Abstract. The combined use of currently used anticancer genotoxins with other drugs is a therapeutic tool for potentially increasing the efficacy of the genotoxins. In the present study, the effects of a RasGAP-derived peptide, P110 (RasGAP<sub>301-316</sub>), designed to target Ras-GTPase activating protein SH3 domain-binding proteins (G3BPs), on the chemotherapeutic agent, cisplatin (DDP), were examined. P110 was demonstrated to enhance the effect of DDP in vitro and in vivo. The results indicate that P110 significantly increased the DDP-induced apoptosis in SGC-7901, HCT-116, HeLa and A-549 cells. Furthermore, P110 combined with DDP significantly suppressed the growth of C26 xenograft tumors in a dose-dependent manner. This synergistic effect may be associated with DDP-induced apoptosis, involving the down-regulation of Bcl-2 and the upregulation of Bax, cytochrome c and caspase-3. The results of the present study indicate that P110, in combination with chemotherapeutics, is likely to represent a potential therapeutic strategy for cancer.

Introduction

In recent years, the human disease spectrum has changed with rapid economic development, and a number of infectious diseases are now controlled effectively. However, cancer remains a major threat to human health. Cancer is the leading cause of mortality in economically developed countries and the second leading cause of mortality in developing countries (1). Cervical carcinoma and gastric, colon and lung cancer are common malignant tumors (2-5) and the incidence and mortality of these types of cancer have increased year by year. Therefore, cancer has become the most serious public health issue worldwide.

Among therapeutic strategies for cancer, radical surgery is the most effective method, however, chemotherapy plays an integral role in patients with advanced tumors by reducing the mortality of cancer. Cisplatin (DDP) is a commonly used chemotherapy agent that has a broad spectrum of antitumor effects, high antitumor activity and a curative effect on cancer. However, the severe side-effects associated with DDP, including bone marrow suppression, neurotoxicity and gastrointestinal reactions (6-9), limit its therapeutic application. Therefore, the identification of anticancer agents with high efficacy and low toxicity, as well as combination therapies, is an important area of study.

Previous studies have reported that overexpression of Ras-GTPase activating protein SH3 domain-binding proteins (G3BPs) in the cytoplasm is closely associated with tumorigenesis and metastasis in colorectal carcinoma and gastric, lung and breast cancer (10,11). Cui et al (12) designed and screened a series of peptides which specifically bind G3BPs. These peptides have been demonstrated to increase the sensitivity of tumor cells to DDP and other anticancer drugs, thus inducing apoptosis in tumor cells. However, this sensitizing effect does not occur in normal cells. P110 (RasGAP<sub>301-316</sub>) is one of the peptides identified in the study. In the present study, the effects of P110 + DDP against various types of cancer cells were examined in vitro and in vivo.

Materials and methods

Peptide synthesis. Peptide P110 (13) (amino acid sequence, FLKGDMFIVHNELEDG) was synthesized at Wuhan KatyGen Pharmaceuticals, Inc. (Wuhan, China). The molecular weight was 4,660.41 Da. It was synthesized by Fmoc technology, purified by HPLC, tested by mass spectrometry and stored at -20°C.

Cell lines and cell culture. Gastric cancer cell line, SGC-7901; colon cancer cell line, HCT-116; cervical carcinoma cell line,
HeLa: lung cancer cell line, A-549: and human umbilical vein endothelial cells (HUVECs) were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Cells were maintained in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal calf serum (Hangzhou, China) at 37°C and 5% CO₂. According to the pre-test (14), the IC₅₀ of P110 in HeLa cells was 20 μM. In this study, the concentration of P110 when combined with DDP was 20 μM. The cells were divided into 4 groups: DDP, P110, P110 + DDP and control. The ultimate concentration of DDP in the cancer cell analyses was 1.1, 3.3, 10, 30 and 90 μM, while in the HUVEC analyses, it was 10, 20, 50, 100 and 150 μM.

Cell cytotoxicity assay. Cell viability following treatment with various concentrations of P110 and DDP was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays performed in triplicate. Briefly, logarithmic growth phase cells of SGC-7901, HCT-116, HeLa, A-549 and HUVEC were selected and digested with 0.25% trypsin into a single cell suspension, adjusting the cell density to 1.8×10⁴ cells/ml. Cell suspension (200 μl) was added to 96-well plates and cultured for 24 h. DDP and P110 + DDP at various indicated concentrations were added. RPMI-1640 was added as a control. Following 24-h incubation, the MTT solution was removed and 200 μl MTT was added to each well and incubated for 4 h. After removal of the MTT solution, 200 μl DMSO was added to each well. Next, the optical density at 570 nm was determined using an iMark Microplate Absorbance reader (Bio-Rad, Hercules, CA, USA). Cell proliferation inhibitory rates were calculated as follows: Inhibition rate (%) = (1 - A₅₇₀ experimental well/A₅₇₀ control well) x 100.

Annexin V/propidium iodide (PI) staining. To quantify the percentage of cells undergoing apoptosis, the Annexin V-FITC kit (Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China) was used according to the manufacturer's instructions. Briefly, HCT-116 cells were incubated for 24 and 48 h with P110 (20 μM) and DDP (10 μM) alone or in combination. Next, the cells were washed twice with cold PBS and resuspended in binding buffer at a concentration of 1×10⁶ cells/ml. Following incubation, 100 μl solution was transferred to a 5-ml culture tube and 5 μl Annexin V-FITC and 10 μl PI were added. The tube was gently centrifuged and incubated for 15 min at room temperature in the dark. At the end of incubation, 400 μl binding buffer was added and the cells were analyzed immediately by flow cytometry (Beckman Coulter, Inc., Fullerton, CA, USA). Flow cytometry was performed using CellQuest software.

Acute toxicity test in mice. Male BALB/c mice, 18-22 g, were obtained from Beijing Vital River Laboratories (Beijing, China). All mice were bred in a specific pathogen-free environment. Four groups of male mice (n=5/group) were treated with P110 in graded doses (125, 250, 500, 1,000 mg/kg body weight) by intraperitoneal injection. Mortality and other physical signs of toxicity, including skin changes, respiratory movements and weight, were observed over 30 days following drug administration and the LD₅₀ was determined.

Xenograft tumors in mice. C26 colon cancer tumor-bearing mice were purchased from the Institute of Biotechnology Research, Chinese Academy of Medical Sciences (Beijing, China). Tissues derived from the C26 colon cancer tumor-bearing mice were processed into single cell suspensions using 0.85% normal saline (1:10). Male BALB/c mice were injected with 0.2 ml tumor cells subcutaneously in the right armpit region and were randomly divided into 8 groups (n=10/group): saline control; DDP 1 mg/kg/every other day; P110 25 mg/kg/day; P110 50 mg/kg/day; P110 100 mg/kg/day; P110 (25 mg/kg/day) + DDP; P110 (50 mg/kg/day) + DDP; and P110 (100 mg/kg/day) + DDP. DDP and P110 were dissolved in 0.85% normal saline. The following day, mice were injected intraperitoneally with the specified doses. On day 11, all mice were sacrificed and the tumor xenografts were removed and weighed. Tumor growth inhibitory rates were calculated using the following formula: Inhibition rate (%) = (1 - mean test tumor weight/mean control tumor weight) x 100. The study was approved by the Institutional Review Board of Renmin Hospital of Wuhan University.

Detection of tumor apoptosis genes. Rabbit anti-Bax and anti-Bcl-2 polyclonal antibodies, anti-cytochrome c (cyc c) and anti-caspase-3 were purchased from Beijing Zhongsan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). The streptavidin peroxidase (SP) anti-rabbit/mouse Universal Immunohistochemistry kit was purchased from Dako (Carpinteria, CA, USA). The removed tumors were paraffinized and cut into sections of 4-5 μm. Expression of Bax, Bcl-2, cyc c and caspase-3 was detected by SP immunohistochemistry. Bax-,- Bcl-2,-, cyc c- and caspase-3-positive protein expression in the cytoplasm appeared brown. Negative samples revealed no brown staining in the cytoplasm and nucleus and light blue staining of the nucleus.

Combined effect of the two drugs. The median effect method of Chou and Talalay (15,16) was used to analyze the interaction between DDP and P110. The drug combination index (CI) was calculated. CI<1 indicated synergy, CI=1 indicated additivity and CI>1 indicated antagonism.

Statistical analysis. Results were analyzed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) and one-way ANOVA (multiple comparisons) and t-tests (two groups comparisons) were performed accordingly. P<0.05 was considered to indicate a statistically significant difference. All data are presented as the mean ± SD.

Results

Effect of single drug exposure on the growth of cancer cells. Cancer cells were treated with various concentrations of P110 or DDP for 24 h and cell viability was determined using the MTT assay (Fig. 1A-D). The growth of SGC-7901, HCT-116, HeLa and A-549 cells was found to be significantly inhibited in a concentration-dependent manner in vitro (P<0.05). The growth inhibitory effect of P110 on the tumor cells was slightly higher than that of DDP, but the difference was not determined to be statistically significant (P>0.05).

Combined effect of P110 and DDP on the growth of cancer cells. When the combined effects were studied, the cells
were exposed for 24 h to the two drugs concurrently. At DDP concentrations of 10, 30 and 90 µM, the inhibitory rate of P110 + DDP was higher than that of DDP or P110 alone and the difference was identified to be statistically significant (P<0.05; Fig. 1A-D). Of note, the inhibitory effect of the combination treatment on the proliferation of HCT-116 and HeLa cells plateaued at DDP concentrations of 10, 30 and 90 µM and the differences between the effects at these concentrations were not observed to be statistically significant (P>0.05; Fig. 1B and D). At the same concentration, the inhibitory effect of the combination treatment in HeLa cells was higher than that in the other cell types (P<0.05; Fig. 1E).

**Growth inhibitory effect on HUVECs.** When the DPP concentration was 10, 20, 50, 100 and 150 µM, the inhibitory rate was 17.2±0.8, 24.0±0.1, 45.4±0.1, 53.5±0.3 and 58.3±0.5%, respectively, indicating that the inhibitory effect was concentration-dependent. The differences between the rates for P110 + DDP and DDP were not identified to be statistically significant, except at 150 µM DDP (P<0.05; Fig. 1F).

**Apoptosis induced by P110 and DDP.** Apoptosis induced by P110 and DDP was confirmed using Annexin V/PI staining to detect the externalization of phosphatidylserine on the cell membrane. As revealed in Fig. 2, the proportion of Annexin V+/PI− cells increased progressively in HCT-116 cells incubated at low concentrations of P110 (20 µM) and DDP (10 µM) for 48 h. P110 and DDP alone were found to significantly promote apoptosis compared with the control (P<0.05) and the combined effect was stronger than the effect of DDP and P110 alone (P<0.05). (A) Control; (B) P110; (C) DDP; (D) P110 + DDP. DDP, cisplatin; PI, propidium iodide.

**Acute toxicity of P110 in vivo.** To evaluate the toxicity of P110 in BALB/c mice, P110 in graded doses (125, 250, 500 and 1,000 mg/kg) was administered to four groups of male mice by intraperitoneal injection. At day 30, all mice were alive. Skin changes, respiratory movements and the weights of the mice in each group were not observed to be significantly different. The LD₅₀ of P110 was calculated to be >1,000 mg/kg.
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P110 + DDP inhibits colon cancer cells in vivo. As demonstrated in Fig. 3, mice treated with P110 (25, 50 and 100 mg/kg) + DDP (1 mg/kg) developed significantly smaller tumors than mice treated with 0.85% normal saline (P<0.05). The tumor growth inhibitory rates in the P110 + DDP group were 23.0, 30.4 and 34.2%, respectively and 22.7% in the group treated with DDP alone. Compared with control, P110 alone had no effect on tumor growth (P>0.05; Table I).

Combined effect of the two drugs. The CIs of P110 (at doses of 25, 50 and 100 mg/kg) + DDP (1 mg/kg) were 0.99, 0.90 and 0.88, respectively. All CIs were <1. These results indicate that the interaction between P110 and DDP is synergistic.

Expression of tumor apoptosis genes in xenograft tumors. Bax, cyt c and caspase-3 expression levels were increased in the xenografts of the P110 + DDP group compared with those in the DDP group (Fig. 4A-F), whereas the Bcl-2 expression level was higher in the xenografts of the DDP-treated group than in the P110 + DDP group (Fig. 4G and H).

Discussion

At present, although antitumor strategies are continually updated and improved, chemotherapy remains the most important method of cancer treatment. DDP is a genotoxic anticancer drug which functions by inducing DNA damage and has been successfully used in tumor treatment for several decades. However, genotoxic drugs are non-selective and require administration at high doses, leading to toxicity in
normal tissue. The issue of toxicity remains a major obstacle for the development of successful cancer chemotherapies (6,7) and the identification of methods for increasing tumor specificity and reducing the side-effects of antitumor drugs is an extremely important area of cancer research.

G3BP is a specific binding protein which binds the SH3 domain of RasGAP via the nuclear transport factor 2 (NTF2)-like domain. Gutard et al (17) reported that G3BP is expressed at high levels in a number of types of human tumor cells. In addition, previous studies have confirmed that G3BP is overexpressed in colon, gastric, lung and breast cancer (18-20). The anti-G3BP antibody selectively kills cancer cells without affecting normal tissues (21). G3BPs have also been found to be closely associated with a negative regulatory protein of RasGAP in the Ras signaling pathway and cell proliferation. G3BPs play a role in signal transduction via an N-terminal NTF2-like domain binding to an N-terminal SH3 domain in RasGAP (22,23). The G3BP protein family is closely associated with cancer and specific fragments of the RasGAP SH3 domain may exhibit a therapeutic effect by combining with G3BPs. Therefore, studies of the protein structure of G3BP and the relationship between Ras-GAP and G3BP are important in the development of methods for screening G3BP-overexpressing cancers and the design of targeted antitumor drugs.

In 2004, Michod et al (24) reported that a specific peptide derived from the RasGAP protein increased the sensitivity of tumor cells to DDP and other anticancer drugs and thus induced the apoptosis of tumor cells. This sensitizing effect did not occur in normal cells. Following this, Cui et al (12) used molecular dynamics simulation to study the potential interaction between G3BP and RasGAP and employed protein homology modeling to determine the three-dimensional structure of the NTF2 domain of G3BP. Protein docking studies were performed to determine the structure of the RasGAP SH3 domain based on the Monte Carlo method and three possible protein-protein interaction models were obtained. Molecular dynamics simulations and binding free energy calculations were performed on the three models to reveal the most stable model of binding. Energy decomposition calculations in each model revealed that the Ile308, Val309, His310, Asn311 and Thr321 residues of the SH3 domain of RasGAP form marked interactions with the Asp28, Met29, His31 and Arg32 residues of the NTF2-like domain of G3BP. Following this, two novel antitumor peptides, P110 and P109, were synthesized and the effects of the two sequences alone and in combination with DDP were demonstrated to be effective for the inhibition of tumor cells and its inhibition rate in HUVECs was lower than that of high doses of DDP and of other concentrations of P110 + DDP. Thus, P10 is able to enhance the antitumor effect of DDP, thereby reducing the dose of DDP required for efficacy and alleviating toxicity in normal tissues to result in an improved clinical chemotherapeutic effect and quality of life.

In conclusion, P110 sensitizes cancer cells to the antitumor effect of DDP, enabling them to be killed selectively, which indicates its potential clinical value.

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References


