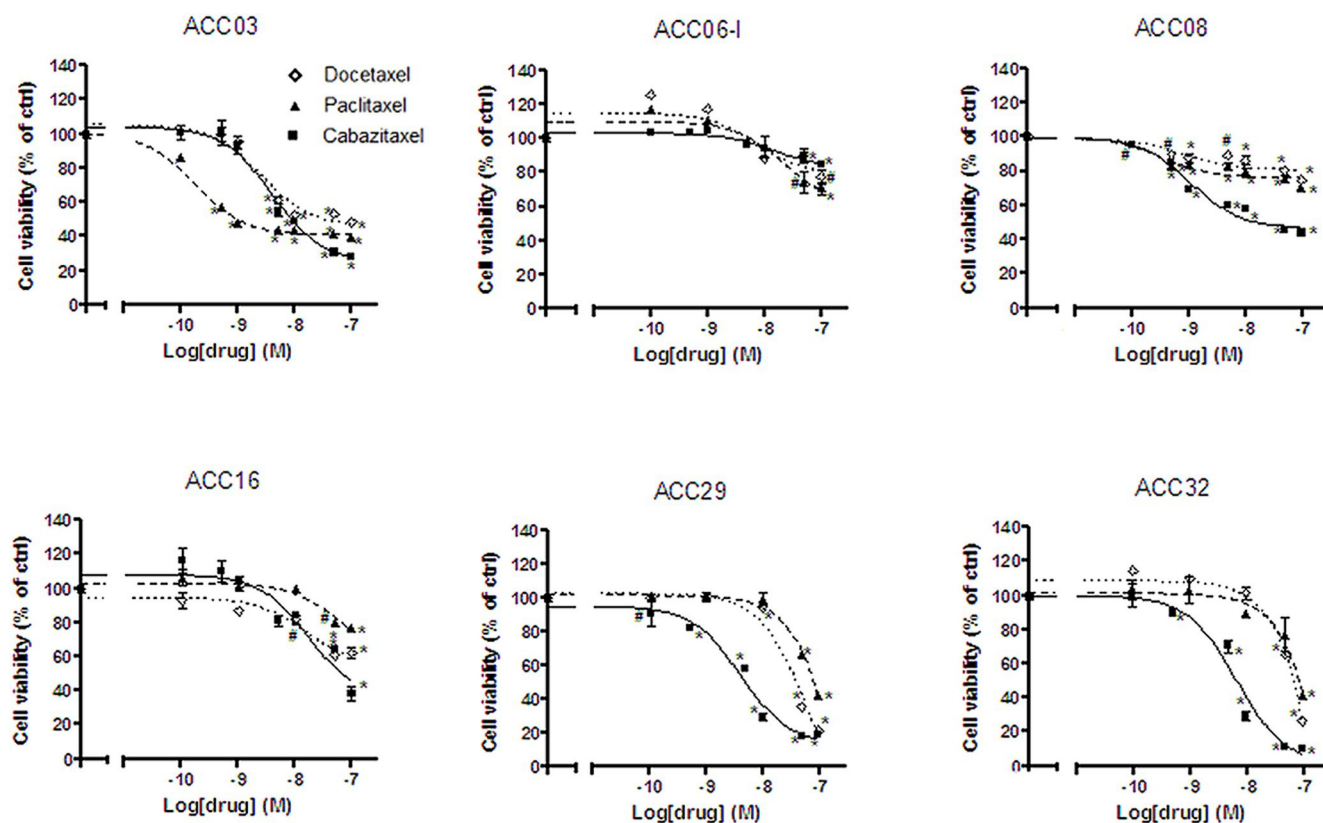


Fig. 1. Cytotoxic effects of taxanes in primary cell cultures derived from ACC patients. Cells were treated with increasing concentration of each taxane (0.1–100 nM) for 4 days. Cell viability was analyzed by MTT assay. Results are expressed as percent of viable cells \pm SEM; * $P < 0.001$ vs untreated cells; # $P < 0.05$ vs untreated cells.

2. Materials and methods

2.1. Cell culture

Human ACC primary cells were derived from six patients submitted to surgical removal of primary or metastatic ACC. Their clinical characteristics are summarized in Table 1 and an immunohistochemistry characterization is shown in Supplemental Fig. 1. The local Ethical Committee approved the project and written informed consent was obtained from all patients. Primary cultures were obtained as previously described (Fiorentini et al., 2016; Fragni et al., 2019), with modifications. Briefly, dissociated tumor cells were purified from cells of other origin using the tumor isolation kit from Miltenyi Biotec (Bologna, Italy). Accordingly to the manufacturer instructions, non-tumor cells were magnetically labeled with a cocktail of antibodies conjugated with MACS™ microbeads, then the cell suspension was loaded onto a MACS column in the MACS separator. The magnetically labeled non-tumor cells were retained within the column, while the tumor cells were collected, washed in PBS and plated in the NCI-H295R cell complete medium (see below). Primary cell cultures were then characterized as of adrenal origin, measuring the Steroidogenic Factor 1 gene expression (Sbiera et al., 2010; Hantel et al., 2016) by q-RT-PCR. The human NCI-H295R, HAC-15 (a subclone of NCI-H295R cells) and SW13 cell lines were obtained from the American Type Culture Collection (ATCC) and cultured as suggested. MUC-1 cell line was kindly given by dr. Hantel and cultured as indicated in Hantel et al., 2016. NCI-H295R cells were established from a secreting human ACC and represented the most widely used experimental cell model to study ACC *in vitro* (Rainey et al., 2004). HAC-15 cells derived from NCI-H295R cell line and retain the ACTH-sensitivity (Parmar et al., 2008). MUC-1 cells derive from a neck metastasis of EDP-M treated patient (Hantel et al., 2016). The SW-13 cell line has been established from a small cell carcinoma in the adrenal cortex. These cells do not produce steroids and their exact histopathologic characteristics are still under investigation (Wang and Rainey, 2012).

2.2. Immunohistochemistry

Human tissue samples were obtained from formalin-fixed and paraffin embedded blocks. Sections (1.5 μ m) were used for routine haematoxylin and

eosin (H&E) staining and immunohistochemistry as previously described (Cominelli et al., 2015). The following primary antibodies and dilutions were used: SF1 (Abcam AB217317, 1:500), P-gp (F4) (Thermo Fisher MAB-13854, 1:50). Heat induced antigen retrieval treatment was performed by incubation for 15' either in microwave or Thermo-bath in EDTA buffer pH 8.0. Depending on the primary antibodies used sections were incubated with Rat-on-Mouse HRP-Polymer (Biocare Medical) or MACH 1™ Universal HRP Polymer Kit (Biocare Medical) and reactions developed in Biocare's Betazoid DAB and nuclei counterstained with haematoxylin. Digital images were acquired by an Olympus XC50 camera mounted on a BX51 microscope (Olympus, Tokyo, Japan) with CellF Imaging software (Soft Imaging System GmbH, Münster, Germany).

2.3. Cell treatments

Cells were treated with increasing concentrations of cabazitaxel, docetaxel and paclitaxel. Preliminary experiments of concentration-response curves and time-course were conducted in the ACC cell lines, in order to establish both the optimal drug concentration range and the treatment length for each cell line, as indicated in the legend of Fig. 2. ACC primary cell cultures were treated for 4 days with a range of concentrations between 0.1 and 100 nM of each taxane. The P-gp inhibitor tariquidar was solubilized in DMSO and tested in the range of concentrations from 10 to 100 nM (Mistry et al., 2001). When required by the experimental design, ACC cells were pre-treated with tariquidar (10 nM) for 1 h and then cabazitaxel or paclitaxel were added to the medium for 4 days. Drugs were purchased from Selleckchem Chemicals (DBA Italia, Segrate, MI, Italy).

2.4. Measurement of cell viability

Cell viability was assessed by 3-(4,5-Dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay. Briefly, untreated and taxane-treated cells were incubated with MTT dye (at final concentration of 0.5 mg/mL) and solubilized by DMSO, as previously described (Arrighi et al., 2011). Absorbance was determined at 540/620 nm by a spectrophotometer (GDV, Rome, Italy).

Cell viability was measured as well using the luminescence assay ATPlite™ (PerkinElmer), that measures the ATP production of viable cells. Cells were plated at the density of 6×10^3 cells/well in 96 wells plate; untreated and drug-treated cell viability was measured accordingly to the manufacturer instructions.

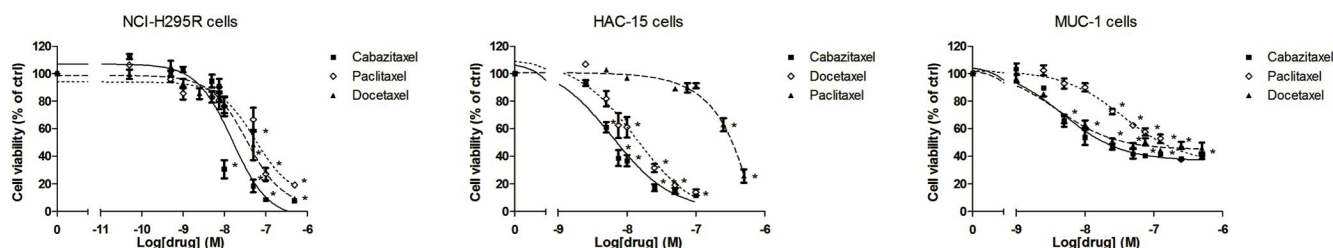


Fig. 2. Concentration-response curves of taxane-induced inhibition of cell viability in ACC cell lines. (a): NCI-H295R cells were treated for 4 days with 0.05–500 nM concentrations of each taxane. * $P < 0.05$ vs untreated cells for each concentration from 5 nM onwards for cabazitaxel and from 10 nM onwards for docetaxel and paclitaxel. (b): HAC-15 cells (a subclone of NCI-H295R cells) were treated for 4 days with 2.5–100 nM cabazitaxel and docetaxel and 5–500 nM paclitaxel. * $P < 0.05$ vs untreated cells for 5 nM cabazitaxel concentration onwards, from 7.5 nM docetaxel concentration onwards, at 250 nM and 500 nM paclitaxel P was significant vs untreated cells. (c): MUC-1 cells were treated for 4 days with 0.05–500 nM concentrations of each taxane. * $P < 0.05$ vs untreated cells for each concentration from 7.5 nM onwards for cabazitaxel and docetaxel and from 10 nM onwards for paclitaxel. Cell viability was measured by MTT assay and/or by ATPlite. Data are the mean \pm SEM of three different experiments, each point run in triplicate.

2.5. ABCB1 gene silencing by interference RNA

A small interfering RNA (siRNA) duplex targeting regions of ABCB1 gene and a non-targeting negative control siRNA (si-control) were used (Silencer® Select Pre-Designed siRNAs, LifeTechnologies, Milan, Italy). The si-ABCB1 sense and anti-sense sequences were: 5'-CGAUACAUGG UUUCCGAUTT-3' and 5'-AUCGGAAAACCAUGUAUCGGA-3' (si-ABCB1 10419). NCI-H295R cells were transfected with si-ABCB1 or si-control using the Lipofectamine RNAiMAX system (LifeTechnologies, Milan, Italy) according to the manufacturer's instructions.

2.6. Quantitative RT-PCR (qRT-PCR)

Gene expression was evaluated by qRT-PCR (ViiA7, Applied Biosystems, Milan, Italy) using SYBRGreen as fluorochrome, as described elsewhere (Sigala et al., 2008). Sequences of oligonucleotide primers were reported in Supplemental Table 1. Reactions were performed under the following conditions: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 62 °C for 1 min. Differences of the threshold cycle (Ct) values between the β actin housekeeping gene and the gene of interest (Δ Ct) were then calculated, as an indicator of difference in the amount of mRNA

expressed (Livak and Schmittgen, 2001).

2.7. Western blot

Whole cells lysate was prepared in ice-cold buffer with protease and phosphatase inhibitor cocktails (Roche, Milan, Italy), as previously described (Fiorentini et al., 2014). Briefly, proteins were separated by electrophoresis on a 4–12% NuPAGE bis-tris gel system (Life Technologies, Milan, Italy) and electroblotted to a PDVF membrane. Membranes were reacted using P-gp (Life Technologies, Milan, Italy), AKT, p-AKT (Cell Signaling Technologies, Milan, Italy), α -tubulin (Sigma Italia, Milan, Italy) and GAPDH (Merk Millipore, Burlington, MA, USA) primary antibodies, according to the manufacturer's instructions. Secondary HRP-labeled anti-mouse and anti-rabbit antibodies (Santa Cruz Biotechnologies, Heidelberg, Germany) were used and the specific signal was visualized using an ECL PLUS Western blotting detection system (Euroclone, Milan, Italy). Densitometric analysis of the immunoblots was performed using GelPro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

2.8. Drug combination experiments

Combination experiments were performed to evaluate the interaction of cabazitaxel with mitotane on cell viability, according to the Chou and Talalay method (Chou and Talalay, 1984). Cells were treated for 4 days with cabazitaxel (NCI-H295R cells: 1.8–120 nM; ACC29 and ACC32 cells: 2–128 nM or MUC-1 cells: 2.5–160 nM) and mitotane alone (NCI-H295R cells: 0.19–12 μ M; ACC29 and ACC32 cells: 0.75–48 μ M; MUC-1 cells: 1.25–80 μ M) or with cabazitaxel in combination with mitotane at a fixed ratio (cabazitaxel: mitotane = 1:100; 1:375; 1:500), as recommended for the most efficient data analysis (Chou, 2006). Cells were analyzed for cell viability using MTT assay and/or ATPlite™. 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). A CI value < 1, = 1 and > 1 indicates synergism, additive effect and antagonism respectively.

2.9. Proteome Profiler Human Apoptosis array

The protein array kit (R&D Systems, Space Import-Export, Milan, Italy) was used to detect simultaneously the expression level of 35 apoptosis-related proteins, as previously described (Fragni et al., 2016). Briefly, membranes containing immobilized antibodies, were blocked with BSA for 1 h at room temperature and then incubated with untreated and taxane-treated NCI-H295R cell lysates (400 μ g/membrane) overnight at 4 °C. After extensive washes, streptavidin-horseradish peroxidase and Chemi reagent mix were used to reveal apoptosis-related proteins by chemiluminescence. Pixel density was analyzed using ImageJ software (National Institutes of Health: Bethesda, MD, USA).

2.10. Double staining AO/EtBr

NCI-H295R cells were treated with 11 nM cabazitaxel for different times. 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 described in Fragni et al. (2019). 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 Image J software.

2.11. Cell cycle analyses

Flow cytometric cell cycle analysis was performed as described in Fragni et al. (2016). Briefly, untreated and cabazitaxel-treated NCI-H295R cells were fixed, treated with RNase A (12.5 μ g/ml), stained with propidium iodide (40 μ g/ml) (Sigma Italia, Milan, Italy) and analyzed by Flow Cytometry using a MACS Quant Analyzer (Miltenyi Biotec GmbH) for cell cycle status. Data were analyzed using FlowJo (TreeStar).

2.12. Statistical analysis

The analysis of the data was carried out by the GraphPad Prism version 5.02 software (GraphPad Software, La Jolla, CA), using the one-way ANOVA, with a post hoc test (Bonferroni's test) for multiple comparisons, considering $P < 0.05$ as threshold for significant difference. IC₅₀ values for each drug were calculated by non-linear regression of the concentration–response curves. Data are expressed as mean \pm SEM of three independent experiments, unless otherwise specified. Cytotoxicity experiments were carried out at least three times, each point run in triplicate. Experiments with primary cell cultures were sometimes carried out twice with each point run in triplicate, due to the limited number of cells available, except for ACC29 and ACC32 cells.

Table 2
Efficacy of taxanes in human ACC primary cell cultures.

| Primary cell cultures | maximum % of cell viability | | |
|-----------------------|-----------------------------|------------|------------|
| | cabazitaxel | paclitaxel | docetaxel |
| ACC03 | 28.4 ± 0.8 | 39.1 ± 1.5 | 48.7 ± 0.9 |
| ACC06-I | 84.4 ± 1.7 | 70.5 ± 3.8 | 77.2 ± 4.6 |
| ACC08 | 43.6 ± 0.9 | 70.4 ± 2.7 | 74.7 ± 0.5 |
| ACC16 | 38.2 ± 4.4 | 76.7 ± 0.6 | 62.1 ± 2.8 |
| ACC29 | 19.1 ± 0.1 | 42.0 ± 0.9 | 21.6 ± 0.4 |
| ACC32 | 10.3 ± 0.3 | 40.8 ± 0.9 | 26.2 ± 0.8 |

Results are expressed as percent of cell viability of each ACC primary culture exposed to 100 nM of each taxane ± SEM.

3. Results

3.1. Cabazitaxel induced cytotoxicity in ACC primary cell cultures

Primary cells were exposed to increasing concentrations of currently available taxanes (paclitaxel, docetaxel and cabazitaxel) for 4 days and analyzed for cell viability. Each taxane exerted a concentration-dependent inhibition of human ACC primary cell viability (Fig. 1), however, due to the different patient tumor stage and ACC cell characteristics, paclitaxel, docetaxel and cabazitaxel displayed a different cytotoxic efficacy in the primary cell cultures (Table 2). Cabazitaxel induced a greater cytotoxic effect than both paclitaxel and docetaxel in all primary cell cultures with the exception of ACC06-I cells, where an effect, although moderate, was observed only at high concentrations. Noteworthy, almost no ACC29 and ACC32 cells viable cells were detected at cabazitaxel concentration of 100 nM. No differences in the sensitivity to cabazitaxel cytotoxicity were found in hormone secreting ACC primary cells (ACC16 and ACC32) versus not secreting cells (ACC03, ACC06-I, ACC08, ACC29).

3.2 Taxanes reduced cell viability of ACC cell lines

The available ACC cell lines NCI-H295R, HAC-15 and MUC-1 cells were exposed to increasing concentrations of each taxane and analyzed by MTT and/or ATP lite assay. Results were reported in Fig. 2 and the calculated IC₅₀ values were shown in Table 3. In each cell line, taxanes induced cytotoxicity, with the calculated IC₅₀ value for the single drug revealing an order of potency that was for NCI-H295R and HAC-15 cell lines: cabazitaxel > docetaxel > paclitaxel, with differences among the cell lines. In particular, paclitaxel-induced cytotoxicity in the HAC-15 cell line was observed only at high concentrations. Experiments conducted with the MUC-1 cell line revealed an order of potency that was: docetaxel ≥ cabazitaxel > paclitaxel, although the efficacy did not reach the 100% of cytotoxicity: indeed, the maximum cytotoxic effect of about 50–60% of cytotoxicity was reached at 100 nM for each taxane and did not increase with higher drug concentrations, thus revealing that taxanes, including cabazitaxel, presented high potency but a limited efficacy in MUC-1 cells. Due to its uncertain histopathological origin, SW13 cell line was used as internal negative control (Wang and Rainey, 2012): each taxane displayed very similar concentration-response curves in SW13 (Supplemental Fig. 2A).

3.3 The cytotoxic effect of cabazitaxel is not influenced by P-gp expression

To assess the role of P-gp in modulating the taxane response, we took advantage of the RNA interference technique to silence the ABCB1 gene in NCI-H295R cells. Results showed that transfection with si-ABCB1 (100 nM) down-regulated ABCB1 mRNA after 2 days of exposure (76 ± 1.9% vs si-control treated cells). ABCB1 mRNA levels were unaffected by treatment with si-control, indicating the specificity of silencing sequences. Exposure of NCI-H295R cells to si-ABCB1 for 6 days resulted in ABCB1 down regulation at the P-gp protein level of about 95 ± 1.7% vs si-control (Fig. 3a). The effect of ABCB1 knock down on cell viability was then investigated in 100 nM si-ABCB1-treated NCI-H295R cells for 2 days and then exposed to cabazitaxel or paclitaxel (chosen as reference drug for the first generation taxanes), at their IC₅₀ value for 4 days. Silencing NCI-H295R cells with si-ABCB1 significantly increased the cytotoxicity induced by paclitaxel: + 37.3 ± 1.3% vs paclitaxel-treated cells without si-ABCB1. Conversely, the cytotoxicity of cabazitaxel did not appear to be affected by ABCB1 knock down (Fig. 3b).

The non-dependence of cabazitaxel on the P-gp protein, observed with the molecular approach of the RNA interference in NCI-H295R cells was confirmed also in other ACC cells, with a pharmacological approach with the P-gp inhibitor tariquidar.

ACC cell lines NCI-H295R and MUC-1 as well as two primary cell cultures, ACC29 and ACC32 cells were exposed to 10 nM tariquidar and then cabazitaxel or paclitaxel (at their respective IC₅₀ for each drug in each ACC cell line or ACC primary cell culture) were added, according to the protocol. The ABCB1 expression was investigated by q-RT-PCR and by Western blot in ACC cells and results are reported in Supplemental Fig. 3. The two primary cell cultures expressed high level of both the ABCB1 gene and the P-gp, while the ΔCt obtained for MUC-1 revealed a low ABCB1 gene expression, which translation into protein was not detectable by Western blot. Preliminary experiments have been conducted in order to

evaluate the effect of tariquidar on cells: exposure of up to 100 nM tariquidar for 4 days did not influence ACC cell viability (Supplemental Fig. 4). The cytotoxic effect of cabazitaxel was independent of P-gp protein expression in NCI-H295R, ACC29 and ACC32 cells; conversely, inhibition of P-gp activity significantly increased the cytotoxicity induced by paclitaxel (Fig. 4A, C, D). The MUC-1 cells did not modify their sensitivity to cabazitaxel, thus suggesting that its effect was independent of P-gp activity, while we observed an increase of cytotoxic effect elicited by paclitaxel in tariquidar-treated cells that did not reach a statistically significant value, in line with the lack of P-gp detection by Western blot (Fig. 4B). Concerning SW13, both our q-RT-PCR and Western blot results (Supplemental Fig. 3) confirmed that these cells did not express ABCB1 gene (Gagliano et al., 2014) and no effect of tariquidar was observed in SW13 cell line exposed to paclitaxel (Supplemental Fig. 2B).

Table 3
Taxanes IC₅₀ values in ACC cell lines.

| Cell line | CABAZITAXEL | PACLITAXEL | DOCETAXEL |
|-----------|-------------------------------|---------------------------------|--------------------------------|
| NCI-H295R | 10.9 nM (95% CI: 8.1 to 14.7) | 58.7 nM (95% CI: 27.2 to 127.8) | 34.5 nM (95% CI: 19.9 to 59.7) |
| HAC-15 | 6.0 nM (95% CI: 4.5 to 8.1) | ambiguous | 13.8 nM (95% CI: 8.7 to 21.0) |
| MUC-1 | 4.4 nM (95% CI: 3.5 to 5.5) | 34.7 nM (95% CI: 21.9 to 55.0) | 3.9 nM (95% CI: 2.9 to 5.3) |

The IC₅₀ is the mean of three independent experiments run in triplicate. The 95% Confidence Interval (95% CI) is reported.

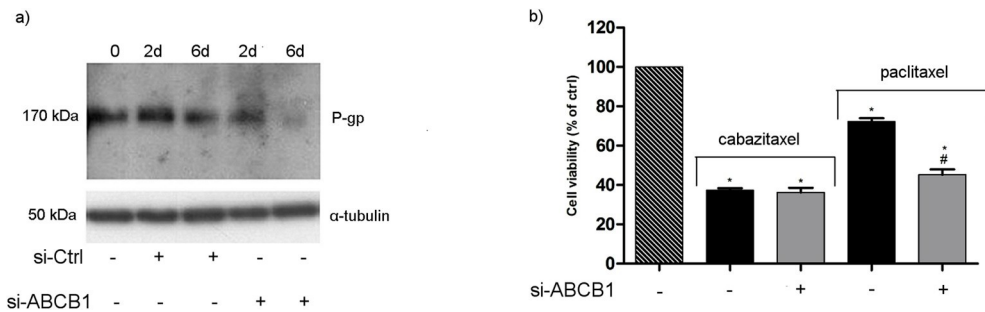


Fig. 3. (a) ABCB1 silencing in NCI-H295R cells was detected by Western blot assays. Human α-tubulin was used as internal control. A representative blot is shown; (b) no-silencing cells and si-ABCB1 treated NCI-H295R cells were exposed to taxanes (IC₅₀ value) for 4 days. Cell viability was measured by MTT assay. Results are expressed as percent of viable cells ± SEM. Data are the mean of three different experiments, each point run in triplicate.

**P* < 0.001 vs untreated cells (ctrl); #*P* < 0.001 vs taxanes-treated cells without si-ABCB1.

3.4 Cabazitaxel enhanced cytotoxicity induced by mitotane in ACC cell models

To evaluate whether cabazitaxel treatment of ACC cells could enhance the cytotoxicity of mitotane, the Chou-Talalay approach to study *in vitro* drug combination was applied and the CI value was calculated. We assessed the combined treatment effect in NCI-H295R cells, MUC-1 cells and in two primary cultures, ACC29 and ACC32 cells. Cells were exposed to cabazitaxel and mitotane alone or in combination, with a fixed molar ratio (cabazitaxel: mitotane = 1:100 in NCI-H295R cells; 1:500 in MUC-1 cells and 1:375 in ACC29 and ACC32 cells) for 4 days. Dose and effect data obtained were then converted to Fa values and analyzed with CompuSyn software. The Fa – CI values for experimental points are reported in Supplemental Table 2. The results indicated that cabazitaxel and mitotane combination exerted synergism with CI range 0.43–0.96 for Fa = 0.55–0.86 and an additive effect for Fa = 0.34 in NCI-H295R cells; synergism with CI range 0.51–0.88 for Fa = 0.18–0.96 and an additive effect for Fa = 0.76 and Fa = 0.88 in ACC29; synergism with CI range 0.64–0.89 for Fa = 0.03–0.99, an additive effect for Fa = 0.91 and a slight antagonism effect for Fa = 0.03 and Fa = 0.75 in ACC32, as shown by the Fa – CI plots (Fig. 5). MUC-1 cells were resistant to mitotane (Fig. 6), thus the combination analysis could not be conducted. The concentration curves of mitotane alone, cabazitaxel alone and the combination obtained in ACC cell lines and in ACC primary cultures are shown in Fig. 6. The isobolograms are shown in Supplemental Fig. 5.

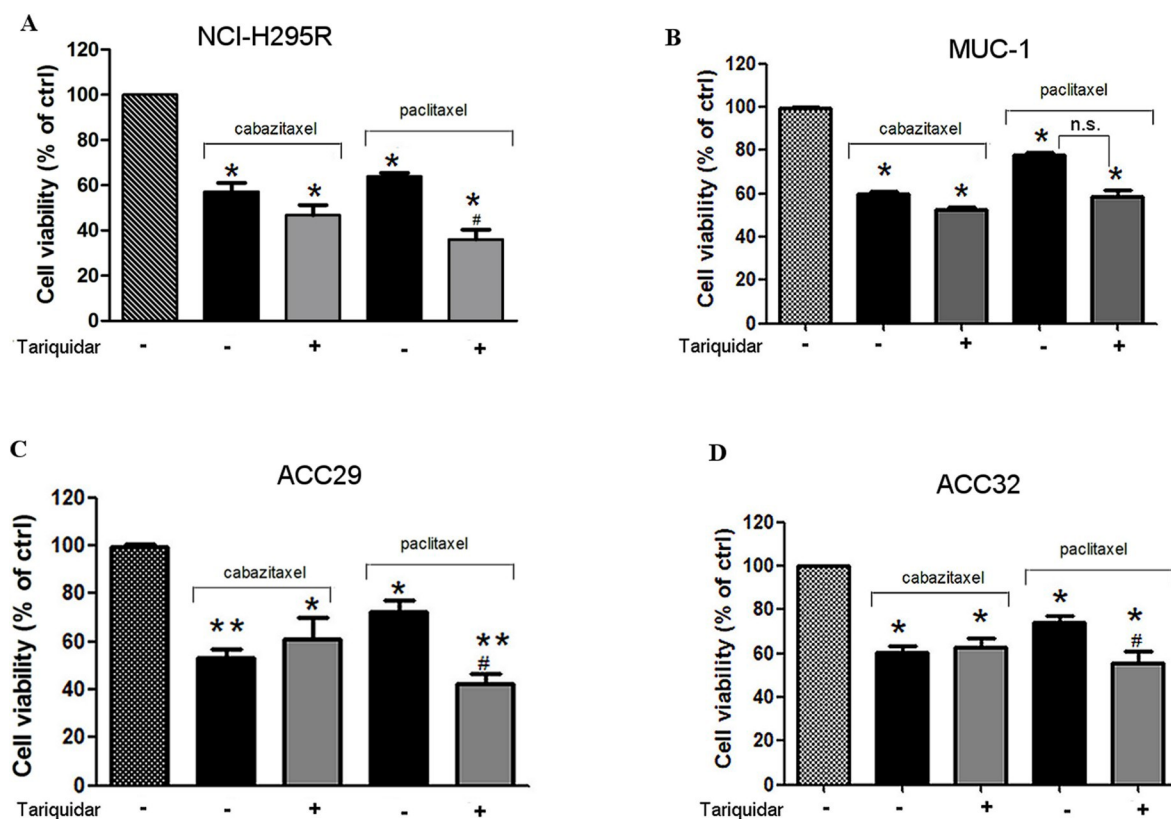


Fig. 4. ACC cells were pre-treated with 10 nM of tariquidar for 1 h and then IC₅₀ value of cabazitaxel or paclitaxel was added to the medium for 4 days. Cell viability was analyzed by MTT assay. Results are expressed as percent of viable cells \pm SEM; * P < 0.001 vs untreated cells (ctrl); # P < 0.001 vs paclitaxel-treated cells without tariquidar.

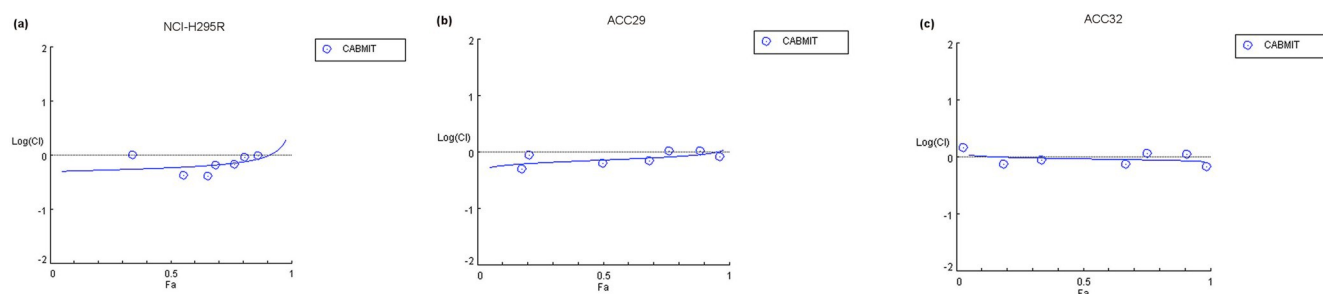


Fig. 5. Combination Index plot. Effect of the combination of cabazitaxel and mitotane on NCI-H295R cell viability (a), ACC29 (b) and ACC32 (c). Cells were treated with increasing concentrations of cabazitaxel and mitotane alone or in combination at fixed concentration molar ratio (cabazitaxel: mitotane; 1: 100 in NCI-H295R cells; 1:375 in ACC29 and ACC32 cells), for 4 days, as described in Materials and Methods. Cell viability was measured by MTT and/or ATPlite™ assay. Dose and effect data obtained were then converted to Fa values and analyzed with CompuSyn software.

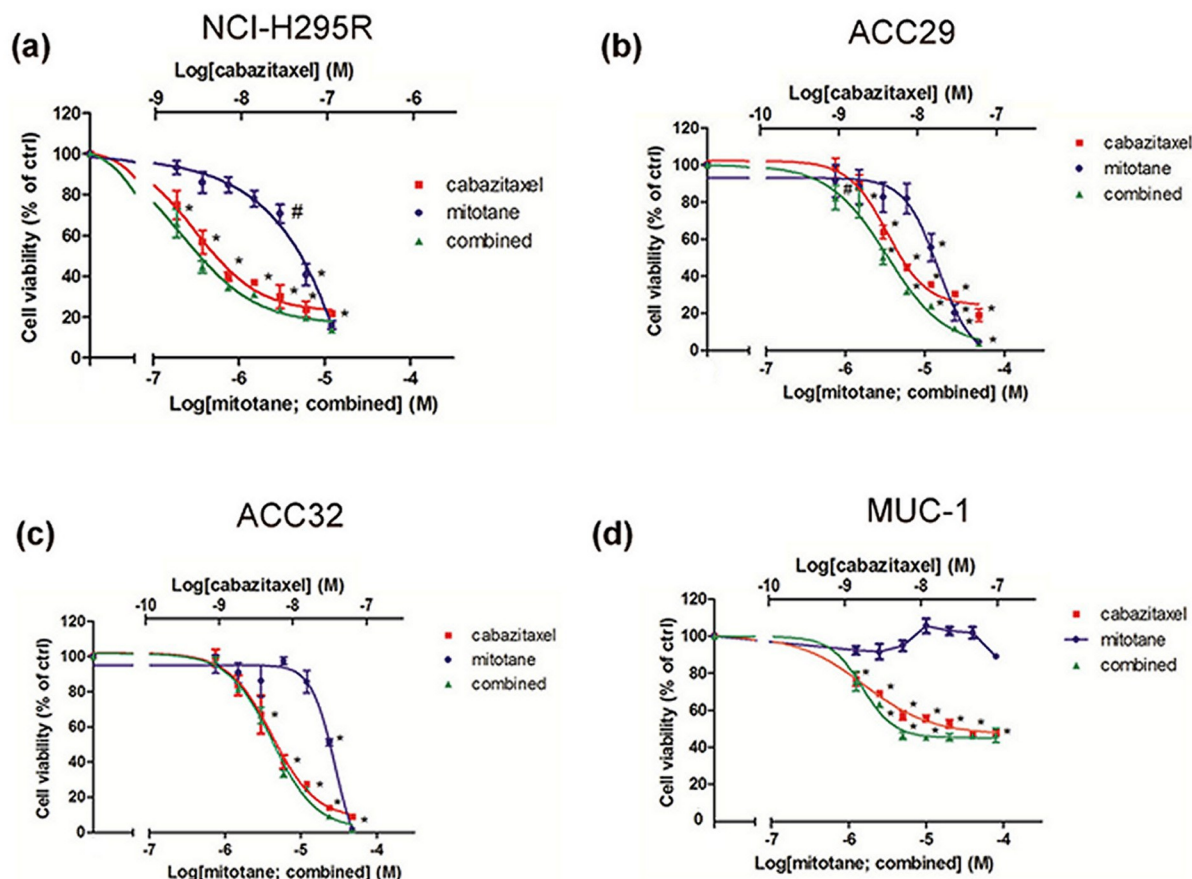


Fig. 6. Concentration-response curves of cabazitaxel, mitotane and drug combination in ACC cells. ACC cells were exposed to increasing concentrations of cabazitaxel and mitotane alone or in combination at fixed concentration cabazitaxel: mitotane: NCI-H295R cells, 1:100 M ratio, ACC29 and ACC32 cells: 1:375 M ratio; MUC-1 cells: 1:500 M ratio, for 4 days. Data are expressed as percent of viable cells vs. control (ctrl) cells. Data are the mean \pm SEM of three experiments performed in triplicate. #P < 0.05 vs untreated cells; *P < 0.01 vs untreated cells.

3.5 Cabazitaxel induced apoptosis of NCI-H295R cells

To investigate molecular basis of cell death induced by cabazitaxel, we carried out the Proteome Profiler Human Apoptosis array. NCI-H295R cells were exposed to IC_{50} cabazitaxel for 1 day, to investigate the early stages of apoptosis induction. A representative blot image is shown in Supplemental Fig. 6. The clustering analysis based on protein function (Table 4), shows that cabazitaxel exposure influenced the expression of proteins involved in various aspect of cellular physiology in NCI-H295R cells. Cabazitaxel induced an increase of pro-apoptotic proteins, such as Bad, Bax, Cytochrome C and (S15)-phospho-p53, involved in the intrinsic apoptotic pathway. Furthermore, the extrinsic apoptosis pathway could as well play an important role in mediating cabazitaxel-induced cell death, as an increase of Death Receptors family proteins has been observed. Cabazitaxel exposure activated as well some anti-apoptotic proteins and molecules involved in adaptative response to stress. The functional effect on apoptosis elicited by cabazitaxel on NCI-H295R cells was investigated by AO/EtBr staining. Fig. 7A and Supplemental Table 3A show that cabazitaxel significantly increased the number of apoptotic cells (yellow columns) compared to viable (green columns) after 4 days of treatment. At that time, an increase of necrotic cells could be observed as well, although the difference was not statistical significant. A representative image of cell acquired fields is showed in Supplemental Fig. 7. Cabazitaxel induced also a significant increase of p21/CIP1/CDKN1A and p27/Kip1 proteins involved in cell cycle arrest, indeed the analyses of the cell cycle progression by flow cytometry in untreated and cabazitaxel-treated NCI-H295R cells revealed a significant cell accumulation in the S phase after 4 days of treatment (Fig. 7B). Furthermore, according to results presented above, after 4 days of cabazitaxel exposure, we reported an increased the proportion of cells in the sub-G0 phase, suggestive of DNA fragmentation (Supplemental Table 3B). Cabazitaxel also reduced the level of the p-Akt ($-45\% \pm 2.37$; $P < 0.01$ vs untreated cell) in NCI-H295R cells, leaving the total Akt unaffected (Fig. 8). This observation suggests that the PI3K/Akt pathway could mediate, at least in part, the cytotoxicity induced by cabazitaxel.

Table 4
Proteome Profiler Human Apoptosis array in untreated (C) and cabazitaxel-treated (T) NCI-H295R cells.

| | CABAZITAXEL Fold Change (T vs C) |
|-------------------------------|-------------------------------------|
| Pro-apoptotic proteins | |
| Bad | 2.6 ± 0.2* |
| Bax | 2.9 ± 0.1* |
| Cleaved caspase-3 | 3.8 ± 0.7* |
| Cytochrome C | 2.5 ± 0.2* |
| Phospho-p53 (S15) | 11.3 ± 0.9** |
| Death Receptors signaling | |
| TRAIL R1/DR4 | 1.7 ± 0.1* |
| TRAIL R2/DR5 | 1.8 ± 0.1* |
| FADD | 2.5 ± 0.1* |
| Fas/TNFRSF6/CD95 | 2.4 ± 0.1* |
| Adaptative response to stress | |
| HO-1/HMOX1/HSP32 | 3.0 ± 0.6* |
| HSP70 | 2.1 ± 0.6* |
| PON2 | 5.4 ± 0.63** |
| Anti-apoptotic proteins | |
| Bcl-2 | 2.0 ± 0.2* |
| cIAP-1 | 2.0 ± 0.2* |
| cIAP-2 | 2.7 ± 0.6* |
| Clusterin | 3.1 ± 0.1* |
| Livin | 1.9 ± 0.9* |
| Cell cycle regulation | |
| p21/CIP1/CDKN1A | 13.0 ± 0.9** |
| p27/Kip1 | 4.6 ± 0.8** |

Data are expressed as fold change ± SEM. Only significant results were considered (* $P < 0.05$; ** $P < 0.01$).

4 Discussion

Mitotane either alone or in association with chemotherapy (EDP-M) is the only systemic therapy with documented efficacy in the management of ACC and is recommended by currently available guidelines (Berruti et al., 2012b; Fassnacht et al., 2018). Over the past years, several drugs have been tested against ACC but discouraging results have been observed up to now (Fassnacht et al., 2018; Ferrari et al., 2016). Although preclinical results obtained in the NCI-H295R cell line demonstrated a cytotoxic and antisecretive effect of paclitaxel (Fallo et al., 1996, 1998), paclitaxel and docetaxel failed to demonstrate a relevant activity in 2 published clinical trials (Berruti et al., 2012a,b; Urup et al., 2013). The histopathological characteristics and at the level of drug accessibility significantly influence the sensitivity and resistance of the tumors to taxanes. Resistance to taxanes is a multifactorial phenomenon that mainly involves P-gp expression, but also includes mutation and alteration in the microtubule. Moreover, other mechanisms have been suggested, such as reactive oxygen species production, activation of DNA repair systems, non-coding RNA and epigenetics (Pucci et al., 2018). Interestingly, adrenal gland physiologically express P-gp (Uhlén et al., 2015), and its maintenance in ACC cells has been proposed as one of the possible causes of resistance of ACC to both paclitaxel and docetaxel (Jordan and Wilson, 2004). To overcome this mechanism, the semisynthetic taxane cabazitaxel was developed on the basis of its poor affinity for P-gp (Galsky et al., 2010) and its high lipophilicity (Vrignaud et al., 2013; Azarenko et al., 2014). The present study demonstrates that cabazitaxel exerts a relevant cytotoxic activity in ACC cell lines and in patient-derived primary cell cultures, although with different sensitivity, that can be due to the

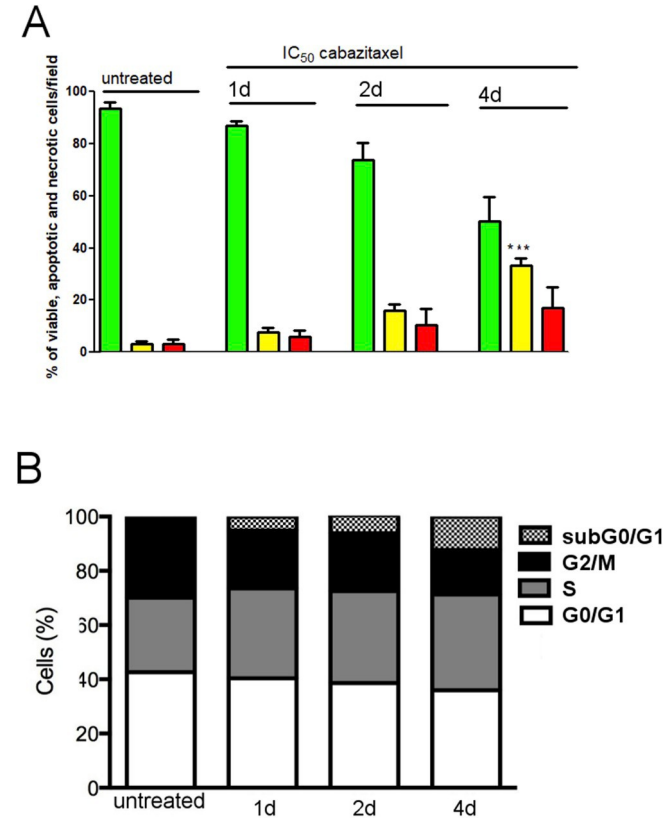


Fig. 7. Cabazitaxel promoted apoptotic cell death and cell cycle arrest in NCI-H295R cells. A) NCI-H295R cells were treated for different time with cabazitaxel at its IC₅₀ and then stained with AO/EtBr. Viable (green), apoptotic (yellow) and necrotic (red) cells were scored under a confocal laser-scanning microscope. Bars represent the percentage of each cell colour vs the total number of cell/field. Magnification, 10x. At least 6 fields were analyzed for each experimental point. *** $p < 0.001$ vs untreated apoptotic (yellow) cells. B) NCI-H295R cells were treated with cabazitaxel at its IC₅₀, 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.

peculiar phenotype of each cell type.

Indeed, cabazitaxel demonstrated higher cytotoxicity compared to the first-generation taxanes, both in ACC cell lines NCI-H295R and in HAC-15 cells (a subclone of NCI-H295R cells) and in 5 out of 6 primary cell cultures. In particular, cabazitaxel, within nanomolar concentrations, displayed a cytotoxicity close to 100% after 4 days of treatment in the ABCB1-expressing NCI-H295R cells and this effect was independent of P-gp. It should be underlined that ACC03, ACC08 and ACC16 appeared to be partially resistant to each taxane, although cabazitaxel was the most efficacious. Cabazitaxel and other taxanes displayed a relatively low cytotoxicity against the ACC cell line MUC-1, which has been established from a patient with disease progression to EDP-M. Similar results were obtained in the ACC06-1 primary cell culture, which derived from a patient who underwent surgery for hepatic metastasis after EDP-M therapy. These results suggest that additional resistance mechanisms may have been activated in these ACC cells. Unfortunately, due to the limited cell availability, we could not perform additional experiments on this primary cell culture. In ACC29 and ACC32 cells primary cells, cabazitaxel was more efficacious and potent than paclitaxel and docetaxel, with concentration-response curves similar to that obtained in NCI-H295R cells. Concerning results obtained with the primary cell cultures, it is noteworthy that the different tumor cell characteristics, together with the size of the biological sample and the dispersion experiment yield, allowed us to obtain sometimes a workable number of cells, i.e. with ACC239 and ACC32 primary cultures,

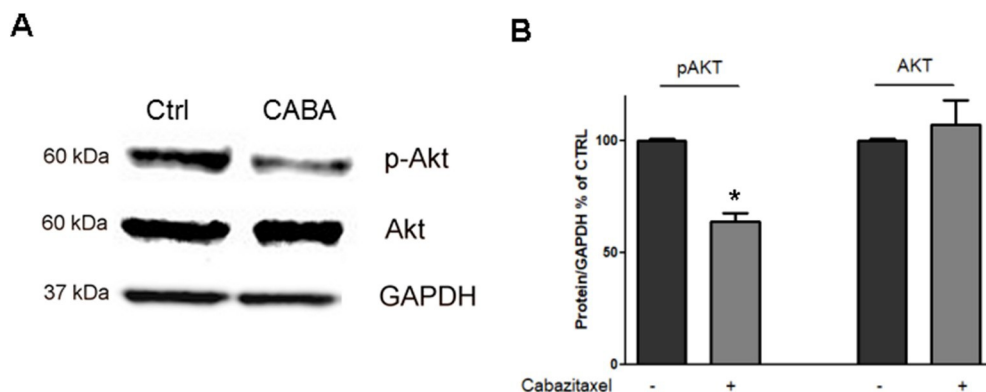


Fig. 8. Effect of cabazitaxel on the expression of AKT and p-AKT proteins by Western blot technique. NCI-H295R cells were exposed to the IC₅₀ value of cabazitaxel for 1 day. A) A representative WB is shown. B) Densitometric analysis of blots of protein of interest were normalized to the corresponding GAPDH levels. Results were obtained from three different biological replicates. Bars represent the mean \pm S.E.M. *P < 0.01 vs untreated cells.

while in other cases, we could perform each taxane concentration-response curve only twice, although always in triplicate. The ACC primary cell cultures were thus an interesting approach to evaluate the *in vitro* drug sensitivity, however, we are aware of the limit of this model. As expected, inhibition of P-gp activity/expression significantly increased the cytotoxicity induced by paclitaxel in ABCB1-expressing cells. Conversely, the cytotoxic effect of cabazitaxel was independent of P-gp protein expression as both the pharmacological inhibition using the P-gp inhibitor tariquidar and ABCB1 gene expression knock-down did not affect the drug cytotoxicity.

The combination of the poor affinity for P-gp together with the high lipophilicity of cabazitaxel suggest that the drug may easily accumulate in ACC cells. This point however is speculative and needs to be extensively studied.

Cabazitaxel in combination with mitotane induced in ACC cells a cytotoxicity that presented an additive/light synergic effect. This effect could have an interesting clinical implication, as the therapeutic potential of the combination can be obtained at low doses of each drug, thus ameliorating the toxicity profile. A limit of this combination could be the fact that cabazitaxel is mainly metabolized by hepatic CYP3A4/5, and mitotane is a CYP3A4/5 inducer. Up to now, however, no cabazitaxel-mitotane interactions are reported (Micromedex Solutions, 2019), although this possibility cannot be excluded. Furthermore, clinical efficacious mitotane concentrations usually are obtained after several weeks to months of treatment (Terzolo et al., 2013). A slow onset of mitotane activity is thus a main limitation, that can possibly be overcome by the contemporary administration of cabazitaxel, with a faster onset of action.

Several studies indicate that taxanes are potent promoters of apoptosis in cancer cells, but their cytotoxic mechanisms are not yet fully understood (Ganansia-Leymarie et al., 2003). Continuous research on the mechanisms of action of cancer drugs, included taxanes, revealed a scenario more complex than initially expected (Komlodi-Pasztor et al., 2011). Furthermore, the alteration of cytoskeleton induced by taxanes may have an impact on a number of intracellular activities such as protein trafficking, signal transduction, cell migration and cell division. Our results are in line with this hypothesis, as we observed that cabazitaxel induced cytotoxicity in ACC cells by an early activation of apoptosis and the increase of cell cycle inhibitors, that lead to cell cycle arrest. However, in ACC cells, we cannot exclude a role of cabazitaxel in the modification of intracellular physiology, with an impact on intracellular metabolism or receptor trafficking, as it has been demonstrated in prostate cancer (Fitzpatrick and de Wit, 2014). This point is of interest and deserves to be deeply investigated in a dedicated study.

In detail, in NCI-H295R cells, cabazitaxel early activated different apoptotic pathways and cell cycle arrest. We observed a significant increase of cytochrome C, an early event implying the loss of the mitochondrial integrity (Wong, 2011), and an increase of pro-apoptotic proteins, such as Bad and Bax, involved in the intrinsic apoptotic pathway, as well as the increase of death receptors, markers of the extrinsic pathway. The induced apoptotic stimulus resulted in the increase of apoptotic cell detection up to 4 days. Cabazitaxel caused also an arrest of the cell cycle, with the accumulation of NCI-H295R cells in the S-phase, modulated by the increase of proteins, such as p21/CIP1/CDKN1A and p27/Kip1. Indeed, p21/CIP1/CDKN1A is a universal cdk inhibitor that blocks progression through all stages of G1/S (Dutto et al., 2015; Fragni et al., 2016), while overexpression of p27/Kip1 blocks progression through S phase (Noomhorm et al., 2014). We observed that cabazitaxel exposure activated as well some anti-apoptotic proteins and molecules involved in adaptive response to stress, that could represent an attempt of cells to protect themselves against the apoptotic insult. Finally, the PI3K/Akt pathway was possibly involved in the cytotoxic effects of cabazitaxel in NCI-H295R cells. The PI3K/Akt signaling network is crucial to different physiological processes, that include cell cycle progression (Potter et al., 2002), differentiation (Lopez-Carballo et al., 2002), transcription (Brazil et al., 2004), translation (Hanada et al., 2004) and apoptosis (Lei et al., 2005) in a wide range of human cells. Akt is an essential component of the PI3K pathway and a gain of function in Akt can lead to uncontrolled cell proliferation and resistance to apoptosis, that are both hallmarks of oncogenic transformation (Li et al., 2012). According to previous studies, and in line with our results, Akt dephosphorylation can activate Bad protein to trigger apoptotic cascades and upregulate p21 and p27 proteins inducing cell cycle arrest (Li et al., 2012; She et al., 2005). A criticism raised by our results was the increase of the S15-p53, as it is known that NCI-H295R cells express a truncated form of p53, lacking the COOH terminus. p53 directly regulates the transcription of over 500 genes and indirectly regulates many additional genes and thereby controls diverse cellular processes, however intriguing results come from the TP53 knock-out mice, where the “expected” spontaneous development

of tumors did not occur (reviewed in Aubrey et al., 2018). The significance of our finding cannot be found at this time and this result deserves to be deeply investigated.

In conclusion, the results of this study demonstrated for the first time that cabazitaxel is active against ACC cells *in vitro* and provided the rationale for the design of a clinical trial entitled: “Cabazitaxel Activity in Patients With Advanced Adrenocortical-Carcinoma Progressing After Previous Chemotherapy Lines” (CabACC study, EUDRACT2017- 001591, ClinicalTrials.gov Identifier: NCT03257891), which has been approved by the local Ethic Committee of Brescia (Italy) and is currently recruiting.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mce.2019.110585>.

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