

Original Research Article

Inhibitory effect of platinum (II) complex of curcumin on non-small cell lung cancer via the PI3K-AKT pathway

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Abstract

Purpose: To study the effect of platinum (II) complex of curcumin (PCC) on non-small cell lung cancer (NSCLC) using human NSCLC cell line A549.

Methods: The anti-proliferative and apoptotic effects of PCC against NSCLC were investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Annexin V-FITC/propidium iodide double staining, quantitative real-time polymerase chain reaction (qRT-PCR), and western blot assays.

Results: First, MTT assay and Annexin V-FITC/propidium iodide double staining results revealed that PCC possesses strong anti-cancer potency and high safety, when compared with cisplatin. Western blotting and qRT-PCR results showed that PCC inhibited the viability of NSCLC cells, decreased the expressions of PI3K, VEGF, AKT and Bcl-2, and upregulated the expression of PTEN.

Conclusion: These results indicate that PCC promotes apoptosis in A549 cells via PTEN/PI3K/AKT signaling pathways.

Keywords: Platinum-curcumin complex, Non-small cell lung cancer, Apoptosis, PI3K-AKT

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INTRODUCTION

Most lung cancer incidents (about 85 %) are due to NSCLC, while squamous cell carcinoma, large cell carcinoma, and adenocarcinoma occur less frequently [1-3]. Currently, surgery, chemotherapy, radiation and immunotherapy are the main courses of treatment for these cancers [4]. Among these, chemotherapy is the most widely used, but its effect is frequently unsatisfactory because of problems such as limited effectiveness, severe toxicity and multidrug resistance [5].

Consequently, natural products have attracted increasing attention in cancer therapy [6]. Curcumin (Cur) is a natural polyphenol (Figure 1) which plays role in inducing tumor differentiation and apoptosis, inhibiting blood vessel formation, and regulating the expressions of tumor suppressor genes and oncogene proteins [7,8]. The expectation is that this is a new type of natural antitumor drug with high efficiency and low toxicity. However the use of Cur has limitations because of its weak anti-tumor effect, low bioavailability and poor water solubility [9]. The gold standard primary therapy for NSCLC patients involves the application of platinum-

linked treatment [10,11]. However, the lack of selectivity between normal cells and cancer cells creates more side effects [12,13]. Therefore, in the present study, platinum (II) complex of curcumin was used (PCC, Figure 1) to investigate if the anti-cancer effects of curcumin could be improved, and also to see if the toxicity of cisplatin could be relieved. The specific mechanism of this complex was also investigated.

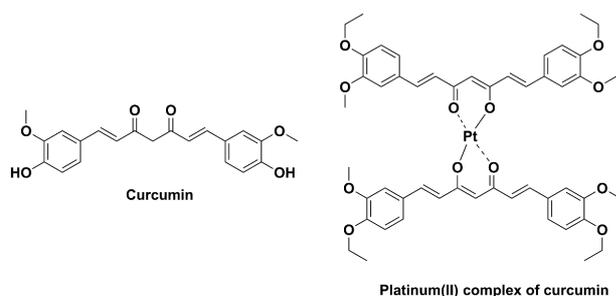


Figure 1: Structures of curcumin and platinum (II) complex of curcumin

EXPERIMENTAL

Materials and reagents

Professor Shuangsheng Zhou of the Anhui College of Traditional Chinese Medicine designed and provided the platinum (II) complex of curcumin. Curcumin and cisplatin (Cis) were products of Chengdu Herbpurify Co., LTD (Chengdu, China) and Sigma Chemical Co. (St. Louis, USA) respectively. They were dissolved in DMSO as stock solution. Sigma Chemical Co (St. Louis, USA) was the source of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Cell lines and cell culture

Human non-small cell lung cancer cell lines (A549 and SPC-A1), and normal lung cell line HBE were bought from KeyGEN BioTECH (Nanjing, China). These cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10 % heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories) and 1 % penicillin–streptomycin antibiotic mixture (Gibco BRL) at 37 °C with 5 % CO₂. The cells were used at the exponential growth phase.

Cell viability assay

Each cell culture was collected and transfected for 24 h before digestion with 0.25 % trypsin. After re-suspending in complete medium, the cells were separately plated in 100- μ L culture medium in 96-well plates at density of 1×10^4

cells/well, and incubated with various concentrations of the different drugs for 48 hours. Four hours before the end of the incubation, MTT dye (10 μ L of 2.5 mg/mL in PBS) was added to the wells, followed by further 4 hour-incubation at 37 °C in the 5 % CO₂ atmosphere.

Next, each plate was centrifuged for 15 min at 1500 rpm before discarding the supernatant without disturbing the well cells and the formazan crystals. The formazan crystals were solubilized in DMSO (150 μ L) before using a plate shaker to agitate the plates for 5 min. A microplate reader (Thermo, USA) was employed to read the optical density (OD) at 490 nm, and the IC₅₀ values were calculated using GraphPad Prism 6.0. All assays were carried out in triplicate.

Determination of cell apoptosis

The A549 cells were seeded at 1×10^5 cells per well in a 6-well microtiter plate. After exposure to PCC (0, 10, and 30 μ M) for 24 h, they were trypsinized before washing twice with chilled PBS and then re-suspending in a binding buffer. Next, each cell was subjected to Annexin V-FITC/PI double staining using apoptosis detection kit (Nanjing Jiancheng Bioengineering Institute) based on the manufacturers protocol. Flow cytometry (BD FACS Calibur, USA) was then used to analyse cellular fluorescence. Each experiment was carried out in triplicate.

PCR analysis

Total RNA was extracted from the cells treated with 0, 10, and 30 μ M PCC, and also from cells treated 10 μ M Cis in different groups. To determine the mRNA expressions of VEGF, PTEN and P13K, the extracted total RNA was reverse-transcribed to cDNA using kits from First strand cDNA Synthesis (TransGen Biotech) in line with the kit manual protocol. The qRT-PCR was done using Top Green qPCR SuperMix (TransGen Biotech) coupled to a 96-well Piko Thermal Cycler system, with β -actin mRNA as internal control. Quantifications of mRNA expressions were carried out with comparative Ct procedure.

Western blot

Total protein was extracted from each cell line by grinding in RIPA buffer containing 1 mM PMSF (Cell-Signalling Tech., US). The protein levels were quantified with BCA protein assay kit (Thermo Fisher Scientific, MA, USA) according to the kit protocol. Primary antibodies for PTEN, P13K and β -actin were bought from Cell Signalling Technology (Beverly, MA, USA), while

the primary antibodies for VEGF, AKT, Bcl-2 were obtained from Abcam (Cambridge, UK). The internal reference used was β -actin. Chemiluminescence was measured with an ECL kit (Pierce Biotechnology) and a Bio-Rad molecular imager was used to visualise and capture the images of the resultant immunoreactive protein bands. The intensity of each band was calculated with ImageJ, and the results were normalised to that of β -actin.

Statistical analysis

Data are presented as mean \pm standard deviation (SD). Statistical analysis was done with one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison. All analysis was done with GraphPad Prism 6. Statistical significance was set at $p < 0.05$.

RESULTS

In vitro cytotoxicity of PCC

The IC_{50} values depicted in Figure 2 reveal that the inhibitory effects of PCC on lung adenocarcinoma cells was comparable to that of the positive control Cis, but superior to that of Cur. Aside from that, in normal HBE cells, PCC

almost exhibited no toxic effects, while Cis showed obvious inhibitory effect on the growth of HBE cells, with IC_{50} of 41.18 μ M. This suggests that the complex sustained the relative safety of Cur and the potent activity of Cis.

Results of flow cytometry for apoptosis

Results from cytometry demonstrated that the pro-apoptotic effect of PCC on A549 cells was dose-dependent. Apoptosis in the low-dose PCC group (45.3 ± 2.8 %) and the high-dose PCC group (72.6 ± 3.5 %) were significantly higher than that in the normal control group ($p < 0.001$), indicating that the pro-apoptotic effect of PCC was comparable to that of Cis.

Effect of PCC on the expressions of P13K, VEGF, and PI3K mRNAs

As shown in Figure 4, data obtained from qRT-PCR revealed that the expression of PTEN mRNA was significantly upregulated in the PCC and Cis groups, when compared to the normal control group, while the expressions of VEGF and PI3K mRNAs in the drug-treated groups were significantly lower ($p < 0.05$).

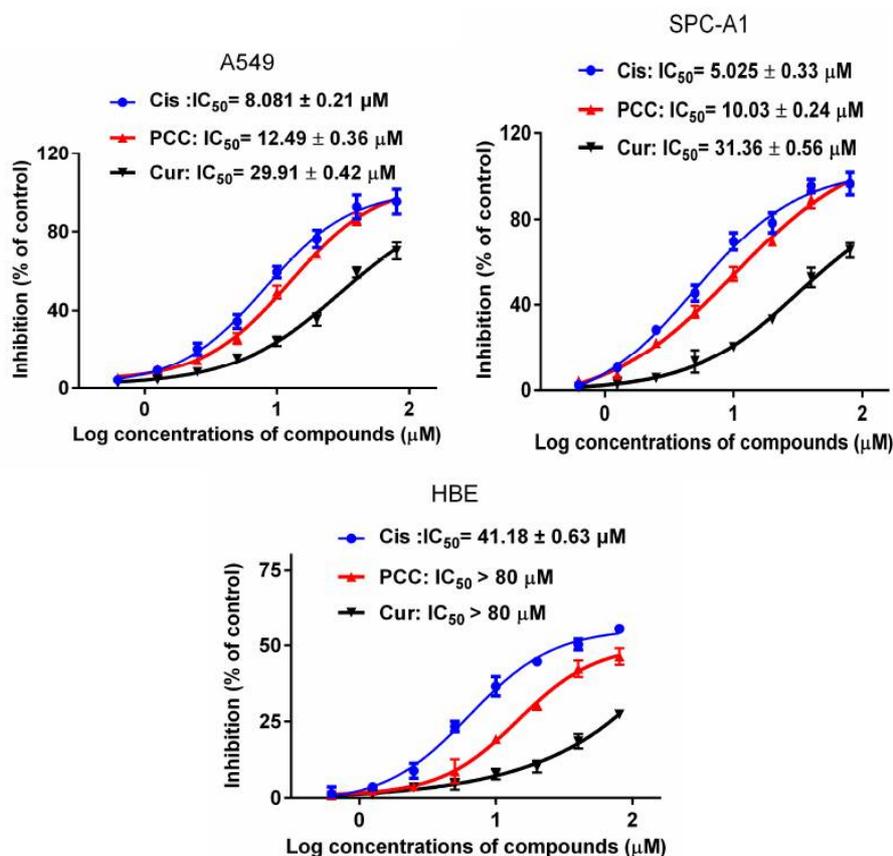


Figure 2: IC_{50} for the potency of PCC on A549, SPC-A1 and HBE cell lines, as determined using MTT assay. Data are presented as mean \pm SD ($n = 3$)

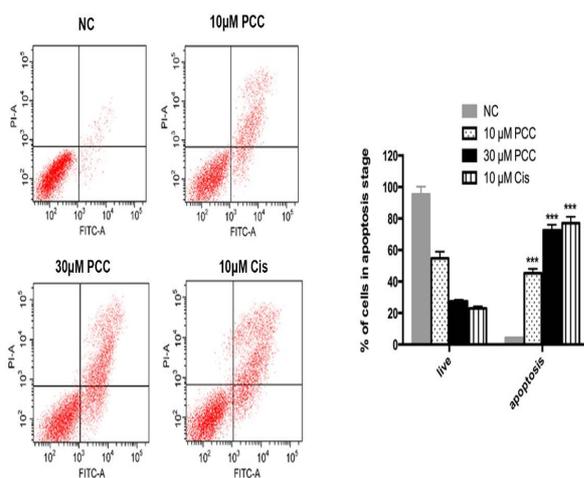


Figure 3: Pro-apoptotic effects of PCC on A549 cells, as determined with Annexin V-FITC/PI staining and flow cytometry. Data are presented as mean ± SD (n = 3)

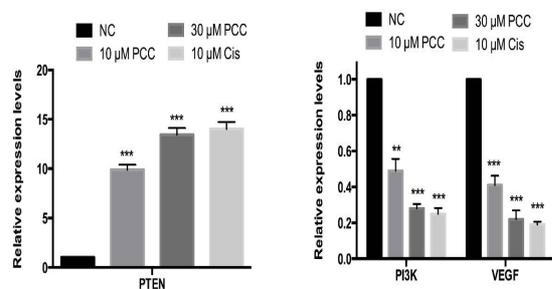


Figure 4: Effect of PCC on mRNA expressions of PTEN, PI3K and VEGF in A549 cells; * $p < 0.05$, compared with the control group

Effect of PCC on the expressions of VEGF and AKT/PI3K pathway-related proteins in A549 cells

Western blotting assay results revealed that in the drug-treated groups, PTEN expression was significantly increased while the PI3K, AKT, VEGF, and Bcl-2 expressions were depressed, relative to corresponding expressions in the normal control group (Figure 5). These results suggest that PCC exerts anti-lung cancer effect via regulation of the PTEN/PI3K/AKT signaling pathway.

DISCUSSION

Although anti-cancer therapies such as radiotherapy, chemotherapy, and targeted therapies have witnessed some advances, the five years survival remains low (< 15 %) [14]. Curcumin occupies a vital position as a paramount source of anti-cancer drugs, but its use is limited because of a weak anti-tumor effect, low bioavailability and poor water solubility [15]. The gold standard therapy for NSCLC is platinum-based chemotherapy, which also has

severe side effects [16]. In the present study, the anticancer effect of a combination of the two drugs was studied. First, the cytotoxicity of PCC on human NSCLC cell lines A549 and SPC-A1, as well as normal human bronchial epithelial cell line HBE was determined. Conclusions were easily drawn that PCC had potent ability to inhibit NSCLC cancer cell growth at a level comparable to that of cisplatin, and superior to that of curcumin. In addition, it had no effect on normal cells, indicating it was safer than cisplatin. Double staining with Annexin V-FITC/PI produced consistent results, showing the ability of PCC to induce significant cell apoptosis in a dose-dependent manner.

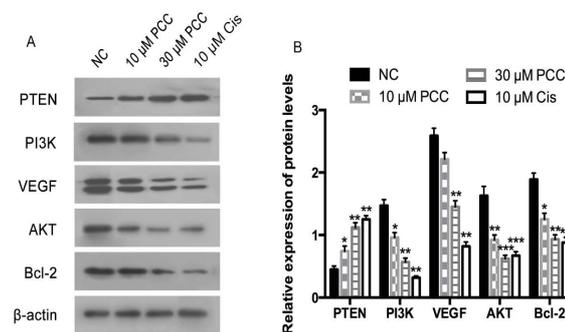


Figure 5: Effect of PCC on expressions of PTEN/PI3K/AKT signaling pathway-associated proteins. Data are expressed as mean ± SD (n = 3); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the normal control group

Researchers have found multiple signaling pathways which are linked to lung cancer. These include the PI3K-AKT [17 - 19], VEGF [20-22], and P53 [23-25] signaling pathways. There is agreement with the fact that PTEN is a dual protein/lipid phosphatase. The central substrate is a product of P13K, i.e. phosphatidylinositol 3,4,5 triphosphate (PIP3) [26]. Cyclins and Bcl-2 proteins correlate closely with cell cycles and apoptosis, respectively [27].

It is known that VEGF mediates the migration and proliferation of endothelial cells, as well as angiogenesis and survival [28-30]. Thus, in order to determine the effect of PCC on the related proteins, the relative expression levels of VEGF, PI3K and PTEN; and the relative protein expression levels of VEGF, PI3K, PTEN, AKT and Bcl-2 were determined using qPCR and western blot, respectively. The results showed that PCC downregulated VEGF and PI3K mRNAs, but upregulated PTEN mRNA. The changes in protein expressions were in agreement with the pattern of expressions of mRNAs.

CONCLUSION

These results suggest that treatment of PCC prevents A549 cell proliferation via regulation of the PI3K/AKT signaling pathway (34-35). PCC exerts anticancer effect on NSCLC, most likely via regulation of PTEN side of PI3K/AKT signaling pathway rather than through PI3K axis. Therefore, PCC is a potential novel anti-lung cancer drug candidate.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

This work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xingxiang Xu; Junjun Yang, Lingfeng Min, Zhenghua Jiang, Wenjing Xu and Hanqing Yao collected and analysed the data, while Yanming Geng and Jiarong Bian wrote the manuscript. All authors read and approved the manuscript prior to publication.

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