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Endocrine; 59(1):213-217; 2018; DOI: 10.1007/s12020-017-1270-0.

**PALBOCICLIB INHIBITS PROLIFERATION OF HUMAN ADRENOCORTICAL TUMOR CELLS**

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**Key terms**: adrenocortical carcinoma, human, Palbociclib, retinoblastoma, CDK4/6

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**Acknowledgments:** This work was supported by University of Brescia local grants; AIRC, project IG17678; Fondazione Camillo Golgi and by a private donation of Mrs Serena Ambrogini and family in memory of her son Guido Cioni.

M.F. was supported by a grant from the Italian Society of Pharmacology.

Palbociclib was kindly provided by Pfizer.

**Introduction**

Adrenocortical cancer (ACC) is a rare malignant tumor [1]. Pharmacological therapy is based on mitotane administered alone or in association with the EDP regimen (etoposide, doxorubicin and cisplatin) [1]. However, the prognosis of ACC patients not amenable to surgery still remains poor and newer treatment strategies are needed [2]. Drugs targeting cell cycle could be a relevant new therapeutic approach for patients with advanced ACC [3].

Cell cycle is controlled by several key proteins, including CDKs (cyclin-dependent kinases), which are the target of recently discovered cell-cycle checkpoint inhibitors. [4]. Palbociclib is a CDK4/6-inhibitor that is active against a broad range of tumors and has an acceptable toxicity, being neutropenia being the most relevant side effect [5]. This drug is actually approved in the management of locally advanced or metastatic breast cancer [6].

In this study, we investigated *in vitro* the effect of Palbociclib in NCI-H295R ACC cells and human ACC primary cultures.

**Methods**

**Cell line.** NCI-H295R cell line was obtained from the American Type Culture Collection (ATCC) and cultured as suggested.

**Primary cell cultures**. Human ACC primary cells were derived from surgical specimens of ACC patients after obtaining a written informed consent. They were identified by a sequential number, based on the date of surgery. The project was approved by the local Ethical Committee. ACC02 cell culture derived from a steroid-secreting ACC; whereas ACC03, ACC06 and ACC08 cell cultures derived from non-secreting ACC. After surgical removal, cells were enzymatically digested with collagenase and cultured in the same medium of NCI-H295R cells.

**Quantitative RT-PCR (qRT-PCR).** Gene expression was evaluated by qRT-PCR (ViiA7, Applied Biosystems), using the SYBR Green as fluorochrome, as described [7]. Primer sequences are shown in Fig.1 (panel A).

**Cell viability assay**. ACC cells were treated up to for 4 days with palbociclib (1 nM -1 μM), solubilized in water. Cell viability was determined by 3-(4,5-Dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay [8]. Absorbance was determined by a spectrophotometer at 540/620 nm (GDV).

**Cell cycle analysis.** Flow cytometric cell cycle analysis was performed as described [9]. Untreated and treated-NCI-H295R cells were fixed, treated with RNase A (12.5 μg/ml), stained with Propidium iodide (40 μg/ml) and analyzed by Flow Cytometry using a MACS Quant Analyzer (Miltenyi Biotec GmbH) for cell cycle status. Data were analyzed using FlowJo (TreeStar).

**Western Blot.** ACC cell lysates were processed as described [8]. The following primary antibodies were used: Rb protein (pRB) (DB Pharmigen; 2.5 μg/mL), CDK4 or CDK6 kinase (Santa Cruz Biotechnologies; both at 1 μg/mL), cyclin D1 (Cell Signalling; 0.1 μg/mL), p107 and p130 (Santa Cruz Biotechnologies, both at 1 μg/mL) and tubulin (Sigma Aldrich; 0.01 μg/mL). The specific signal was visualized and the densitometric analysis was performed using the GelPro-Analyzer version 6.0 (MediaCybernetics).

**Statistical analysis.** Data analysis was conducted using GraphPad Prism 5 software (GraphPad Software). Statistical analysis was carried out using one-way ANOVA and Bonferroni's Multiple Comparison test. A p value $< 0.05$ was considered as statistically significant**.**

**Results**

**CDK 4/6 and Rb protein (pRb) expression.** The mRNAs encoding for CDK4/6 and pRb were examined in both NCI-H295R cells and ACC primary cultures using qRT-PCR. Differences in the threshold cycle (Ct) value between the β–actin housekeeping gene and CDK4, CDK6 and Rb genes (ΔCt) were calculated, as an index of the amount of mRNA expressed. Both NCI-H295R cells and ACC primary cultures expressed high amount of mRNA encoding CDK4/6. pRb mRNA was highly detected in ACC primary cultures while it was almost undetectable in NCI-H295R cells (Fig.1a). CDK4, CDK6 and pRb were then analyzed by Western Blot, showing that both the CDK kinases were equally expressed. The ~ 106 KDa band, corresponding to the predicted molecular weight of pRb, was detected in ACC02, ACC03 and ACC06 primary cells, but it was almost absent in NCI-H295R and ACC08 cells (Fig.1b).

**Effect of Palbociclib on NCI-H295R cells and ACC primary cultures cell viability.** The MTT assay revealed that Palbociclib induced a concentration-dependent decrease of cell viability in each cell culture (Fig.1c).

**Effect of Palbociclib on NCI-H295R cell cycle**. NCI-H295R cells were then treated with the Palbociclib $IC_{50}$ and the cell cycle distribution was analyzed by flow cytometry at different times. Results indicated an increase in the proportion of cells at the G0/G1 phase after 48 hours of treatment (untreated cells: 51.3%±1.5; Palbociclib-treated cells: 60.8%±6.7), that was maintained at 72 hours (untreated cells: 47.4%±0.2; Palbociclib-treated cells: 59.5%±5.1). Furthermore, Palbociclib significantly decreased the expression levels of the cell cycle-related proteins cyclin D1 (34%±2,3 and 46%±1,1 of reduction, at 48 and 72 hours of treatment, respectively, $p<0.001$) (Fig 1d), consistent with a G0/G1 cell cycle arrest [10]. CDK4/6 expression did not change after treatment both at mRNA and protein level of (data not shown). Interestingly, a~ 130 kDa band, likely corresponding to the p130/RBL2 Rb-like family protein [11,12], was clearly detected in NCI-H295R cells (Fig.1e).

**Discussion**

This study explored for the first time the *in vitro* activity of the CDK4/6 antagonist Palbociclib in ACC, using the commercially available NCI-H295R cell line and primary cultures derived from ACC patients.

It is known that CDK4/6 inhibition by Palbociclib correlates with greater CDK4/6 inhibitor therapeutic activity, whereas the loss of RB1 is linked to CDK inhibitor resistance [4]. Inactivating mutations or homozygous deletions of the *RB1* gene were found in 7% of ACC [13,14], indicating that a small proportion of ACC may be potentially resistant to CDK4/6 inhibitors. In this study, while NCI-H295R cells and all the primary ACC cells expressed CDK4 and CDK6, pRb protein was found in 3 out of 4 ACC primary cultures. In the ACC08, in fact, only the pRb transcript was clearly

detected, suggesting the presence of a mutation in the translational mechanisms of this protein. In addition, both pRb mRNA and protein were hardly detected in NCI-H295R cells.

Palbociclib significantly affected cell viability in a concentration-dependent way in all ACC cells used in this study, although with a different sensitivity. Overall, the $IC_{50}$ values obtained were close to the C*max* of about 51 ng/ml measured after one single dose of 125 mg Palbociclib in humans [15], corresponding to 0.11 μM. Flow cytometric analysis revealed that Palbociclib treatment lead to cell accumulation in G0/G1 phase in NCI-H295R cells, combined with a significant decrease of cyclin D1 levels, while CDK4/6 expression was unchanged. These data confirm also in ACC cells that the cell cycle arrest is likely the main mechanism of the cytotoxic effect of Palbociclib [10].

Interestingly, despite the lack of pRb expression, both NCI-H295R cells and ACC08 primary culture were sensitive to the anti-tumoral effect of Palbociclib. It is well known that pRb is just one of the multiple targets of the CDK4/6/Cyclin D pathway [11] and a Rb-like family including p107/RBL1 and p130/RBL2 proteins (all substrates for CDK/6 inhibitors) was identified [12]. One of these proteins (p130/RBL2) was detected in NCI-H295R cells in this study. A recent study showed that pRb negative human hepatoma cell lines are sensitive to palbociclib due the expression of both p107/RBL1 and p130/RBL2 [16].

Several molecular targets have been recently identified for novel treatment approaches in the management of ACC, including mTOR [17,18], Wnt-beta catenin signaling pathway [13], receptors for different growth factors and the ACAT1 [2] and CYP17A1 [19] steroidogenic enzymes.

In this study, we provide a preclinical preliminary evidence that CDK4/6 targeting agents could be effective in the ACC treatment, irrespective pRb expression. Palbociclib, whose toxicity profile seems to be advantageous over the EDP regimen [20], deserves to be further explored as a new therapeutic option in ACC.

**Compliance with ethical standards**

**Conflict of interest:** The authors declare that they have no conflict of interest in relation to the topic of the manuscript

**Funding:** This work was supported by University of Brescia local grants; AIRC, project IG17678; Fondazione Golgi and a private donation of Mrs Serena Ambrogini and family in memory of her son Guido Cioni.

**Ethical approval:** The project was approved by the local Ethical Committee.

**Informed consent:** Informed consent was obtained from all individual participants included in the study.

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**Fig.1 Gene expression of CDK4/6 and pRb in NCI-H295R cells and ACC primary cultures. a,** Aliquots of each cDNA, chosen after standard curves experiments, were used in 25 µl reaction containing 12.5 µl SYBR Green Mix (Bio-Rad Laboratories) and 12.5 pmol each of sense and antisense oligonucleotide primers of selected genes and human β-actin as reference gene. Reactions were performed under the following conditions: 1 cycle 95◦C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 1 min. Results are presented as a mean ΔCt ± SEM. **b, Analyses of CDK4, CDK6 and pRb expression of NCI-H295R cells and ACC primary cultures.** Lysates from NCI-H295R cells (line 1) and primary cultures of ACC03 (lane 2) ACC02 (lane 3), ACC08 (lane 4) and ACC06 (lane 5) were analysed for CDK4, CDK6 and Rb protein (pRb) expression by using Western Blot technique. The human α-tubulin was used as internal control. The specific signal was visualized by the ECL-PLUS system. **c, Effect of Palbociclib on NCI-H295R cells and ACC primary cultures cell viability.** ACC cells were treated as described in Methods. Results are expressed as percent of viable cells versus untreated cells (control) ± SEM of at least three different experiments, run in triplicate. NCI-H295R cell line, ACC03, ACC08 and ACC06: for concentrations starting from 250 nM to 1 µM, *$p$<0.001 vs.untreated cells; ACC02: for concentrations starting from 500nM to 750nM, #$p$<0.01 vs.untreated cells, and for 1µM concentration, *$p$<0.001 vs.untreated cells. $IC_{50}$ value were: 0.84 µM (95% CI: 0.4-1.7) for NCI-H295R cells, 0.67 µM (95% CI: 0.66-0.69) for ACC02, 0.29 µM (95% CI: 0.15-0.57) for ACC03, 0.50 µM (95% CI: 0.28-0.88) for ACC06, and 0.18 µM (95% CI: 0.10-0.32) for ACC08. **d, Cyclin D1 expression in untreated and Palbociclib-treated NCI-H295R cells**. Cells were treated with palbocilcib and analysed for cyclin D1 using Western Blot technique. The human α-tubulin was used as internal control. The specific signal was visualized by the ECL-PLUS system. **e, Analyses of p107/RBL1 and p130/RBL2 expression in NCI-H295R cells.** Lysates from NCI-H295R cells were analysed for p107/RBL1 and p130/RBL2 expression by using Western Blot technique. The human α-tubulin was used as internal control. The specific signal was visualized by the ECL-PLUS system.