Brief Communication

Potent inhibitory effect of the cyclolignan picropodophyllin (PPP) on human adrenocortical carcinoma cells proliferation

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Abstract: Adrenocortical carcinoma (ACC) is a very aggressive tumor with a poor prognosis. Available treatments for this type of cancer are far from being satisfactory. The IGF signalling pathway represents an important mechanism for ACT growth and constitutes a relevant therapeutic target. We investigated the effect of picropodophyllin (PPP), a member of the cyclolignan family and a new inhibitor of IGF-1R, on proliferation of human adrenocortical cell lines H295R and SW-13. PPP inhibits proliferation and induces an important accumulation in G2/M phase and apoptosis of H295R and SW-13 cells. Our data suggest that PPP may be a promising candidate for drug development for adrenocortical carcinoma.

Keywords: Adrenal cortex, cancer, IGF receptor, cell lines
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of IGF-1R without interfering with the highly homologous insulin receptor or tyrosine kinases of other relevant growth factor receptors relevant for cancer cells [11]. PPP induces tumor regression and inhibition of metastasis in several models of human cancer and its administration is well tolerated in vivo [12]. These data prompted us to investigate the effect of PPP on the growth of two established human ACC cell lines (H295R and SW-13).

Materials and Methods

Chemicals

PPP was synthetized in an ultrapure form as described [11]. NVP-AEW541 [13] was provided by Novartis. Stock solutions of both compounds were prepared in DMSO (50 mM and 10 mM, respectively).

Cell culture and proliferation assays

H295R cells were cultured in DMEM/F-12 supplemented with 2% NuSerum, 1% ITS Plus and antibiotics, as described [14]. SW-13 cells were cultured in DMEM/F12 supplemented with 10% FCS and antibiotics. To measure proliferation, cells were seeded in duplicate in 24-well plates at the density of 3x10^4 cells/well and cultured in complete medium in the presence of the indicated concentration of the different compounds or DMSO added to the culture medium. Cells were counted after 3 days of culture using the COUNTESS automate instrument (Invitrogen).

Immunoblots

H295R and SW-13 cells were treated with the indicated concentrations of different compounds or with DMSO vehicle. Protein extracts were prepared by harvesting cells in RIPA buffer ([50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 50mM NaF, Protein Inhibitor Cocktails 1 and 2 (Sigma)]. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot was performed using a chemiluminescence system for protein detection (ECL Plus, GE Healthcare). Antibodies used were as follows: anti-IGF1Rβ; anti-Akt (total) and phospho-Akt(Ser473); anti-p44/p42 mitogen-activated protein kinase and anti-phospho-p44/p42 mitogen-activated protein kinase (all from Cell Signaling Technology); anti-phosphotyrosine PY20 (Sigma).

Flow cytometry

H295R and SW-13 cells were fixed in 70% ethanol and then treated with RNase A (50 µg/mL) for 30 min at 37°C. DNA was stained with propidium iodide (50 µg/mL) and cells were analyzed for cell-cycle distribution with a FACScan instrument (Becton Dickinson).

Results

In this study, we used two established human ACC cell lines: the well-differentiated H295R cells, that retain the ability to synthetize steroid hormones, and SW-13 derived from a stage IV tumor that are not steroidogenic. Both cell lines represent suitable models to study the effects of IGF-1R inhibitors since they express high levels of IGF-1R. However, H295R cells, but not SW-13, produce high levels of IGF2, which acts in an autocrine manner to trigger their proliferation [8, 9].

We first evaluated the effect of different doses of PPP on H295R and SW-13 cell proliferation and compared it with the NVP-AEW541 IGF-1R inhibitor. After 3 days of treatment, PPP inhibited cell growth of H295R (IC50 1.8 x 10^-7M) and SW-13 (IC50 1.4 x 10^-7M) cells in a dose-dependent manner, having a more potent effect than NVP-AEW541 on both cell lines (IC50 4.6 x 10^-7M for H295R cells and IC50 1.6 x 10^-6M for SW-13 cells; Figure 1A).

We next evaluated the effect of PPP on cell cycle distribution after a 24h treatment. Exposure of H295R cells to 1 mM PPP increased the fraction of cells in G2/M-phase (from 22% of DMSO control to 38%) and sub-G1 (hypodiploid apoptotic cells; from 2% of DMSO control to 8%) with a corresponding decrease of the fraction of cells in the G1-phase (from 47% of DMSO control to 22%). The PPP-induced G2/M accumulation (from 22% of DMSO control to 74%) and apoptosis (from 2% of DMSO control to 8%) were also observed in SW-13 cells (Figure 1B).

Surprisingly, PPP did not significantly modulate phosphorylation of IGF-1Rβ in H295R cells (Figure 2A). The apoptotic effect of PPP has been shown to be associated with an important inhibition of PI3K/Akt pathway and a moderate effect on the ERK pathway in other cell types [11, 15]. After a 48h serum starvation, H295R and SW13 cells were treated with or without 10^-5M PPP for 2h and finally stimulated with 10%
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serum. We observed that PPP exerts no significant effect on Akt and ERK1/2 phosphorylations whereas NVP-AEW541 exerts a substantial inhibition of Akt and a moderate effect on ERK1/2 phosphorylations. These results suggest that PPP effect does not involve inhibition of the Akt and ERK1/2 effect in adrenocortical cell lines (Figure 2B).

Discussion
The factors responsible for the incidence of benign adrenocortical tumors and its malignant transformation are not well understood. In addition to IGF2 overexpression, increased levels of the IGF-1R have been found in adrenocortical carcinomas, suggesting an important role for the IGF-system in adrenocortical carcinogenesis [9, 10]. These results indicated that IGF-1R may represent an important target for cancer therapy. Recently some studies using IGF-1R inhibitors such as NVP-AEW541 or an anti-IGF-1R monoclonal antibody produced inhibition of ACC cell growth in vitro and in vivo [9, 10]. The results of a phase I clinical study of an anti-IGF-1R monoclonal antibody in patients with advanced ACC have been reported [16].

In this report we have studied the effect of PPP, a member of the cyclolignan family described as a specific inhibitor of the IGF-1R, on ACC cells proliferation [11]. PPP has been shown to block the phosphorylation of the IGF-1R without affecting the homologous insulin receptor [11]. This represents an obvious advantage over some other IGF-1R inhibitors. Moreover, PPP has been shown to be well tolerated in vivo after oral administration [11, 12]. For these reasons,
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PPP may represent a compound potentially interesting for drug development for ACC.

We have shown that PPP inhibits growth of two different human ACC cell lines (H295R and SW13) in vitro. Cell cycle analysis revealed that a 24h treatment with PPP drastically increased the fraction on G2/M and sub-G1 phases. These effects on cell cycle have been also observed in multiple myeloma cells after PPP treatment [17]. PPP exerts an important effect on proliferation at lower concentrations than NVP-AEW541. Nevertheless, we could not detect any effect of PPP on rapid phosphorylation of Akt and ERK1/2, whereas it has been largely associated with an inhibition of PI3K/Akt pathway in other cell lines [15]. These results suggest that in ACC cell lines, the inhibitory effect of PPP does not primarily involve these signaling pathways. Recently, other reports revealed that PPP may act with mechanisms different from Akt/Erk inhibition. PPP is a stereoisomere of podo-phyllotoxin (PPT), an established inhibitor of microtubule assembly leading to mitotic arrest presenting a general toxicity [18, 19]. However, PPP used here is an ultrapure compound. It was shown that PPP does not bind to tubulin at concentrations up to 50 µM while PPT does. The effect of PPP observed in our cellular model is observed at a concentration of 0.1 µM.

Further studies are necessary to further characterize the molecular mechanism of the inhibitory action of PPP on adrenocortical cell lines proliferation. This compound may represent an interesting therapeutic tool to be associated to newer drugs in ACT chemotherapy [14, 20-22], in order to develop more selective and specific treatments for clinical use.

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Abbreviations: ACC, adrenocortical carcinoma; PPP, picropodophyllin; IGF-1R, type 1 IGF receptor.

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