Original Article

Tumstatin 185-191 increases the sensitivity of non-small cell lung carcinoma cells to cisplatin by blocking proliferation, promoting apoptosis and inhibiting Akt activation

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Abstract: Purpose: This study aimed to investigate the synergistic anti-tumor effects of tumstatin 185-191 and cisplatin in non-small cell lung carcinoma cells (NSCLC) (A549 cells and cisplatin resistant A549/DDP cells), and the potential role of Akt signaling pathway was also explored. Methods: A549 or A549/DDP cells were treated with Tum185-191 or Tum185-191 plus cisplatin. Cell viability was assessed by modified MTT assay. 50% inhibiting concentration (IC50) and reversing drug-resistance index (RI) of chemotherapeutics were determined by MTT assay. Cell apoptosis was measured by Hoechst 33258 staining and flow cytometry. The activation of Akt signaling pathway was evaluated by immunocytochemistry and Western blot assay. Results: Tum185-191 inhibited the proliferation of A549 cells and A549/DDP cells. In the presence of Tum185-191 (20 and 40 µM), IC50 of cisplatin reduced significantly in A549 cells and A549/DDP cells. Combined use of tumstatin 185-191 and cisplatin exerted synergistic effects in promoting apoptosis. A549 and A549/DDP cells had a high expression of p-Akt, and Tum185-191, but not cisplatin, significantly inhibited p-Akt expression. Combined use of cisplatin and Tum185-191 failed to further inhibit p-Akt expression. After Tum185-191 treatment, the increased p-Akt expression was observed at 15 min, peaked at 30-60 min, but disappeared at 120 min. Conclusion: Tum185-191 increases the apoptosis, inhibit the proliferation, enhance the sensitivity of A549 cells to cisplatin and also partly reverse the resistance of A549-DDP cells to cisplatin, which is at least partially mediated by inactivating Akt pathway. These findings provide evidence for the chemotherapy of NSCLC with Tum185-191 and cisplatin.

Keywords: Non-small cell lung carcinoma cells, Tumstatin 185-191, cisplatin, Akt

Introduction

Lung cancer is the leading cause of cancer-related death in both men and women worldwide [1, 2]. Approximately 75-80% of lung cancer is non-small cell lung carcinoma (NSCLC). Radical resection is the treatment of choice for NSCLC. However, many lung cancer patients lose the opportunity to operation at the initial diagnosis due to lack of specificity of early diagnosis. More than 75% of patients with NSCLC are potential candidates for chemotherapy [3, 4], but chemotherapy usually produces rather poor response rates in NSCLC patients with rare complete remissions [5, 6]. Thus, it is imperative to develop new treatments to improve the overall disease-free survival of NSCLC patients.

Type IV collagen, a major component of basement membrane, is organized as a network of heterotrimers composed of three α (IV) chains among six possible, α1 (IV) to α6 (IV) [7]. NC1 α3 (IV) chain, also known as tumstatin, was first identified by Maeshima et al [8], to display antiangiogenic and anti-tumor properties through
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two distinct peptide sequences [9-11]. In recent years, several investigators have studied the role of tumstatin in melanoma, glioma, colon carcinoma, Lewis lung carcinoma and prostate cancer [12-17]. Results show that tumstatin and peptides from tumstatin might be used as potent antitumor agents against a wide range of human tumors. To date, however, few studies have investigated the anti-tumor activity of tumstatin combined with conventional anti-tumor drugs in carcinomas, and the anti-tumor activity of tumstatin is still poorly understood in NSCLC.

Studies have suggested that tumstatin-induced apoptosis of cells requires its binding to αVβ3 integrin [18, 19]. Of the pathways activated by integrin, the phosphatidylinositol 3-kinase (PI3K)/Akt is essential for the integrin mediated regulation of cell proliferation and survival [20-23]. PI3K/Akt pathway plays key roles in the tumorigenesis and therapeutic resistance of cancer cells. Inhibition of the PI3K/Akt pathway may sensitize the small cell lung cancer (SCLC) and NSCLC cells to traditional chemotherapeutic agents [24-26]. In addition, adherent SCLC cells that are selected after prolonged subculturing show increased activation of Akt and greater resistance to traditional chemotherapy or radiation [26-28]. Tumstatin has been shown to inhibit the activation of PI3K/Akt in endothelial cells and glioma cells [12, 29]. However, whether combined use of tumstatin and chemotherapeutics exert synergistic anti-tumor effects, whether tumstatin is still effective on the resistance of NSCLC cells to chemotherapy, and whether tumstatin affects the Akt activity are still largely unclear.

Studies have shown that tumstatin 185-191 and tumstatin 185-203 have a β-turn at the YSNS (188-191), which is crucial for biological activity [30, 31] and both tumstatin 185-191 and tumstatin 185-203 have anti-tumor activity. In this study, we investigated the anti-tumor effects of tumstatin 185-191 alone or in combination with cisplatin on A549 cells and A549/DDP cells. In addition, the status of Akt pathway was evaluated after exposure to tumstatin 185-191 and cisplatin in these cell lines.

Materials and methods

Reagents

Phospho-specific antibodies directed against Ser473 (S473) of Akt were purchased from Cell Signaling Technology. Insulin-like growth factor (IGF)-1 was obtained from PeproTech. Cisplatin (Qilu Pharmaceutical Co., LTD.), and penicillin, streptomycin, trypsin, methylene diphosphonate and percoll were from Sigma Aldrich. Other reagents were local products of analytic grade.

Peptide

The NC1 α3 (IV)-(185-191) peptide, CNYYYSNS, was obtained by solid-phase synthesis using a Fmoc (N-(9-fluorenyl) methoxycarbonyl) derivative procedure, purified further by reverse phase high performance liquid chromatography with a C18 column for elution with a gradient of acetonitrile in trifluoroacetic acid, and then lyophilized. The peptide was dissolved in dimethyl sulfoxide (DMSO) (final DMSO concentration was lower than 0.2% [v/v] in all experiments) for the determination of its biological activity.

Cell culture

A549 cells, a human adenocarcinoma cell line, and A549/DDP cells, a cisplatin resistant cell line, were obtained from the Xiangya Central Experiment Laboratory (Changsha, China). Cells were grown in a humidified environment with 95% air and 5% CO2 at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells in the logarithmic phase of growth were used for following experiments.

Cell viability assay

Cells were seeded at 5×10³ cells per well in 96-well plates (Costar, Corning INC., Coring, NY) followed by incubation for 24 h, and then exposed to various concentrations of agents for 48 h. Then, cells were quantified by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT solution was added to each well at a final concentration of 0.5 mg/ml. After 4-h incubation at 37°C, the supernatant was removed, and 100 µl of DMSO was added to each well. The absorbance at 490 nm was recorded by using an enzyme-linked immunosorbent assay reader. Six replicated wells were included in each group, and means were calculated. At least three independent experiments were done.

Apoptosis assay

Apoptosis of A549 cells and A549/DDP cells was detected by two methods: (1) Hoechst...
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33258 staining: Cells were seeded on sterile cover glasses in the 6-well plates, followed by incubation for 24 h, and then treated with various concentrations of agents for 48 h. Cells were fixed, washed twice with D-Hank’s and stained with Hoechst 33258. The nuclei were observed under a fluorescence microscope. (2) Flow cytometry: Cells were treated with various concentrations of agents for 48 h, harvested after digestion with 0.25% trypsin and washed with PBS. Cells at a density of 1×10^6 were fixed in 70% ice-cold EtOH/PBS for 20 min on ice, washed with PBS and then incubated in PI solution (69 mM PI, 388 mM sodium citrate, 100 µg/ml RNase A) for 15 min at 37°C. Cells were immediately detected with FAC Scan flow cytometry (Becton Dickinson, San Jose, USA).

Immunocytochemistry

Cells were seeded on coverslips in 6-well plates followed by incubation for 24 h, maintained in serum free medium for 24 h, and then treated with various concentrations of agents for 30 min. Samples were washed in PBS thrice (5 min for each), fixed in 4% paraformaldehyde for 30 min, incubated in 3% H_2O_2 for 10 minutes, and blocked for 60 min in 5% BSA at room temperature. Then, cells were incubated with rat monoclonal anti phospho-Akt (p-Akt) antibody (1:100) at 4°C over night, followed by addition of other reagents according to the manufacturer’s instructions. Finally, 0.5% DAB-H_2O_2 was added, followed by incubation for 30 min for visualization. The positive cells had yellow, tan, or brown granules in the cytoplasm or nucleus.

Western blot assay

A549 cells and A549/DDP cells were independently incubated overnight in 6-well plates in RPMI 1640 supplemented with 10% FCS. Then, cells were maintained in serum-free medium for 24 h. Following addition of 50 ng/ml IGF-1, incubation was done for 15 min, and then cells were harvested. Cells were pretreated with various concentrations of cisplatin and tumstatin for different durations before IGF-1 treatment. Total protein was collected after cell lysis with lysis buffer [20 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml Leupeptin], and the protein concentration was determined by using Lowry’s modified method. Then, 50 µg of proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto nitrocellulose Hybond C membrane using a semidy transblot apparatus. The membranes were blocked for 1 h at room temperature in blocking buffer [20 mM Tris-HCL (PH 7.6), 137 mM NaCl, 0.1% Tween-20 with 5% w/v nonfat milk], subsequently incubated overnight at 4°C with primary antibody, and then for 1 h at room temperature with a second horseradish peroxidase (HRP) conjugated anti-IgG antibody. Visualization was done with a HRP-DAB chemiluminescence detection kit (Tiangen Biochem, Beijing, China). Densitometry (Molecular Dynamics, Sunnyvale, CA) was performed to quantify the band intensities by using volume/area integration.

Statistical analysis

All data were presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) with a one-tailed Student’s t-test was used for multiple comparisons among groups. A value of P<0.05 was considered statistically significant.

Results

Inhibitory effect of tumstatin 185-191 on cell proliferation

To investigate the anti-tumor activity of tumstatin 185-191 in lung cancer, A549 cells and A549/DDP cells were independently treated with tumstatin 185-191 at multiple concentrations. MTT assay revealed that tumstatin 185-191 at 24 h and then increased with the prolongation of treatment. According to the linear regression equation, the half inhibitory concentration (IC_50) of tumstatin 185-191 in A549 cells at 48 h was 73.67 µM (Figure 1A).
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Figure 1. A: Effects of Tum185-191 and cisplatin on the proliferation of A549 cells (48 h); B: Effects of Tum185-191 and cisplatin on the proliferation of A549/DDP cells (48 h).

Figure 2. Apoptosis of A549 cells treated with Tum185-191 and cisplatin for 48 h. (x 200, Arrow: apoptotic cells, Hoechst 33258 staining). A: Control group; B: 40 µM Tum185-191 group; C: 5 µM cisplatin; D: 40 µM Tum185-191 in combination with 5 µM cisplatin.

Similar to those of A549 cells, tumstatin 185-191 at low concentrations had no obvious inhibitory effect; when the tumstatin 185-191 concentration was greater than 40 µM, tumstatin 185-191 significantly inhibited the A549/DDP cells viability. According to the growth
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curve within 24-72 h, A549/DPD cells showed a rapid growth and cells at 48 h were used for further experiments. The IC_{50} of tumstatin 185-191 in A549/DPD cells at 48 h was 80.25 µM (Figure 1B).

Synergistic anti-tumor activity of tumstatin 185-191 and cisplatin

Combined use of tumstatin 185-191 and cisplatin produced a significant antitumor effect on A549 cells. Cisplatin at 1.25 µM inhibited the viability of A549 cells by 6.17%, while 1.25 µM cisplatin in combination with 20 and 40 µM tumstatin 185-191, inhibited the viability of A549 cells by 19.11% and 40.52%, respectively. Similarly, 2 µM cisplatin inhibited the viability of A549 cells by 18.67%, while 2 µM cisplatin in combination with 20 and 40 µM tumstatin 185-191 inhibited it by 39.63% and 58.86% respectively. The viability of A549 cells was inhibited by 48.75% by cisplatin at 5 µM, while cisplatin at 5 µM in combination with 20 and 40 µM tumstatin 185-191 inhibited it by 66.00% and 78.89%, respectively. According to the linear regression equation, the IC_{50} of cisplatin in A549 cells was 5.24 µM, but was 3.48 µM and 1.39 µM when 20 and 40 µM tumstatin 185-191 was present, respectively (Figure 1A).

Similar findings were also observed in A549/DDP cells. Cisplatin significantly inhibited the growth of A549/DDP cells, which became more evident in the presence of tumstatin 185-191. Moreover, the higher the tumstatin 185-191 concentration, the more obvious the inhibitory effects were. The IC_{50} of cisplatin in A549/DDP cells was 77.16 µM, but in the presence of 20 and 40 µM tumstatin 185-191, it was 57.89 µM and 26.40 µM, respectively. The RI was 1.33 in 20 µM Tum185-191 treated cells and 2.92 in 20 µM Tum185-191 treated cells (Figure 1B).

Apoptosis induced by tumstatin 185-191

In untreated cells, only a few apoptotic cells were observed. However, more apoptotic cells were observed in 40 µM Tum185-191 group
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Figure 4. Apoptosis of A549 cells treated with Tum185-191 and cisplatin for 48 h (flow cytometry). A: Control group; B: 40 µM Tum185-191 group; C: 5 µM cisplatin; D: 40 µM Tum185-191 in combination with 5 µM cisplatin.

and 5 µM cisplatin group as compared to untreated cells, and the apoptotic cells increased significantly after combined treatment (Figures 2 and 3).
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Apoptotic cells were further detected by flow cytometry. Tumstatin 185-191 significantly induced the apoptosis of A549 cells and A549/DDP cells in a dose-dependent manner.

Figure 5. Apoptosis of A549/DDP cells treated with Tum185-191 and cisplatin for 48 h (flow cytometry). A: Control group; B: 40 µM Tum185-191 group; C: 80 µM cisplatin; D: 40 µM Tum185-191 in combination with 80 µM cisplatin.
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Moreover, combined treatment with Tum185-191 and cisplatin further increased the apoptosis rate (Figures 4 and 5).

**Inhibition of Akt activation by tumstatin 185-191**

To investigate whether tumstatin 185-191 could inhibit the Akt pathway in lung cancer cells, the p-Akt at S473 (an indicator of Akt activation) was detected in A549 cells and A549/DDP cells. As shown in immunocytochemistry, tumstatin 185-191 reduced the p-Akt positive cells in a dose dependent manner (Figures 6 and 7).

Western blot assay showed tumstatin 185-191 decreased p-Akt expression in A549 cells and A549/DDP cells at doses as low as 40 µM, whereas cisplatin alone had no influence on the p-Akt expression. Combined treatment with Tumstatin 185-191 and cisplatin could not further increase p-Akt expression as compared to tumstatin 185-191 alone. Furthermore, the decrease in p-Akt expression following Tum185-191 treatment was observed as early as 15 min, and became more evident with the prolongation of treatment. This inhibition reached a peak at 30-60 min, but disappeared at 120 min (Figures 8 and 9).

**Discussion**

**Inhibitory effect of Tum185-191 on A549 cells and A549/DDP cells**

Tumstatin was believed to be a specific inhibitory factor of endothelial cells [8], but it was found later in experiments *in vitro* and animal models that Tumstatin could effectively inhibit the growth of various tumor cells such as melanoma, glioma and laryngocarcinoma, and promote apoptosis of tumor cells, exerting anticancer effects [12-16]. Our results revealed that the inhibitory effect of Tum185-191 against A549 cells was not obvious at low doses, and
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The growth of A549 cells was only significantly inhibited when the dose of Tum185-191 reached 40 µM or higher, showing a concentration dependent manner. After treatment with high-dose Tum185-191, the apoptosis rate of A549 cells increased significantly. Thevenard et al found that, in melanoma cells, an approximate inhibitory rate of 45% could be achieved when the tumstatin concentration was 20 µM [30]. Cao et al reported that the effective dose of tumstatin for colorectal cancer was far lower than that for A549 cells [32], which may be ascribed to the lower sensitivity of A549 cells to Tum185-191 as compared to melanoma cells. Our results demonstrate that Tum185-191 has anti-tumor effect against A549 cells, and may become a promising drug for the therapy of lung adenocarcinoma.

Chemotherapy resistance is a major reason for chemotherapy failure and disease aggravation in NSCLC patients. Non-sensitivity of tumor cells to chemotherapy induced apoptosis is an important mechanism under the drug resistance of cancers [33]. Cisplatin has favorable anti-tumor effects against solid tumors, and has been used as common chemotherapeutic for lung cancer. Cisplatin resistance usually refers to multi-drug resistance (MDR), i.e., the insensitivity to multiple chemotherapeutics besides cisplatin. Therefore, clinicians are often difficult to determine an alternative treatment regimen for patients with cisplatin resistance. In our study, results showed that Tum185-191 exerted similar anti-tumor effects on drug resistant A549/DDP cells and common A549 cells. Our results showed that the anti-tumor effect of Tum185-191 was independent of drug resistance for A549 lung adenocarcinoma cells, and there is no cross resistance between cisplatin and Tum185-191 for A549/DDP cells. It also suggests that the mechanism of anti-tumor activity of cisplatin is different from that of Tum185-191. Therefore, patients

Figure 7. p-Akt expression of in A549/DDP cells treated with Tum185-191 at different concentrations for 30 min (×200, immunocytochemistry, positive cells having brown granules). A: Blank control group; B: Control group; C: 20 µM Tum185-191 group; D: 40 µM Tum185-191 group.
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Effects of Tum185-191 on Akt activation

It has been confirmed that Akt signal pathway participates in the onset and development of lung cancer. Akt activity increases in endothelial cells in long term smokers, high expression of p-Akt is observed in bronchial endothelial cells in malignant and precancorous lesions, and it is believed that Akt signal pathway is correlated with prognosis of patients with lung cancer [34-36]. Therefore, Akt signal pathway may become a potential target for the treatment and prevention of lung cancer.

In this study, the effects of Tum185-191 on p-Akt expression were investigated in A549 cells and A549/DDP cells, aiming to explore the potential mechanism of anti-tumor effect of Tum185-191. Results indicated that Tum185-191 could significantly inhibit the expression of p-Akt. It has been found that Akt signal pathway can inactivate some components of apoptosis cascade, such as caspase-9, forehead and proapoptotic Bad, thus blocking apoptosis. In addition, Akt signal pathway may also affect the cell cycle in cyclin D1 and p27Kip1 dependent manner [27, 37, 38]. As shown in our study, high dose Tum185-191 significantly inhibited the proliferation of A549 cells and A549/DDP cells and promoted their apoptosis, which may be ascribed to the inhibition of p-Akt expression.

However, cisplatin had no influence on the p-Akt expression, which suggests that the anti-tumor activity of cisplatin has no relationship with Akt signaling pathway. In addition, the p-Akt expression was comparable between drug resistant and common A549 cells. This suggests that the drug resistance of A549 cells is not ascribed to the Akt signaling pathway.

Moreover, the decrease in p-Akt expression was observed at as early as 15 min after Tum185-191 treatment, and the inhibition became more obvious with the prolongation of treatment, but it disappeared at 120 min. This suggests that the change in p-Akt expression is an early event in A549 cells and A549/DDP cells after Tum185-191 treatment, and whether Akt signaling pathway is related to the anti-

with cisplatin resistant lung adenocarcinoma may also benefit from Tum185-191 therapy.

**Figure 8.** Western blot assay of p-Akt expression in A549 cells. A: A549 cells treated with 40 µM Tum185-191; B: A549 cells treated with Tum185-191 for 30 and 60 min; C: A549 cells treated with Tum185-191 and cisplatin for 30 min.
tumor effect of Tumstatin 185-191 and what is the mechanism of anti-tumor effect of Tumstatin 185-191 at late stage are required to be further elucidated.

Synergistic effects of Tumstatin 185-191 and cisplatin

NSCLC shows a poor sensitivity to multiple chemotherapeutics. In some patients sensitive to chemotherapeutics, the secondary drug resistance will also be induced during the long-term chemotherapy. Chemotherapeutics at a high-dose not only increase the toxic and adverse effects, but are easily and quickly to induce drug resistance [33]. Therefore, it is necessary to develop chemotherapy sensitizers and reversal agents of drug resistance, which has been a hot topic in studies on tumors [39].

In the present study, for A549 cells, the IC_{50} was 5.24 µM for cisplatin alone, and 3.48 µM and 1.39 µM in the presence of 20 µM and 40 µM Tumstatin 185-191, respectively. Moreover, the inhibitory effects on cell proliferation and inductive effect on cell apoptosis were significantly improved after combined treatment. For A549/DDP cells, in the presence of 20 µM and 40 µM Tumstatin 185-191, the IC_{50} of cisplatin reduced to 57.97 µM and 26.40 µM, respectively. These results suggest that, anti-tumor effect against A549 cells and A549/DDP cells is observed after combined treatment, and Tumstatin 185-191 is able to lower the effective dose of cisplatin. Because chemotherapeutics are mostly cytotoxic, and different chemotherapeutics have distinct toxic reactions, the dose and course of chemotherapeutics in the combined therapy are restricted, which significantly limits the anti-
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The tumor effect of these drugs. Unlike traditional chemotherapeutics, Tumstatin is an endogenous peptide without cytotoxicity. Therefore, combined use of Tum185-191 and cisplatin may be tolerable. The results in this study will provide a new idea for combination of cytotoxic drugs and some signal pathway inhibitors in clinical practice.

There were limitations in this study. Akt inhibitor was not used in this study, and thus we could not confirm whether the anti-tumor effect of tumstatin 185-191 is related to Akt signaling pathway. In addition, the anti-tumor effect of tumstatin 185-191d is required to be validated in animal models.

In summary, our findings demonstrate that Tum185-191 can exert anti-tumor effect by down-regulating p-Akt expression in A549 cells and A549/DDP cells, and Tum185-191 may also increase the sensitivity of A549 cells to cisplatin, which partially reverses the resistance of A549/DDP cells to cisplatin. These findings suggest that Tum185-191 alone or in combination with cisplatin may provide a new strategy for the therapy of lung adenocarcinoma.

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Disclosure of conflict of interest

None.

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