

# Inhibition of the met receptor tyrosine kinase signaling enhances the chemosensitivity of glioma cell lines to CDDP through activation of p38 MAPK pathway

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## Abstract

The Met receptor tyrosine kinase is known to be overexpressed in many solid tumors and plays a crucial role in tumor invasive growth and metastasis. In this study, we showed that hepatocyte growth factor-induced Met activation as well as Met-dependent downstream signaling of AKT and p44/42 mitogen-activated protein kinase (MAPK) could be efficiently blocked by TAT-coupled carboxyl-terminal tail peptide of Met receptor (TCTP), and inactivation of Met signaling significantly enhanced the sensitivity of T98G and U251 glioma cells to *cis*-diaminedichloroplatinum (CDDP, cisplatin). However, neither phosphoinositide 3-kinase/AKT inhibitor LY294002 nor p44/42 MAPK inhibitor PD98059 alone or combined could imitate the effect of TCTP on chemosensitivity enhancement of T98G cells to CDDP, indicating that Met-dependent inactivation of AKT and p44/42 MAPK signaling was not the main cause for the increased chemosensitivity to CDDP. Further studies revealed that TCTP significantly activated p38 MAPK in T98G and U251 cell lines. Activation of p38 MAPK by sorbitol pretreatment resembled the sensitization effects, whereas inhibition of p38 MAPK activation by its inhibitor SB202190 counteracted the sensitization effects induced by TCTP. Therefore, p38 MAPK activation was one of the major causes for the increased chemosensitivity to CDDP induced by

Met inactivation. Taken together, the study indicated that Met receptor played an important role in regulating cell response to chemotherapy and suggested that inhibition of Met signaling could be used in combination with other chemotherapeutic regimens in treatment of tumor patients. [Mol Cancer Ther 2009;8(5):1126–36]

## Introduction

Chemotherapy has been one of the major regimens in treating most solid tumors. Despite its excellent anticancer activities, the clinical applications of chemotherapeutics are often limited by their undesirable side effects such as severe nephrotoxicity and hepatotoxicity caused by *cis*-diaminedichloroplatinum (CDDP, cisplatin; refs. 1, 2). Moreover, malignant cells are either intrinsically resistant to chemotherapy or able to obtain resistance during the course of treatment (3, 4). One of the most feasible methods to overcome these clinical obstacles of chemotherapy is increasing the chemosensitivity of tumor cells. Although major efforts have been on the chemosensitivity enhancement, the underlying molecular mechanism is not well understood. Therefore, further investigation is needed for the future improvement of effective chemotherapy.

Currently, the receptor tyrosine kinases (RTK) have become the important therapeutic targets in a variety of malignancies (5). They are not only the key regulators of normal cellular processes but also play a critical role in the development and progression of many types of cancer. Overexpression and abnormal activation of RTKs are usually associated with increased tumor growth, metastasis, resistance to chemotherapeutic agents, and poor prognosis. Therefore, a series of strategies, including down-regulation of their expression, blockade of their downstream signaling, and abrogation of their activation using antisense oligonucleotides, monoclonal antibodies, or inhibitors, have been developed to block the function of the RTKs and improve the overall clinical outcomes. In fact, designing RTK inhibitors is considered to be an emerging new paradigm in the development of cancer therapies (6, 7).

The Met RTK is a high-affinity receptor of hepatocyte growth factor (HGF; refs. 8, 9). Both Met receptor and HGF are expressed in numerous tissues, although their expressions are confined predominantly to cells of epithelial and mesenchymal origin, respectively (10). They play significant roles in cellular physiology such as motogenesis or morphogenesis (11–13). Met receptor is often overexpressed and activated in several human solid tumors including brain tumors, and the expression levels of Met receptor are frequently correlated with tumor grade and tumor blood vessel density (14, 15). Met activation induced by HGF affects

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multiple downstream signaling pathways including phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinases (MAPK), which have been identified as the important oncogenic pathways in tumors (16). The PI3K/AKT pathway mediates cell survival and growth (17), whereas the Ras/MAPK pathway correlates with cell differentiation, proliferation, and antiapoptosis (18).

Various studies have shown that inactivation of RTKs such as ErbB2 (19) or other important functioning proteins such as Grb7 (20) can significantly enhance the chemosensitivity. Here, a novel peptide-based sensitizer specifically targeting the Met inactivation was supposed to be investigated. There are at least three ways to block the Met activation: antagonism between ligand/receptor interaction, inhibition of tyrosine kinases catalytic activity, and blockade of intracellular receptor/effector interactions. Previous study (21) showed that a peptide derived from the carboxyl-terminal tail of the Met receptor was able to inhibit the kinase activity and invasive growth in normal or transformed epithelial cells *in vitro*. This carboxyl-terminal tail of the Met receptor was coupled to a cell-penetrating peptide (CPP) called TAT fragment [TAT-coupled carboxyl-terminal tail peptide of Met receptor (TCTP)], and its potential effect on CDDP sensitization as well as its possible mechanism in glioma cell lines *in vitro* were studied in detail.

## Materials and Methods

### Reagents

Peptides were synthesized by HD Biosciences and their sequences were TCTP (YGRKKRRQRRR-IGEHYVHVNA-TYVNVKCV) and TAT (YGRKKRRQRRR). MEM/nonessential amino acids, MEM/Eagle's balanced salt solution, and glucose (400 mmol/L) were obtained from the cell centre of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. RPMI 1640, DMEM, fetal bovine serum, and 0.25% trypsin were purchased from Hyclone. Protease inhibitor mixture was from Roche. Antibodies to P-Met (Tyr<sup>1234</sup>/Tyr<sup>1235</sup>), P-AKT (Ser<sup>473</sup>), AKT, P-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>), p38 MAPK, P-p44/42 MAPK, p44/42 MAPK, P-SAPK/JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>), and SAPK/JNK and the PI3K/AKT inhibitor LY294002 were from Cell Signaling Technologies. Antibody to total Met was kindly provided by Dr. Zhao Min (Chinese National Human Genome Center). Horseradish peroxidase-conjugated secondary antibodies were from Amersham. HGF were purchased from R&D Systems. CDDP was from Mayne Pharma. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were obtained from Ameresco. The p44/42 MAPK inhibitor PD98059 and the p38 MAPK inhibitor SB202190 were from Calbiochem Merck Bioscience and Sigma, respectively.

### Cell Culture

Five human glioma cell lines were evaluated: U251, T98G, A172, U87MG, and CCF-STGG1. All cell lines were kindly provided by Dr. Xiaozhong Peng (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College). U251, A172, T98G/U87MG, and CCF-STGG1 cell lines were grown in MEM/Eagle's bal-

anced salt solution, DMEM, MEM/nonessential amino acids, and RPMI 1640 supplemented with 10% fetal bovine serum, respectively. All cells were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% room air.

### Western Blot Analysis

Cells were pretreated as described for the indicated time and washed three times in PBS and lysed by adding 1× SDS sample buffer [62.5 mmol/L Tris-HCl (pH 6.8 at 25°C), 2% (w/v) SDS, 10% glycerol, 50 mmol/L DTT, 0.01% (w/v) bromophenol containing 1× protease inhibitors]. Immediately, cells were scraped off the plate and transferred the extract to a microcentrifuge tube and kept on ice for 30 min and then sonicated for 15 s to shear DNA and reduce sample viscosity. Samples were heat to 95°C to 100°C for 5 min and cooled on ice and then centrifuged at 13,000 × g at 4°C for 10 min. Supernatants (10 μL) were loaded for SDS-PAGE and then carefully transferred to the polyvinylidene difluoride membranes. The membranes were blocked in 5% bovine serum albumin in TBST [500 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 0.1% Tween 20] for 1 h at room temperature and incubated overnight at 4°C with the appropriate antibodies followed by secondary antibody linked to horseradish peroxidase (also in 5% bovine serum albumin in TBST) and then developed by the enhanced chemiluminescence detection system on the film.

### MTT Cytotoxicity Assay

Cells were plated at  $1.5 \times 10^3$  to  $2.5 \times 10^3$  per well in 96-well culture plates, incubated for 24 h, and pretreated by different stimuli for the indicated time. PBS or DMSO only was used as control. At the end of the time point, MTT was added to each well at the final concentration of 0.5 mg/mL and incubated for 4 h at 37°C. The medium was then removed, and 100 μL DMSO was added to each well. Absorbance at 570 nm was measured by a microplate reader. Mean cell viability was calculated by the ratio of absorbance units of treated cell samples to the mean absorbance units of the control cell samples. All the experiments were carried out in triplicate and each experiment was repeated at least three times.

### Flow Cytometry Analysis for Cell Apoptosis

Apoptosis and cell death were assessed by flow cytometry using FITC-labeled Annexin V and propidium iodide (PI) apoptosis detection kit (BD Biosciences Pharmingen). Cells were seeded in 6-well plates at the appropriate densities and subsequently preincubated by the different peptides or agents for the indicated periods. The fraction of apoptosis cells was determined by the Annexin V-FITC/PI detection kit according to the instructions of the manufacturer. Briefly, all cells were collected after trypsinization and washed by cold 1× PBS for two times and then resuspended in 100 μL binding buffer. Annexin V-FITC (5 μL) and PI (5 μL) were added to the cell suspensions followed by incubation at room temperature in the dark for 15 min. The cell suspensions were diluted with 400 μL binding buffer and immediately analyzed with flow cytometry. In total, 10,000 cells were analyzed. The experiment was carried out at least three times.

## Results

### TCTP Blocked HGF-Induced Met Autophosphorylation and Inhibited Met-Dependent Downstream Signaling of AKT and p44/42 MAPK in Glioma Cell Lines

Activation of Met receptor by HGF results in the autophosphorylation of specific tyrosine residues located in both the kinase activation loop (Tyr<sup>1234</sup>-Tyr<sup>1235</sup>) and the carboxyl-terminal tail (Tyr<sup>1349</sup>-Tyr<sup>1356</sup>; refs. 8, 22, 23). These specific tyrosine phosphorylation sites regulate kinase activity and act as binding sites for cellular signaling proteins, respectively. The expression level and activation ability of Met receptor are altered during tumorigenesis. In this study, the HGF/Met pathway of five glioma cell lines were tested and three cell lines, which are of different responses to HGF stimulation, were selected: T98G (moderately sensitive to HGF stimulation), U251 (highly sensitive to HGF stimulation), and CCF-STGG1 (no response to HGF stimulation; data not shown). The results confirmed that TCTP efficiently blocked HGF-induced Met autophosphorylation in glioma cell lines of T98G and U251, whereas the control peptide TAT did not (Fig. 1A). Dose-response experiments showed that the inhibitory potency was dependent on the concentration of TCTP, indicating that the effect was specifically caused by TCTP (Fig. 1B). Time course assay showed that the blockage lasted at least 24 h in T98G cells, with rarely visible reversible recovery (Fig. 1C).

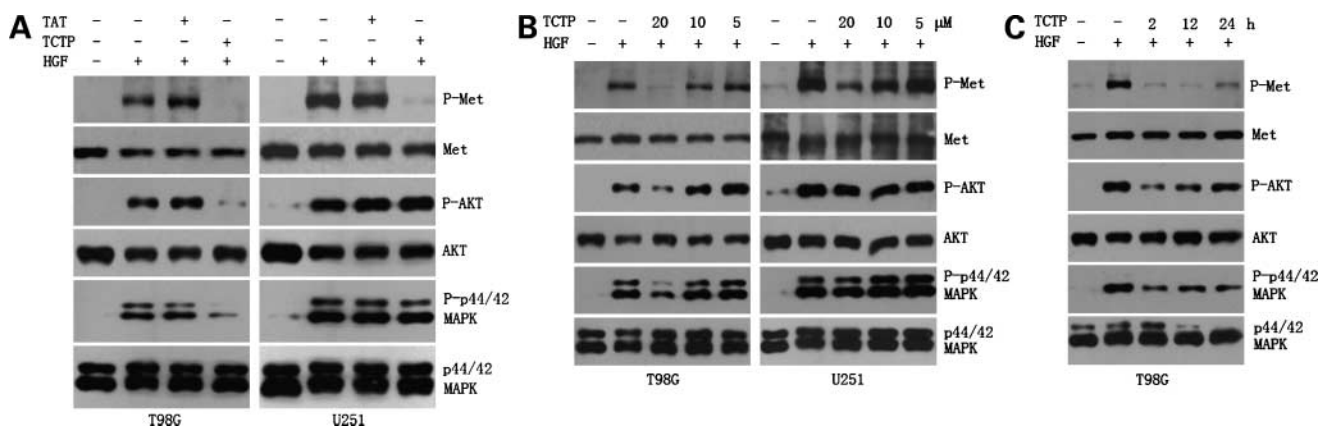
The HGF/Met pair triggers invasive growth by activating a cascade of downstream signaling events. After phosphorylation, Met binds and phosphorylates the multiadaptor protein like Gab1, which in turn recruits and activates several SH2-containing effectors. The signal is then transmitted to the nucleus via activation of various pathways including the MAPK cascade and PI3K/AKT pathway (16). The re-

sults of this study showed that Met-dependent downstream signaling of AKT and p44/42 MAPK was also impaired by TCTP in T98G (Fig. 1A). TCTP led to the inhibition of HGF-induced Met-dependent AKT and p44/42 MAPK pathway activation in a dose- and time-dependent manner in T98G cell line (Fig. 1B and C), but the effect was also not significant in U251 cell line (Fig. 1A and B). Therefore, Met inactivation induced by TCTP also interrupted the downstream signaling initiated by HGF stimulation.

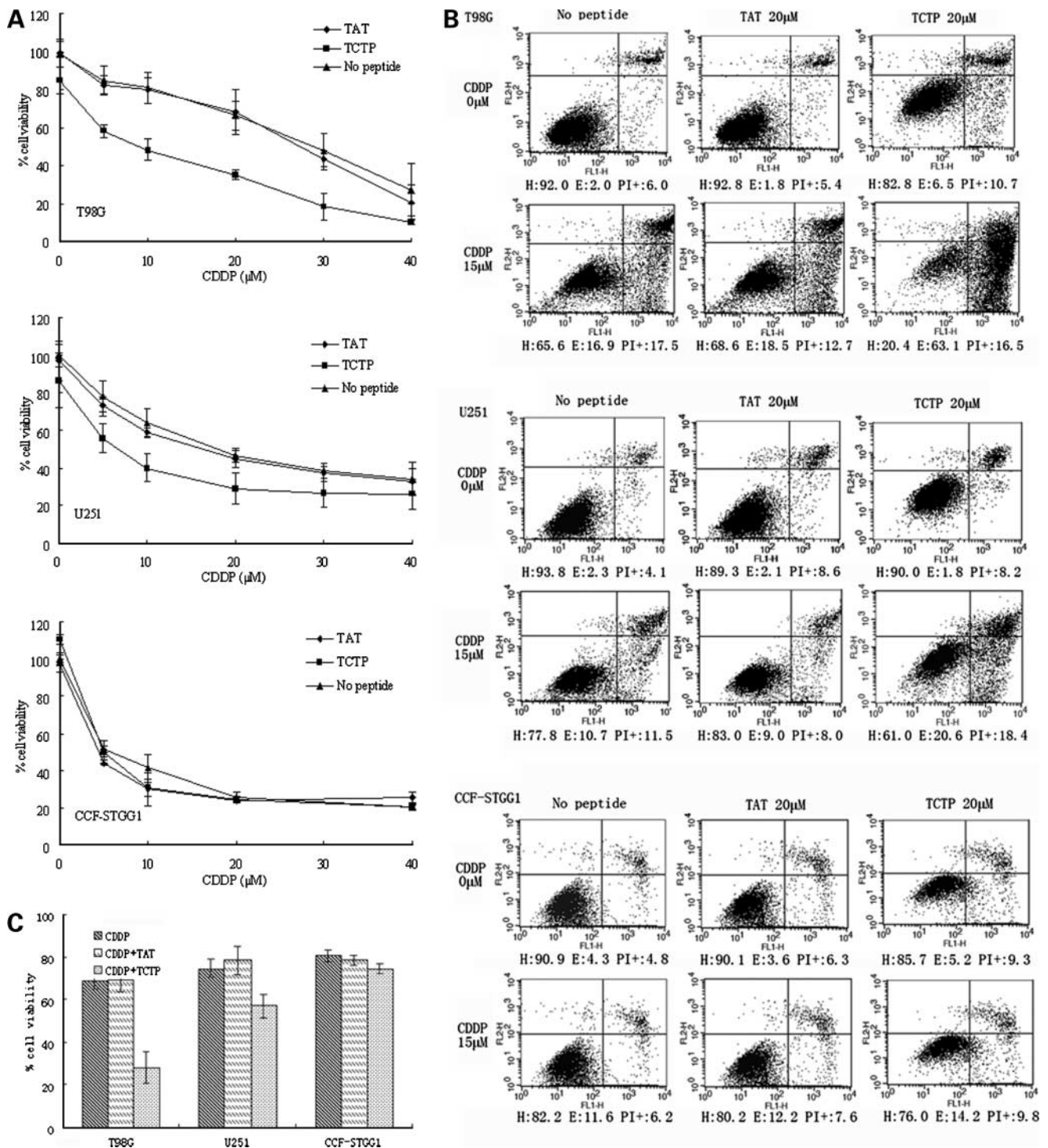
### Inhibition of Met Signaling by TCTP Increased the Apoptotic Response to CDDP in T98G and U251 Cell Lines

RTKs are among the most critical targets for tumor therapy, and studies have been employed to inactivate RTKs as an effective strategy to enhance the chemosensitivity (19, 20). Met overexpression and subsequent activation is correlated with tumor invasive growth, so it was tested that whether the sensitivity of Met-expressing tumor cells toward chemotherapeutic drugs could be modulated by the Met inhibition. TCTP treatment alone resulted in either slight (<10% in CCF-STGG1) or moderate (~10-15% in T98G and U251) cell viability changes *in vitro* (Fig. 2). However, when treated with different chemotherapeutic agents in addition to TCTP, cells responded differently. TCTP significantly increased the cell apoptosis induced by CDDP and, to a much less extent, by Taxol, doxorubicin, etoposide, and methotrexate in T98G or U251 cell lines (data not shown).

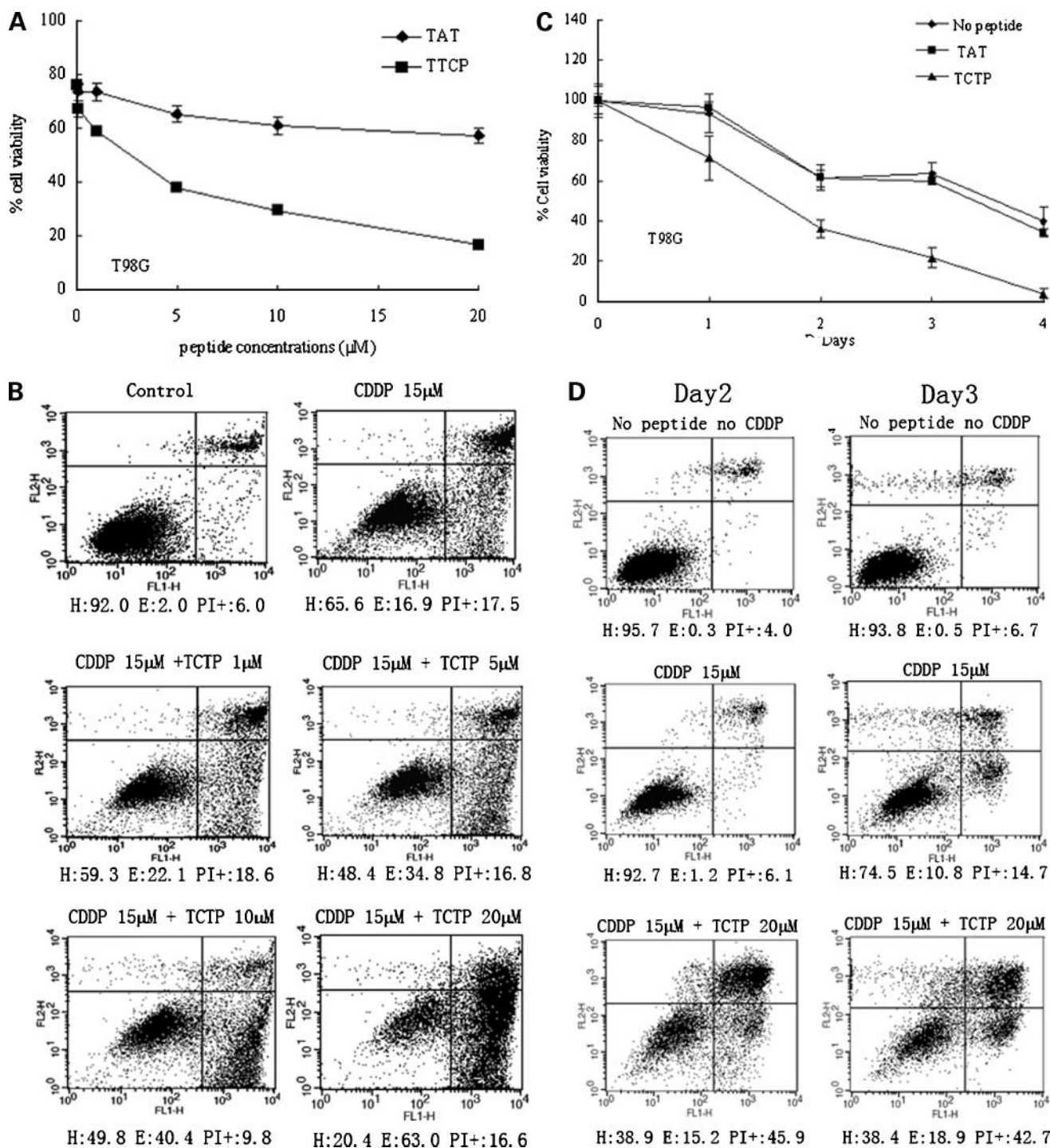
To further investigate TCTP-induced chemosensitivity enhancement to CDDP, we first used MTT assay to evaluate the effect *in vitro*. A synergistic cytotoxic effect was observed when both T98G and U251 cells were cotreated with 20  $\mu\text{mol/L}$  TCTP and different concentrations of CDDP ( $P < 0.001$ ; Fig. 2A). Exposure 20  $\mu\text{mol/L}$  TCTP and



**Figure 1.** Inhibition of HGF-induced Met autophosphorylation and Met-dependent downstream signaling of AKT and p44/42 MAPK pathway activation by TCTP. **A**, effect of TCTP on HGF-induced Met phosphorylation and Met-dependent downstream signaling of AKT and p44/42 MAPK in T98G and U251 cell lines. Cells were serum starved for 24 h and treated with the indicated peptides at a final concentration of 20  $\mu\text{mol/L}$ . Two hours later, cells were treated with 40 ng/mL recombinant HGF for 15 min in the presence of indicated peptides and then lysed. Phosphorylated and total proteins were evaluated by Western blotting with the corresponding antibody. **B**, dose-response activity of TCTP on HGF-induced Met phosphorylation and Met-dependent downstream signaling of AKT and p44/42 MAPK in T98G and U251 cell lines. Serum-starved T98G and U251 cells were treated with TCTP at indicated concentrations for 2 h and then stimulated with 40 ng/mL HGF for 15 min in the presence of indicated peptides. Phosphorylation and total proteins were assessed as described in **A**. **C**, time course activity of TCTP on HGF-induced Met phosphorylation and Met-dependent downstream signaling of AKT and p44/42 MAPK activation. Serum-starved cells were treated with 20  $\mu\text{mol/L}$  TCTP. At the indicated time points, cells were stimulated with 40 ng/mL HGF for 15 min in the presence of indicated peptides and then lysed. Phosphorylation and total proteins were assessed as described above. Representative of three independent experiments.



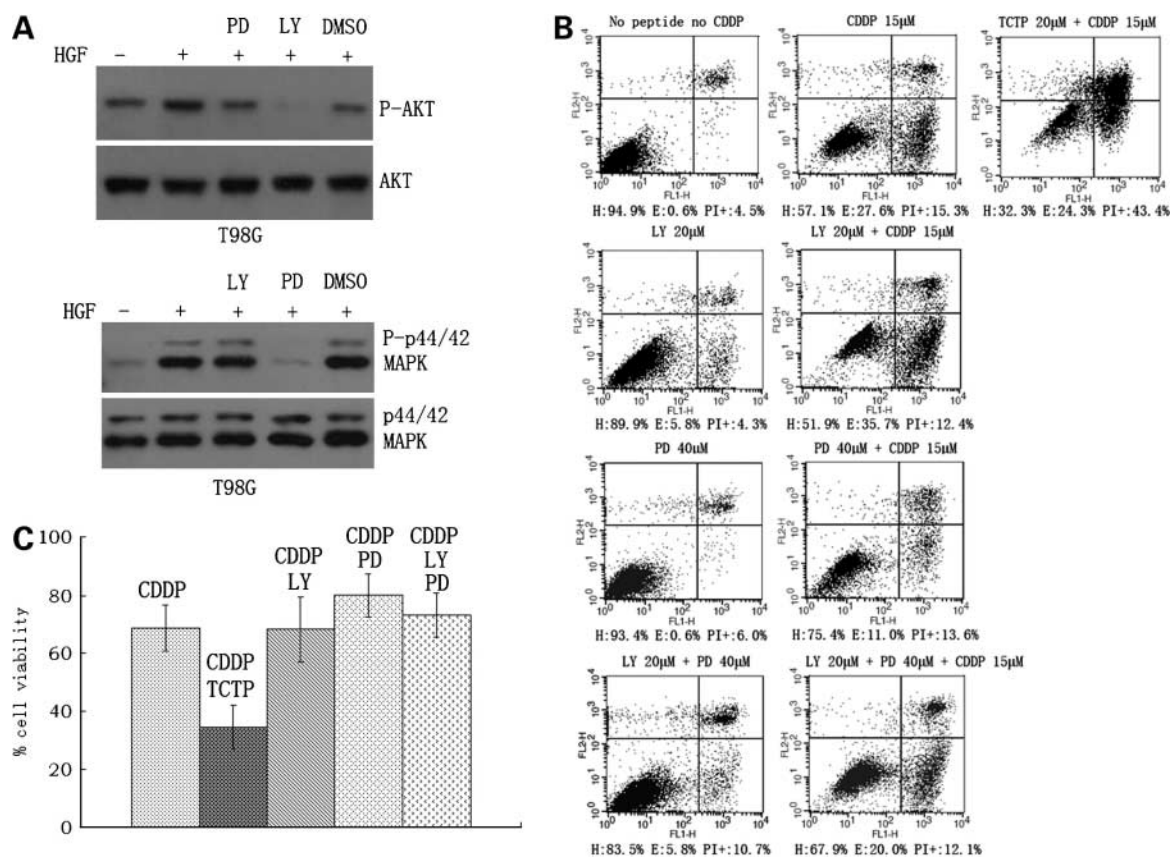
**Figure 2.** TCTP-induced chemosensitivity enhancement to CDDP. **A**, sensitivity curve of T98G, U251, and CCF-STGG1 cell lines toward CDDP in the presence and absence of TCTP or TAT via MTT assay. Different concentrations of CDDP alone or treated with either 20  $\mu\text{mol/L}$  TCTP or TAT for 72 h, the cell viability of each group was examined by MTT assay as described previously. **B**, effects of TCTP on cell apoptosis induced by CDDP in T98G, U251, and CCF-STGG1 via flow cytometry. Cells were exposed to 15  $\mu\text{mol/L}$  CDDP in the presence and absence of 20  $\mu\text{mol/L}$  TCTP or TAT for 72 h and analyzed via flow cytometry. Annexin V-FITC versus PI; *bottom right quadrant*, cells in early stage of apoptosis (*E*) that only have phosphatidylserine on their surface. PI-permeable (*PI+*) cells include the cells in late stage of apoptosis, which have phosphatidylserine on their surface and also with the ability of PI-permeable cells (*top right quadrant*) and the dead cells (*top left quadrant*). Representative of three independent experiments. **C**, cell viability analysis of flow cytometry data. Cell viability was graphed for treatment of 15  $\mu\text{mol/L}$  CDDP only, 15  $\mu\text{mol/L}$  CDDP combined with 20  $\mu\text{mol/L}$  TAT, and 15  $\mu\text{mol/L}$  CDDP combined with 20  $\mu\text{mol/L}$  TCTP for 72 h in T98G, U251, and CCF-STGG1, respectively. Three independent experiments were analyzed. Mean  $\pm$  SD percentage of cell viability.



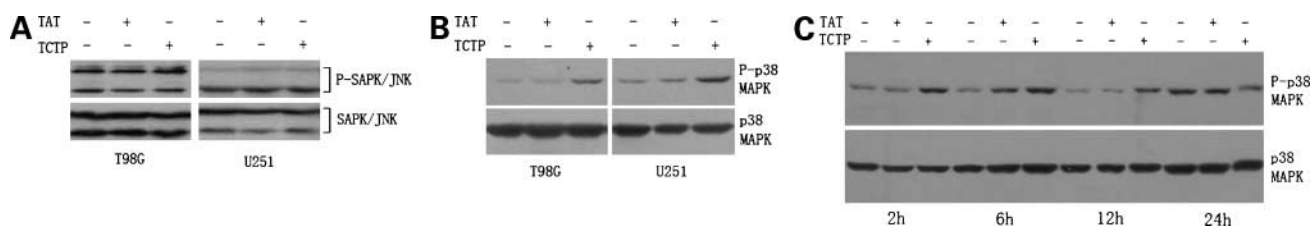
**Figure 3.** Dose- and time-response of TCTP on chemosensitivity enhancement to CDDP in T98G cells. **A**, dose-response of TCTP on chemosensitivity enhancement to CDDP in T98G cells via MTT assay. Cells were treated with 15  $\mu\text{mol/L}$  CDDP in the presence of indicated concentrations of TCTP or TAT for 72 h, and then the cell viability was examined by MTT assay as described previously. **B**, dose-response of TCTP on chemosensitivity enhancement to CDDP in T98G cells via flow cytometry. Cells were treated with 15  $\mu\text{M}$  CDDP combined with different concentrations of TCTP for 72 h, and then the cell populations were analyzed by multiparametric fluorescence of Annexin V-FITC versus PI via flow cytometry, and the percentages of cell populations in each quadrant are described as indicated in Fig. 2B. **C**, time-response of TCTP on chemosensitivity enhancement to CDDP in T98G cells via MTT assay. Cells were treated by 15  $\mu\text{M}$  CDDP in the presence and absence with 20  $\mu\text{mol/L}$  TCTP or TAT, the cell viability of T98G was examined by MTT assay at the indicated times. **D**, time-response of TCTP on chemosensitivity enhancement to CDDP in T98G cells via flow cytometry. Cells were treated by 15  $\mu\text{mol/L}$  CDDP in the presence and absence with 20  $\mu\text{mol/L}$  TCTP or TAT at the indicated times, and then the cell populations were analyzed by multiparametric fluorescence of Annexin V-FITC versus PI via flow cytometry, and the percentages of cell populations in each quadrant are described as indicated in Fig. 2B. Representative of three independent experiments.

10  $\mu\text{mol/L}$  CDDP to T98G cells achieved a  $51.8 \pm 5.6\%$  reduction in cell viability, whereas 10  $\mu\text{mol/L}$  CDDP alone and 10  $\mu\text{mol/L}$  CDDP combined with 20  $\mu\text{mol/L}$  TAT reduced cell viability by  $18.8 \pm 8.0\%$  and  $20.1 \pm 6.6\%$ , respectively. TCTP (20  $\mu\text{mol/L}$ ) combined with 10  $\mu\text{mol/L}$  CDDP to U251 cells showed a  $59.9 \pm 7.2\%$  reduction in cell viability, whereas 10  $\mu\text{mol/L}$  CDDP alone and 10  $\mu\text{mol/L}$  CDDP combined with 20  $\mu\text{mol/L}$  TAT caused reductions of  $36.2 \pm 7.7\%$  and  $41 \pm 2.2\%$ , respectively. Meanwhile, the  $\text{IC}_{50}$  of CDDP to T98G and U251 cell lines at 72 h was also reduced significantly (from  $\sim 30$  to 10  $\mu\text{mol/L}$  in T98G and from 20 to  $\sim 5$   $\mu\text{mol/L}$  in U251) once 20  $\mu\text{mol/L}$  TCTP was present, indicating that TCTP was an efficient sensitizer of CDDP. CCF-STGG1 cells, which were not sensitive to HGF stimulation, showed no significant difference of reduction in cell viability among each group ( $P > 0.05$ ; Fig. 2A).

Similar results were also obtained through flow cytometry by detection of cell apoptosis via FITC-labeled Annexin V and PI assay. The result showed that 20  $\mu\text{mol/L}$  TCTP itself only caused  $\sim 10\%$  more apoptosis in T98G compared with the negative control. When combined 20  $\mu\text{mol/L}$  TCTP with 15  $\mu\text{mol/L}$  CDDP, the percentage of cell apoptosis caused by CDDP was increased from 34.5% to 79.6%, whereas the TAT control peptide had no influence on apoptosis of T98G cell and did not affect the CDDP chemosensitivity. It was also shown that, in U251 cell line, 20  $\mu\text{mol/L}$  TCTP enhanced the chemosensitivity to CDDP, and the apoptosis was enhanced by  $\sim 20\%$ , whereas TCTP or TAT alone had little effects on apoptosis (Fig. 2B). In contrast, chemosensitivity of CCF-STGG1 cells to CDDP was not affected significantly by TCTP (Fig. 2B). Three independent flow cytometry experiments of TCTP-induced



**Figure 4.** Effect of AKT and p44/42 MAPK signaling impairment on the sensitivity of T98G cells to CDDP. **A**, HGF-mediated activation of AKT and p44/42 MAPK signaling was blocked by their inhibitors, LY294002 and PD98059, respectively. Cells were serum starved for 24 h and incubated with medium containing LY294002 (20  $\mu\text{mol/L}$ ), PD98059 (40  $\mu\text{mol/L}$ ), or DMSO only for 1 h. The cells were treated with 40 ng/mL recombinant HGF for 15 min in the presence of indicated inhibitor and lysed. Phosphorylated and total proteins were evaluated by Western blotting with the corresponding antibody. **B**, T98G cells were treated with 15  $\mu\text{mol/L}$  CDDP, 20  $\mu\text{mol/L}$  TCTP with 15  $\mu\text{mol/L}$  CDDP, 20  $\mu\text{mol/L}$  LY294002, 20  $\mu\text{mol/L}$  LY294002 combined with 20  $\mu\text{mol/L}$  CDDP, 40  $\mu\text{mol/L}$  PD98059, 40  $\mu\text{mol/L}$  PD98059 combined with 20  $\mu\text{mol/L}$  CDDP, 20  $\mu\text{mol/L}$  LY294002 with 40  $\mu\text{mol/L}$  PD98059, 20  $\mu\text{mol/L}$  LY294002 combined with 40  $\mu\text{mol/L}$  PD98059, and 20  $\mu\text{mol/L}$  CDDP for 72 h, and cell apoptosis of each group was analyzed by multiparametric fluorescence of Annexin V-FITC versus PI via flow cytometry, and the percentages of cell populations in each quadrant are described as indicated in Fig. 2B. Representative of three independent experiments. **C**, cell viability analysis of flow cytometry data. Cell viability was graphed for treatment of 15  $\mu\text{mol/L}$  CDDP only, 15  $\mu\text{mol/L}$  CDDP combined with 20  $\mu\text{mol/L}$  TCTP, 15  $\mu\text{mol/L}$  CDDP combined with 20  $\mu\text{mol/L}$  LY294002, 15  $\mu\text{mol/L}$  CDDP combined with 40  $\mu\text{mol/L}$  PD98059, 15  $\mu\text{mol/L}$  CDDP combined with 20  $\mu\text{mol/L}$  LY294002, and 40  $\mu\text{mol/L}$  PD98059 for 72 h in T98G cells. Three independent experiments. Mean  $\pm$  SD percentage of cell viability. LY, LY294002; PD, PD98059.



**Figure 5.** Effect of TCTP on activation of SAPK/JNK and p38 MAPK. **A**, effect of TCTP on SAPK/JNK activation in T98G and U251 cell lines. Cells were incubated with the indicated peptides at a final concentration of 20  $\mu\text{mol/L}$  for 2 h and then lysed. Phosphorylated and total proteins of SAPK/JNK were evaluated by Western blotting with the corresponding antibody. **B**, effect of TCTP on p38 MAPK activation in T98G and U251 cell lines. Cells were incubated with the indicated peptides at a final concentration of 20  $\mu\text{mol/L}$  for 2 h and then lysed. Phosphorylated and total proteins of p38 MAPK were evaluated by Western blotting with the corresponding antibody. **C**, time course activity of TCTP on p38 MAPK phosphorylation. T98G cells were treated with 20  $\mu\text{mol/L}$  TCTP for the indicated time points, cells were then lysed, and protein levels were assessed by the corresponding antibodies. Representative of three independent experiments.

chemosensitivity enhancement to CDDP were graphed (Fig. 2C).

Both MTT assay and flow cytometry indicated that TCTP enhanced CDDP induced apoptosis in T98G and U251 cell lines in a dose- and time-dependent manner, indicating that the chemosensitivity enhancement was caused by TCTP specifically (Fig. 3).

#### **AKT and p44/42 MAPK Signaling Impairment by TCTP Was Not the Main Cause for Sensitization to CDDP in T98G Cell Line**

PI3K/AKT and p44/42 MAPK are the two definite downstream targets of Met receptor. Inhibition of PI3K/AKT signaling has proven to be an efficient way to attenuate the resistance of chemotherapy (24), whereas inhibition of p44/42 MAPK activation can also enhance the chemosensitivity (25). It was confirmed that TCTP could impair HGF-induced Met-dependent signaling of AKT and p44/42 MAPK pathways (Fig. 1A). To investigate whether impairment of Met-dependent AKT and p44/42 MAPK signaling by TCTP was important in the enhanced chemosensitivity to CDDP in T98G cell line, LY294002 and PD98059 were used to interrupt the signaling transduction of PI3K/AKT and p44/42 MAPK, respectively. LY294002 is a selective inhibitor of PI3K, which has been widely used for the suppression of PI3K and AKT activation, whereas PD98059 is the specific p42/44 MAPK inhibitor, which has been commonly used for the suppression of p44/42 MAPK activation (Fig. 4A). It was found that LY294002 or PD98059 alone failed to induce any visible apoptosis in T98G cells via flow cytometry. Either LY294002 or PD98059 combined with CDDP did not cause the chemosensitivity enhancement at all (Fig. 4B). It is possible that inhibition of both pathways simultaneously may contribute to the TCTP-induced chemosensitivity enhancement. To address this possibility, we inhibited both pathways by LY294002 and PD98059 simultaneously and found that combination with both inhibitors did not influence the cell apoptosis induced by CDDP either (Fig. 4B). Three independent flow cytometry experiments were graphed to show the effects of LY294002 or/and PD98059 on chemosensitivity enhancement of T98G cells to CDDP (Fig. 4C). These data suggested that impairment of AKT and p44/42

MAPK signaling by TCTP was not the main contributor of the increased sensitivity to CDDP induced by Met inactivation in T98G cells.

#### **Activation of p38 MAPK Was a Main Contributor to the Chemosensitivity Enhancement Caused by TCTP**

MAPK cascade is one of the most well-defined pathways downstream of the Met receptor. The serine/threonine kinases of MAPK family mainly include the primarily anti-apoptotic extracellular signal-regulated kinases, JNK, and proapoptotic p38 MAPK. Because p44/42 MAPK inhibition was not the main cause for chemosensitivity enhancement induced by Met inactivation, this study was focused on the other two pathways, JNK and p38 MAPK. TCTP-induced Met inactivation had no effect on the JNK-related pathway signaling (Fig. 5A); thus, the investigation was centered on p38 MAPK pathway. p38 MAPK pathway is usually activated by cell stress and is involved in cancer cell apoptosis induced by several chemotherapeutic agents including platinum drug (26, 27). The results showed that TCTP significantly activated the p38 MAPK phosphorylation in both T98G and U251 cell lines (Fig. 5B), and the induction of p38 MAPK by TCTP in T98G cells lasted at least 12 h without any recovery (Fig. 5C). TCTP-induced p38 MAPK activation was not so obvious in CCF-STGG1 (data not shown).

In attempting to determine whether p38 MAPK activation played an important role in the TCTP-induced chemosensitization to CDDP, the effect of p38 MAPK activation on chemosensitivity was firstly tested. Osmotic stress induced by sorbitol treatment activated the p38 MAPK phosphorylation (Fig. 6A). Little effect on the apoptosis of T98G cell line was obtained by pretreatment with 400 mmol/L sorbitol alone for 1 h then followed with normal medium incubation, whereas significant chemosensitivity enhancement (25.5% to 55.3% at 72 h) was observed by pretreatment of 400 mmol/L sorbitol combined with 15  $\mu\text{mol/L}$  CDDP for 1 h then followed with normal medium containing 15  $\mu\text{mol/L}$  CDDP (Fig. 6C). This result suggested that activation of p38 MAPK might be an important factor of TCTP-induced chemosensitivity enhancement to CDDP. Furthermore, we used the specific inhibitor of p38 MAPK, SB202190, which inhibited TCTP-induced p38 MAPK

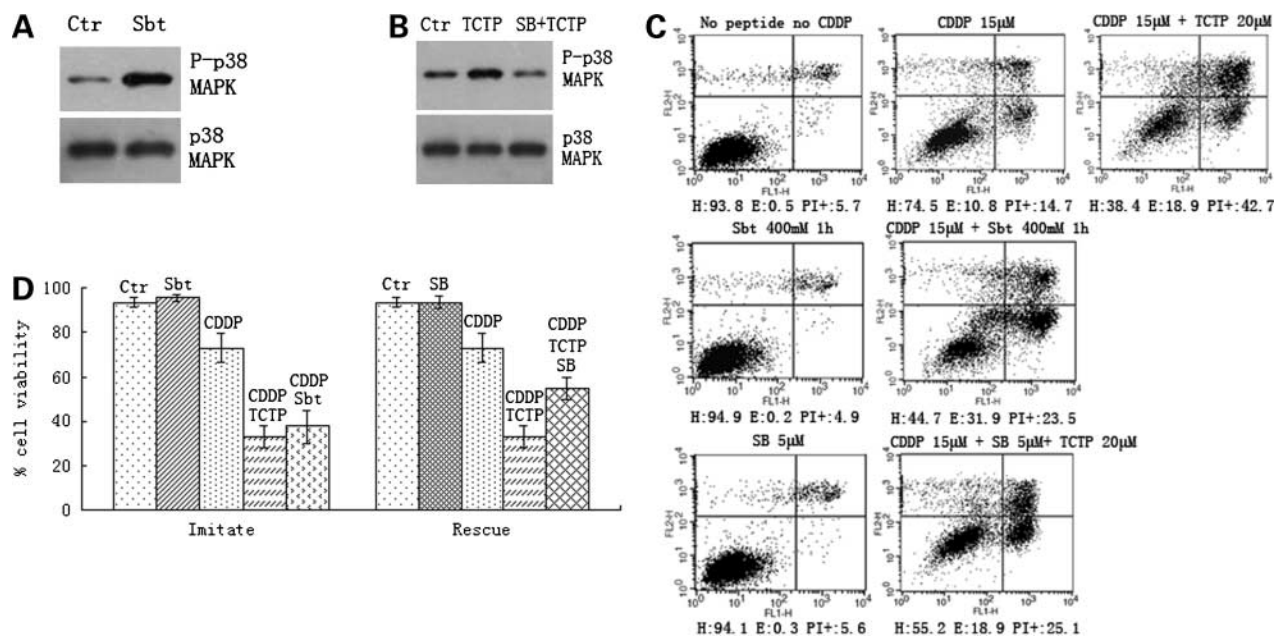
activation (Fig. 6B), to counteract the effect of TCTP on p38 MAPK activation without interrupting the influence of other pathways induced by TCTP. SB202190 itself had little effects on T98G apoptosis, but it significantly rescued the TCTP-induced chemosensitivity enhancement to CDDP in T98G cell line (Fig. 6C). Three independent flow cytometry experiments were graphed to display that p38 MAPK activation was an important factor for chemosensitivity enhancement to CDDP (Fig. 6D). These data suggested activation of p38 MAPK signaling by TCTP was one of the main contributors of the increased sensitivity to CDDP induced by Met inactivation in T98G cells. Taken all together, Met inhibition by TCTP induces chemosensitivity enhancement to CDDP mainly through p38 MAPK activation but not through inhibition of AKT or p42/44 MAPK pathways.

## Discussion

Chemosensitivity enhancement is one of the major challenges to overcome the multidrug resistance and undesirable side effects of chemotherapy. Chemoresistance is frequently correlated with overexpressed and abnormally activated RTKs. Recent advances in tumor therapies, especially targeting toward the specific RTKs, have offered hope

of improved therapeutic effects by interfering the cell signaling events that govern the growth, migration, and survival of cancer cells. Inhibition of abnormally activated RTKs is a potential and valuable strategy to enhance tumor cells' sensitivity to the chemotherapy agents and thus improving the effects of chemotherapy.

In the present study, we showed that inhibition of Met signaling by TCTP could significantly enhance the chemosensitivity to CDDP. It is known that the major obstacles in cancer chemotherapy are the severe side effects that limit the dosage of the anticancer drugs because of lacking selectivity for tumor versus normal cells and the chemoresistance established by the cell mutation under the treatment of drugs. During the past decades, many impressive advances concerning the molecular description of intracellular signaling pathways, their aberrations in tumor cells, and their contributions to the drug response have been explored (28–30). It is an important goal of molecular cancer research to integrate these insights into therapeutic strategies and use the essential differences in the genetic and biochemical properties of normal and cancer cells for therapeutic purposes. The targeting of drugs at such molecules might result in a high therapeutic index minimizing the undesirable side effects of cancer treatment on normal cells and implement the idea of individual treatment of cancer patients. The



**Figure 6.** p38 MAPK activation was a major contributor of TCTP-induced chemosensitivity enhancement to CDDP. **A**, effect of sorbitol on p38 MAPK activation. Cells were treated by 400 mmol/L sorbitol for 1 h and then lysed. Phosphorylated and total proteins of p38 MAPK were evaluated by Western blotting with the corresponding antibody. **B**, effect of SB202190 on p38 MAPK inhibition. Cells were treated with either TCTP 20 μmol/L alone or combined with 5 μmol/L SB202190 for 1 h and then lysed. Phosphorylated and total proteins of p38 MAPK were evaluated by Western blotting with the corresponding antibody. **C**, effect of p38 MAPK signaling on the chemosensitivity enhancement caused by TCTP in T98G cell line. Cells were treated with 15 μmol/L CDDP, 20 μmol/L TCTP combined with 15 μmol/L CDDP, 400 mmol/L sorbitol pretreatment for 1 h and followed by normal medium incubation, 400 mmol/L sorbitol combined with 15 μmol/L CDDP pretreatment for 1 h and then followed by 15 μmol/L CDDP in normal medium incubation, 5 μmol/L SB202190, 20 μmol/L TCTP combined with 5 μmol/L SB202190, and 15 μmol/L CDDP for 72 h. Cell apoptosis of each group was analyzed by multiparametric fluorescence of Annexin V-FITC versus PI via flow cytometry, and the percentages of cell populations in each quadrant are described as indicated in Fig. 2B. Representative of three independent experiments. **D**, cell viability analysis of flow cytometry data. Cell viability was graphed to display the results obtained in C. Three independent experiments were analyzed. Mean ± SD percentage of cell viability. *Ctrl*, control; *Sbt*, sorbitol; *SB*, SB202190.



results of current work showed that interference with specific protein-protein interactions in glioma cell lines where the Met receptor was overexpressed by TCTP led to an enhancement in chemosensitivity to CDDP. The system could be extended to other types of malignancies with Met abnormally expressed and activated.

The mechanism of sensitization in cancer therapy is very complicated and not clearly understood. The results also provided an approach to understand the pathways related to the sensitization of glioma cell lines to CDDP. To better understand of the mechanism involved in sensitization of gliomas to CDDP, the highly selective or specific inhibitors were used to determine which pathways were involved in the sensitization to CDDP by TCTP. TCTP could impair Met-dependent AKT and p44/42 MAPK signaling in T98G cells. The PI3K/AKT pathway activation can mediate protection from apoptotic stimuli in several cancer types and may relate to the chemosensitivity resistance of multiple chemotherapy agents. Inhibition of PI3K/AKT signaling has been proven to be an efficient way to attenuate the resistance of chemotherapy (24). Same to PI3K/AKT signaling, activation of p44/42 MAPK pathway usually promotes cell proliferation, migration, and differentiation and delivers antiapoptotic signals following cell stimuli with a variety of growth factors and may also relate to the resistance of chemotherapy. Inhibition of p44/42 MAPK activation can enhance the chemosensitivity (25). However, it was found these two signaling pathways were not the major cause for sensitization to CDDP by TCTP because neither LY294002 nor PD98059 alone or combined could mimic the effect of sensitization to CDDP caused by TCTP.

Further study revealed that TCTP could significantly induce the p38 MAPK activation. To investigate whether p38 MAPK activation by TCTP was the major cause of the sensitization, the sorbitol, which has been considered as the activator of p38 MAPK, was used first to imitate the effects of TCTP on sensitization of T98G cell line to CDDP. The results indicated that activation of p38 MAPK by sorbitol treatment led the similar effect of TCTP, suggesting that activation of p38 MAPK might be an important factor of TCTP-induced chemosensitivity enhancement to CDDP. However, sorbitol has numerous effects other than p38 MAPK activation. To better illustrate the role of p38 MAPK activation in TCTP-induced chemosensitization, we used the specific inhibitor of p38 MAPK, SB202190, to counteract the effect of TCTP on p38 MAPK activation without interrupting the influence of other pathways induced by TCTP. SB202190 could counteract (retrieve) the sensitization induced by TCTP. These data supported that p38 MAPK activation was one of the main contributors for the TCTP-induced enhancement in CDDP chemosensitivity. The potency of p38 MAPK signaling to apoptosis has long been studied (31–33). Previous studies showed that activation of p38 MAPK could enhance the chemotherapy-induced apoptosis (34, 35) and attenuation of p38 MAPK signaling could protect cells from cytotoxicity induced by chemotherapy agents (36, 37). However, the relationship between Met receptor and p38

MAPK is not clear at present. p38 MAPK could be activated by the Met receptor phosphorylation under HGF stimulation (34, 38), whereas there was a report indicating that inhibition of Met receptor signaling by a tyrphostin called Adaphostin exhibited the activation of p38 MAPK (39). In this study, it was also shown that impairment of Met signaling could activate the signaling of p38 MAPK, and the p38 MAPK activation was a main contributor to the sensitization of CDDP induced by TCTP. However, a further research is needed to investigate the mechanism on how TCTP activates p38 MAPK.

CPPs, also named Trojan peptides or protein transduction domains, are a class of short polypeptide sequences usually containing <30 amino acids. They are always cationic and/or amphipathic and have ability to carry the large macromolecules across membranes efficiently both *in vitro* and *in vivo* and possess low cellular toxicity. It is regarded as a good carrier of target proteins, peptide, DNA, small interfering RNA, etc. Although the internalization mechanism of CPPs is still extensively debatable, the applications of them are becoming more and more popular (40). Here, we used a well-known CPP TAT as the vehicle for the peptide delivery. Once internalized, the tail peptide blocked the ligand-dependent Met autophosphorylation, altered the Met-dependent downstream signaling, and enhanced the chemosensitivity to CDDP. The finding provided us a feasible method to develop the strategies based on the small peptide fragment that could block the activation of RTKs to enhance the chemosensitivity. Moreover, using CPPs as the vehicle for peptide delivery would possibly deliver specific therapeutic proteins from cell surface to the intracellular therapeutic targets. It is possible that, coupled with a CPP sequence, the therapeutic peptides or proteins would pass through the blood-brain barrier. *In vivo* results showed that, after intravenous injection, CPP-conjugated proteins could be found in a variety of organs and cell types within an organism including the brain (41). If delivery of therapeutic proteins can be targeted toward specific cell types via recognizing the cellular surface markers, side effects of such drugs will be further minimized.

In this research, glioma cells were chosen to evaluate the effect of TCTP on chemosensitivity enhancement. Glioblastoma multiforme is an aggressively invasive malignant tumor with a median survival period of ~12 months (42) and for which the Met receptor overexpression is involved in their invasion and angiogenesis (43, 44). Currently, the first-line chemotherapy drug for gliomas is temozolomide. Other traditional chemotherapy agents such as CDDP were somehow restricted by their limited ability to cross the blood-brain barrier and could not reach the effective concentration in the tumor sites. CDDP alone or combined with other chemotherapy agents has been used to treat the low-grade, newly diagnosed, or recurrent glioblastoma (45–47). The obstacle of CDDP being the first-line drug for glioma chemotherapy may only be hindered by its ability to reach the effective concentration at tumor site, because local chemotherapy of glioblastoma multiforme with CDDP

followed by irradiation is proven to be well tolerated and effective (48). Therefore, chemosensitivity enhancement induced by Met inactivation is a potential application to improve the therapeutic effects of CDDP in tumor therapy including gliomas.

Several chemotherapeutic drugs such as Taxol, doxorubicin, etoposide, methotrexate, and CDDP were chosen to establish the sensitization effect of TCTP, and it was found that the peptide only significantly increased the sensitivity to CDDP. The results suggested that the specific RTK inhibition might only correlate with sensitivity of specific drug (drugs), and this specificity required devising a specific regimen to optimize combination chemotherapy in different clinic applications according to their molecular characteristics.

Given the recent clinical availability of Met inhibitors, our study provided a new strategy (indication) in clinical development of Met inhibitors. Although the results were encouraging and showed the potential use of TCTP in sensitizing the tumor cells to CDDP, many steps should be optimized. *In vivo* properties of mice will have to be investigated and optimized first.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Inhibition of the met receptor tyrosine kinase signaling enhances the chemosensitivity of glioma cell lines to CDDP through activation of p38 MAPK pathway

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