Regulation of cellular growth, apoptosis, and Akt activity in human U251 glioma cells by a combination of cisplatin with CRM197

Lifei Wang[a,b], Ping Wang[a,b], Yunhui Liu[c] and Yixue Xue[a,b]

The aberrantly activated antiapoptotic phospatidyl-3-inositol-kinase (PI3K)/Akt signaling induced by cisplatin limits the effectiveness of chemotherapy; inhibition of this pathway may augment the sensitivity of tumor cells to cisplatin-induced toxicity and promote apoptosis. Cross-reacting material 197 (CRM197), the nontoxic mutant of diphtheria toxin, could act as an heparin-binding epidermal growth factor inhibitor and has been shown to have some anticancer effects, but the effect of CRM197 on glioma cells remains unclear. The aim of this study was to investigate the effects of a combination of cisplatin with CRM197 on the growth and apoptosis of human U251 glioma cells and the possible mechanism. In this study, we demonstrated that cisplatin or CRM197 induced a dose-dependent growth inhibition in U251 cells, but cisplatin at 5 μg/ml and CRM197 at 1 μg/ml did not affect the viability of human astrocytes. Cisplatin induced a time-dependent growth inhibition in U251 cells, whereas the growth-inhibitory effects induced by CRM197 alone or combined with cisplatin reached a peak at 24 h after treatment. Compared with the administration of cisplatin or CRM197 alone, CRM197 combined with cisplatin significantly enhanced U251 cell growth inhibition and apoptosis. Cisplatin induced sustained activation of Akt, whereas CRM197 markedly suppressed the Akt phosphorylation induced by cisplatin. The effects of growth inhibition and apoptosis were markedly enhanced after a combination of cisplatin with CRM197 plus the PI3K inhibitor LY294002 or wortmannin. Therefore, CRM197 combined with cisplatin could enhance growth inhibition and apoptosis of glioma cells by inhibiting the cisplatin-induced PI3K/Akt pathway. *Anti-Cancer Drugs* 23:81–89 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Glioma is one of the most common malignant brain tumors in adults. It is difficult to treat because of resistance to conventional radiotherapy and chemotherapy [1]. Despite treatment with conventional therapies such as surgery, radiation and chemotherapy, the prognosis of patients with such tumors remains poor [2,3].

Cross-reacting material 197 (CRM197) is the product of a single missense mutation (Gly^{52} to Glu) within the fragment A region of diphtheria toxin. The mutation yields a nontoxic mutant of diphtheria toxin and shares immunological properties with the native molecule to bind to the diphtheria toxin receptor, which is also known as the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF) [4,5]. CRM197 has already been used as a well tolerated and effective carrier protein in human vaccines since the mid-1980s [6]. Recently, CRM197 has been proven to deliver horseradish peroxidase (HRP) (serving as a 40 kDa 'model' protein drug) across the blood–brain barrier. Increased caveolae-mediated transcytosis played a critical role in promoting CRM197-targeted delivery to the brain [7–9]. In addition, the anticancer effects of CRM197 have been demonstrated. Administration of CRM197 inhibited the growth of ovarian cancer, breast cancer, adrenocortical carcinoma, and gastric cancer [10–14]. The combination of CRM197 with paclitaxel exhibited synergistic anticancer effects in ovarian cancer and gastric cancer [14,15]. The anticancer effects of CRM197 are mainly because of the inhibition of HB-EGF functions to block its mitogenic activity [11]. Its anticancer properties also depend on the toxicity of the molecule and its inflammatory–immunological properties [16,17]. Therefore, a combination of conventional chemotherapeutic agents with CRM197 would potentially be efficacious in the treatment of patients with glioma.

Cisplatin is a potent inducer of growth arrest and/or apoptosis in most cell types and is among the most effective and widely used chemotherapeutic agents used for the treatment of human cancers such as glioblastomas [18,19]. Unfortunately, the development of resistance to cisplatin-based chemotherapy is a major obstacle for its successful clinical application. Previous studies have indicated that the activation of Akt promoted tumor cell survival and proliferation [20]. The serine/threonine protein kinase Akt is a downstream phospatidyl-3-inositol-kinase (PI3K) target and can also be activated

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by a variety of growth factors, cytokines, G-protein-coupled receptors, and adhesion molecules. Activated Akt kinase regulates cell survival, cell migration, and suppresses apoptosis. In many human cancers, both PI3K and Akt are frequently activated and/or overexpressed [21,22]. Cisplatin activates Akt in several cancer cell lines [23,24]. Inhibition of Akt activity decreases the survival of the cells exposed to cisplatin, suggesting that cisplatin-induced Akt activation may lead to cisplatin resistance. Recent data demonstrated that CRM197 blocked Akt activation by means of the suppression of epidermal growth factor receptor transactivation [9,12,14]. The effects of combination of cisplatin with CRM197 on the growth and apoptosis in tumor cells remain to be demonstrated.

The objective of this study was to investigate the effects of a combination of cisplatin with CRM197 on human U251 glioma cells and the possible mechanism. We found that CRM197 combined with cisplatin could enhance growth inhibition and apoptosis of glioma cells by inhibition of the PI3K/Akt pathway induced by cisplatin.

Materials and methods

Reagents

CRM197, cisplatin, PI3K inhibitor LY294002 and wortmannin, dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). The fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (Annexin V-FITC and PI) apoptosis detection kit and the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbo-cyanine iodide (JC-1) apoptosis detection kit were purchased from Becton Dickinson Bioscience (California, USA). The primary polyclonal antibody against phospho-Akt (Ser473), Akt, and β-actin antibody and the secondary antibodies conjugated with HRP were purchased from Santa Cruz Biotechnology (California, USA). All other chemicals were of analytical grade and purchased from different commercial sources.

Cell culture

The human astrocytes (HA) were purchased from ScienCell Research Laboratories (California, USA) and maintained in astrocyte medium with 2% fetal bovine serum, 1% penicillin/streptomycin solution, and 1% astrocyte growth supplement (ScienCell Research Laboratories). The human U251 glioma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin (Hyclone, Utah, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Growth inhibition assays

Cells were seeded in 96-well plates at a density of 1×10^4 cells/ml, with each well containing 200 μl of culture medium, and incubated for 24 h. Then these cells were treated with different factors at different time points. Blank wells contained the same amount of culture medium without cells. At the end of each time point, MTT was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 h at 37°C. Then the medium was removed and dimethyl sulfoxide (100 μl) was added to dissolve the formazan crystals by shaking the plate weakly for 10 min. The optical density (OD) was measured at 570 nm using a microplate reader. The cell inhibition rate was calculated using the following formula: Inhibition rate (%) = (OD570 of control cells – OD570 of treated cells)/(OD570 of treated cells – OD570 of blank cells) × 100%.

Annexin V-FITC and PI assay for flow cytometry

Cells were seeded in six-well plates at a density of 1×10^4 cells/ml, with each well containing 2 ml of culture medium, and incubated for 24 h. Then these cells were subjected to different treatments for 24 h. Cells were collected and resuspended in 100 μl of binding buffer. Annexin V-FITC (5 μl) and PI (5 μl) were added to the cell suspension, followed by incubation at room temperature in the dark for 15 min. After this period of incubation, 400 μl of binding buffer was added and the samples were immediately measured using a flow cytometer. The results were analyzed using CellQuest software (BD, San Jose, California, USA). The dots in the lower left quadrant, upper left quadrant, upper right quadrant, and lower right quadrant represent live cells (Annexin V–, PI–), damaged cells (Annexin V–, PI+), late apoptotic and necrotic cells (Annexin V+, PI+), and early apoptotic cells (Annexin V+, PI–), respectively.

JC-1 assay for flow cytometry

Cells were seeded in six-well plates at a density of 1×10^4 cells/ml, with each well containing 2 ml of culture medium, and incubated for 24 h. Then these cells were subjected to different treatments for 24 h. After incubation with 10 μl of 200 μmol/l JC-1 (diluted with PBS to a final concentration of 2 μmol/l) at 37°C for 30 min, cells were washed once by PBS and resuspended with 500 μl of PBS. The samples were immediately measured using a flow cytometer. JC-1 accumulates in the mitochondria to form red fluorescent aggregated in live cells, whereas it exists mainly in the form of a monomer showing green fluorescence in apoptotic cells because of the depolarization of the mitochondrial membrane potential (MMP). The results were analyzed using CellQuest software. JC-1 green and red fluorescences were recorded on FL1 and FL2 channels, respectively. The dots in the upper right quadrant and lower right quadrant represents live cells (FL-1+, FL-2+) and apoptotic cells (FL-1+, FL-2–), respectively.

Western blot

U251 cells treated with various treatments for different durations of time were lysed in lysis buffer [EDTA

(2 mmol/l), ethylene glycol tetraacetic acid (10 mmol/l), 0.4% NaF, Tris-HCl (20 mmol/l), 1% NP-40, 1% Triton X-100, pH 7.5] supplemented with a proteinase inhibitor [aprotinin (10 mg/ml), phenyl-methyl-sulfonyl chloride (10 mg/ml) and sodium orthovanadate (50 mmol/l)]. Whole-cell extracts were quantified using the Coomassie G250-binding method and equal amounts of proteins (30 μg) were separated by SDS-PAGE. Gels were transferred onto a polyvinylidene flouride membrane, blocked with 5% nonfat dry milk in tris buffered saline with tween 20 (TBST) and incubated with the primary antibody solution. Alternatively, primary phospho-antibodies were diluted in 5% BSA in TBST at 4°C overnight. The membrane was washed with TBST and incubated with an HRP-conjugated secondary antibody solution. After subsequent washes, immunoblots were visualized by enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology). Autoradio-graphic images were scanned and integrated density values of protein bands were quantified using ChemiImager 5500 software (Alpha Innotech, San Leandro, California, USA).

Statistical analysis

All results were expressed as the mean ± standard deviation for each group. One-way analysis of variance, followed by the Bonferroni test was used to determine significant differences between groups. A statistical significance was inferred at P values less than 0.05.

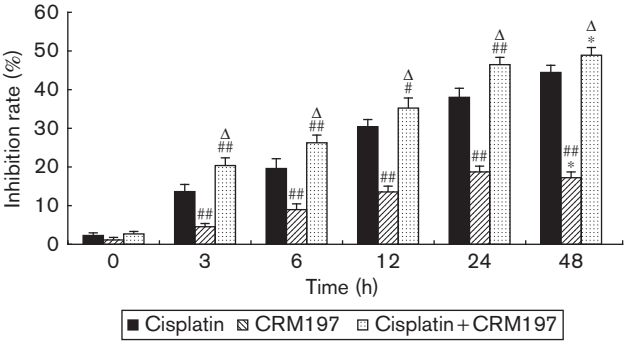
Results

Effects of cisplatin and CRM197 on cell growth

Cisplatin or CRM197 inhibited U251 cell growth in a dose-dependent manner (Fig 1a and b). In HA cells, 5 μg/ml of cisplatin or 1 μg/ml of CRM197 did not significantly affect the growth inhibition rate compared with the untreated groups, but these doses could markedly induce growth inhibition effects on U251 cells. Cisplatin (5 μg/ml) combined with CRM197 (1 μg/ml) significantly increased the growth inhibition rate in U251 cells compared with HA cells (versus 7.2 ± 1.92%, Fig. 1c) and these two doses were used as the optimal dosage in the subsequent experiments.

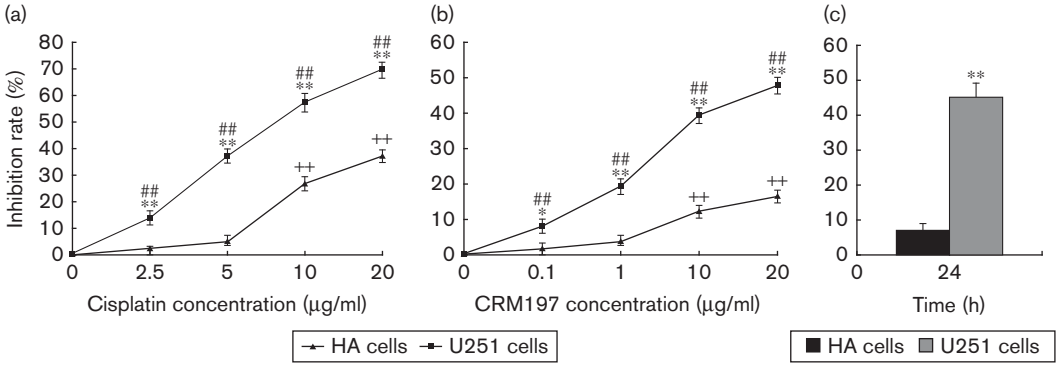
Administration of cisplatin alone, CRM197 alone or a combination of these two agents inhibited U251 cell growth in a time-dependent manner. The CRM197-induced growth inhibitory effects increased to a peak at 24 h, and remained at 48 h (Fig. 2). The combination of cisplatin with CRM197 significantly increased the growth

Fig. 2



The time-dependent influence of cisplatin and cross-reacting material 197 (CRM197) on U251 cell growth. Cells were treated with cisplatin (5 μg/ml), CRM197 (1 μg/ml), and cisplatin (5 μg/ml) combined with CRM197 (1 μg/ml) for different durations of time and then growth-inhibitory effects were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data are given as mean ± standard deviation from four independent experiments ($n=5$ duplicate wells per group). $^{\#}P<0.05$; $^{\#\#}P<0.01$ versus the cisplatin group at the same time point; $^{\Delta}P<0.01$ versus the CRM197 group at the same time point; $^{*}P>0.05$ versus the same drug at the former time point.

Fig. 1



The dose-dependent influence of cisplatin and cross-reacting material 197 (CRM197) on cell growth in human astrocytes (HA) and U251 cells for 24 h. The inhibition rate of different concentrations of (a) cisplatin and (b) CRM197 was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). $^{*}P<0.05$; $^{**}P<0.01$ versus the same concentration of cisplatin or CRM197 in HA cells; $^{++}P<0.01$ versus untreated HA cells; $^{\#\#}P<0.01$ versus untreated U251 cells. (c) The inhibition rate of combination of 5 μg/ml cisplatin with 1 μg/ml of CRM197 was assayed by MTT. $^{**}P<0.01$ versus HA cells. Data are given as mean ± standard deviation from four independent experiments ($n=5$ duplicate wells per group).

inhibition rate compared with cisplatin alone ($P < 0.05$) or CRM197 alone ($P < 0.01$) at the same time point, but no significant difference was observed at 48 h between groups of cisplatin combined with CRM197 and cisplatin alone ($P > 0.05$). Therefore, the twenty-fourth hour after administration was chosen as the optimal time point in the subsequent experiments.

Effects of combination of cisplatin with CRM197 on apoptosis

The U251 cell apoptosis was analyzed with Annexin V-FITC and PI double staining after various treatments for 24 h. As shown in Fig. 3, the percentages of Annexin V-FITC-positive cells were as follows: the control group, 4.73 ± 1.98%; cisplatin group, 34.61 ± 2.63%; CRM197 group, 18.88 ± 2.46%; and the group with a combination of cisplatin with CRM197, 45.62 ± 2.96%. Cisplatin, CRM197, and a combination of cisplatin with CRM197 significantly induced apoptosis compared with the control group ($P < 0.01$); the combination of cisplatin with CRM197 significantly induced apoptosis compared with cisplatin alone ($P < 0.05$) or CRM197 alone ($P < 0.01$).

Reduction of MMP is one of the molecular events for early apoptosis. Changes of MMP in U251 cells after various treatments were determined by JC-1 staining. As shown in Fig. 4, flow cytometric analysis demonstrated that the percentage of cells testing positive for depolarized mitochondria (green) was 6.83 ± 1.91% in the control group; 36.22 ± 2.52% in the cisplatin group; and 20.78 ± 2.12% in the CRM197 group and increased to 47.03 ± 2.73% in the two-agent combination group. The combination of cisplatin with CRM197 significantly induced apoptosis compared with control ($P < 0.01$), cisplatin alone ($P < 0.05$), or CRM197 alone ($P < 0.01$; Fig. 4). These results were similar to those of the apoptosis assay using Annexin V-FITC and PI double staining.

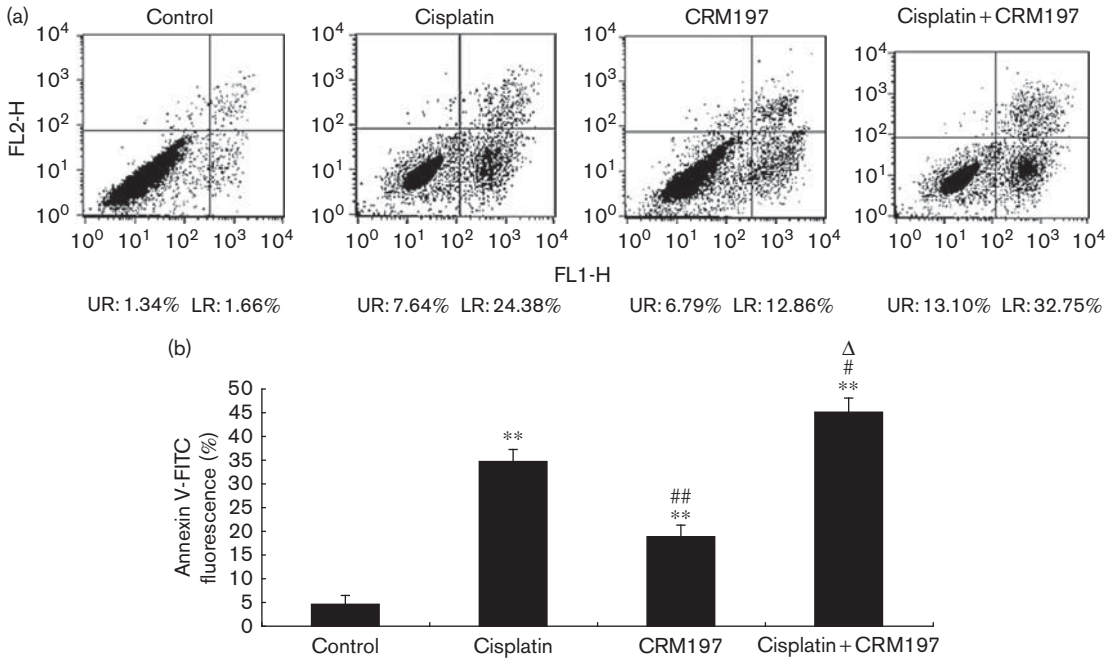
CRM197 inhibited the p-Akt expression induced by cisplatin

U251 cells were treated with cisplatin, CRM197 and a combination of cisplatin with CRM197 across various time points and the expression levels of p-Akt and Akt protein were assayed by a western blot. As shown in Fig. 5, cisplatin treatment for 24 h induced the upregulated expression of p-Akt and CRM197, and a combination of cisplatin with CRM197 markedly inhibited Akt phosphorylation compared with the control group ($P < 0.05$ and $P < 0.01$).

Effects of the combination of cisplatin with CRM197 plus LY294002 or wortmannin on cell growth and apoptosis

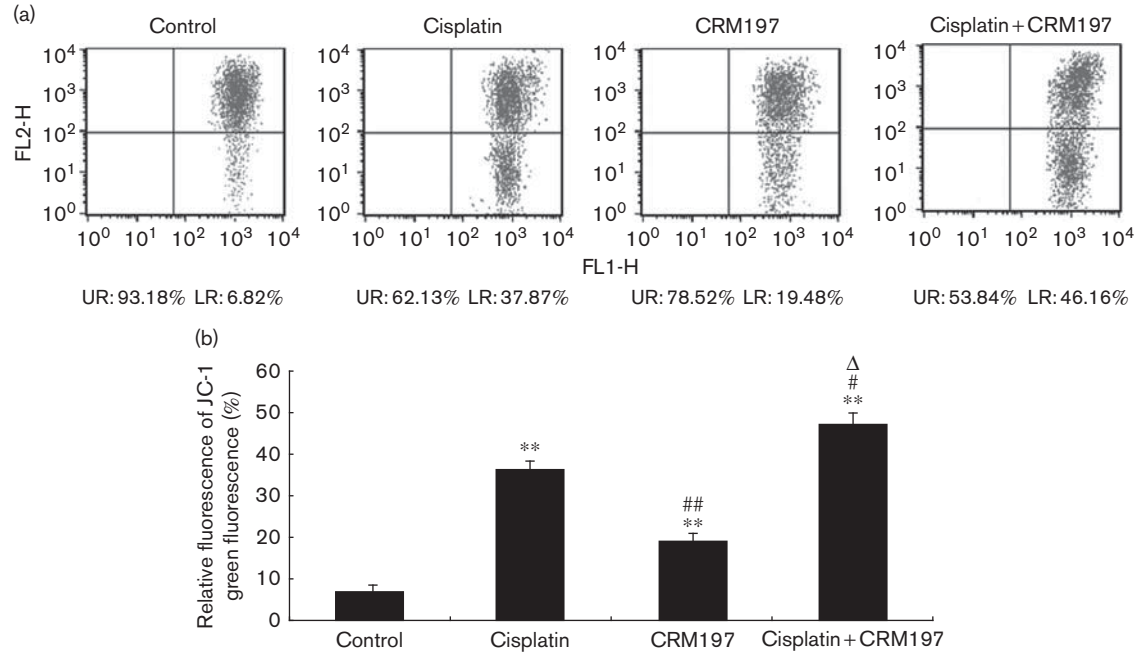
As shown in Fig. 6, 10 μmol/l of LY294002, 1 μmol/l of wortmannin, cisplatin combined with CRM197, and a

Fig. 3



Cisplatin and cross-reacting material 197 (CRM197)-induced U251 cell apoptosis was assayed by fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (Annexin V and PI) staining for flow cytometry for 24 h. (a) Quadrant dot plot by flow cytometry following Annexin V and PI staining. (b) Quantitative analysis of the early and late apoptosis rate. Data are given as mean ± standard deviation of $n = 3$ individual experiments. $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus cisplatin; $^{\Delta}P < 0.01$ versus CRM197. LR, lower right quadrant; UR, upper right quadrant.

Fig. 4



Cisplatin and cross-reacting material 197 (CRM197)-induced changes of MMP in U251 cells were assayed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbo-cyanine iodide (JC-1) staining for flow cytometry for 24 h. (a) Quadrant dot plot by flow cytometry following JC-1 staining. (b) Quantitative analysis of the percentage of cells with depolarized mitochondria (green). Data are given as mean ± standard deviation of $n=3$ individual experiments. $^{**}P<0.01$ versus control; $^{\#}P<0.05$; $^{\#\#}P<0.01$ versus cisplatin; $^{\Delta}P<0.01$ versus CRM197. LR, lower right quadrant; UR, upper right quadrant.

combination of cisplatin with CRM197 plus 10 μmol/l of LY294002 or 1 μmol/l of wortmannin significantly inhibited Akt phosphorylation compared with the control ($P < 0.01$). The combination of cisplatin with CRM197 plus LY294002 or wortmannin significantly inhibited Akt phosphorylation compared with the combination of cisplatin with CRM197 ($P < 0.01$).

MTT results showed that the inhibition rate of cisplatin combined with CRM197 plus LY294002 or wortmannin was significantly increased compared with the combination of cisplatin with CRM197 ($P < 0.01$; Fig. 7).

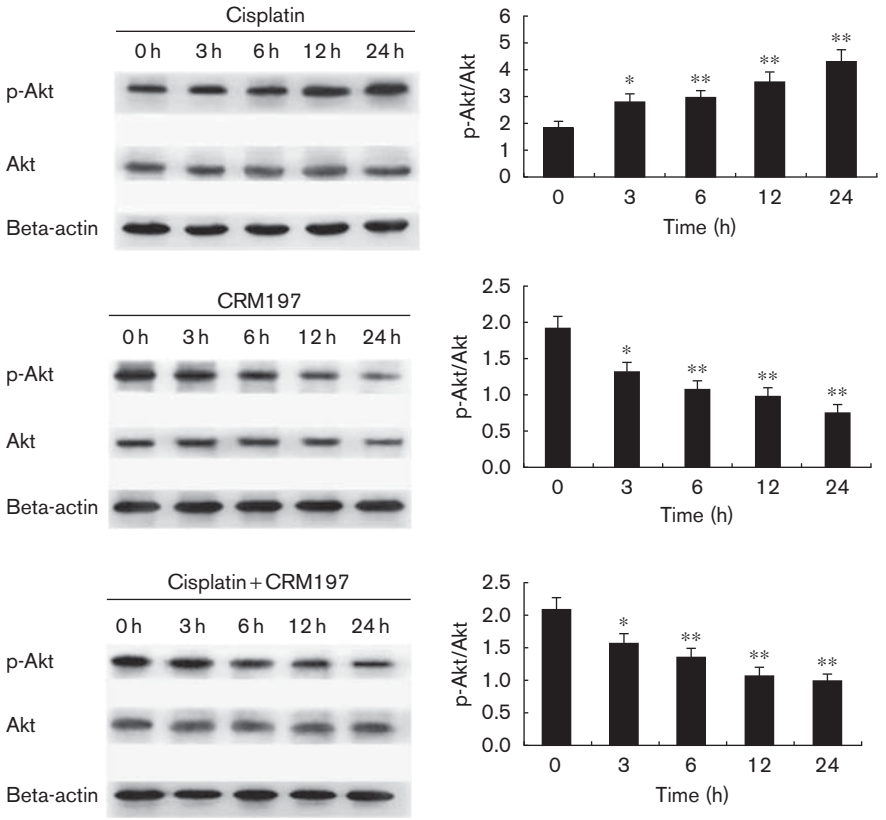
Annexin V and PI staining results showed that the percentage of Annexin V-FITC-positive cells was 5.47 ± 1.57% in the control group, 20.87 ± 2.39% in the LY294002 group, 18.31 ± 2.48% in the wortmannin group, 45.96 ± 3.19% in the group with a combination of cisplatin with CRM197, 64.38 ± 3.20 or 68.75 ± 3.22% in the group of cisplatin with a combination of CRM197 plus LY294002 or plus wortmannin. As shown in Fig. 8, LY294002, wortmannin, cisplatin combined with CRM197, cisplatin combined with CRM197 plus LY294002 or wortmannin significantly induced U251 cell apoptosis compared with the control ($P < 0.01$); the combination of cisplatin with CRM197 plus LY294002 or wortmannin significantly induced U251 cell apoptosis compared with the combination of cisplatin with CRM197 ($P < 0.01$).

Discussion

Some reports demonstrated that combination cancer therapy might induce synergistic anticancer effects and improve the chemotherapy outcomes. In our research, we found that CRM197 combined with cisplatin could inhibit the cisplatin-induced PI3K/Akt pathway to enhance growth inhibition and apoptosis of U251 glioma cells.

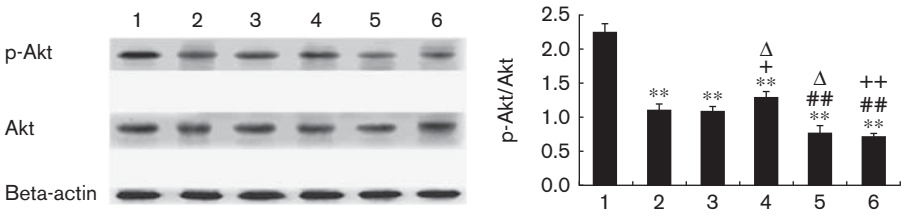
To investigate the effects of cisplatin or CRM197 on HA cells and U251 cells, various doses of the two drugs were tested. The results showed that cisplatin at 5 μg/ml or CRM197 at 1 μg/ml was highly toxic to U251 cells, but did not affect the viability of HA cells. Our data demonstrated that CRM197 could induce U251 cell growth inhibition in a dose-dependent and time-dependent manner, although less effective in comparison with cisplatin. The administration of 1 μg/ml CRM197 inhibited the U251 cell growth and reached a peak at 24 h (Fig. 2). CRM197 also significantly induced U251 cell apoptosis (Figs 3 and 4). The growth inhibition rate induced by CRM197 was 17.97 ± 1.54% assayed by MTT and the percentage of apoptotic cells was 18.88 ± 2.46 or 17.78 ± 2.12% assayed by Annexin V and PI staining or JC-1 staining for flow cytometry at 24 h. Martarelli *et al.* investigated the growth inhibition effects on H295R and SW-13 cell (human adrenocortical carcinoma cell lines) induced by CRM197 for 72 h by

Fig. 5



Western blot analysis of the Akt phosphorylation levels of different durations of time induced by cisplatin, cross-reacting material 197 (CRM197), or cisplatin combined with CRM197 on U251 cells. Data are given as mean ± standard deviation of $n=4$ individual experiments. $^{*}P<0.05$; $^{**}P<0.01$ versus 0 h.

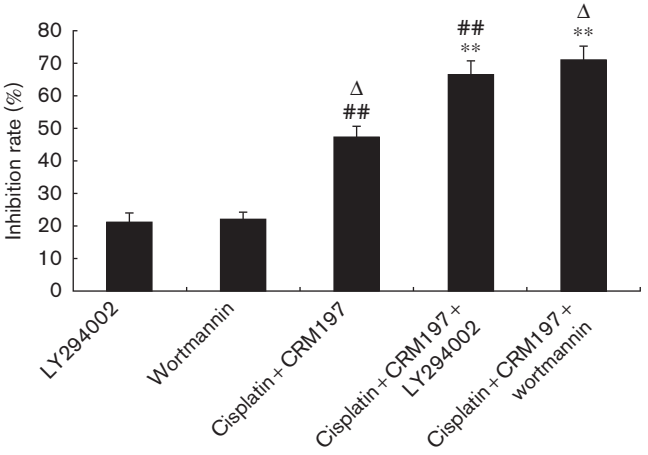
Fig. 6



Western blot analysis of the Akt phosphorylation level in U251 cells treated with different treatments for 24 h. 1–6 represents control, LY294002, wortmannin, cisplatin combined with cross-reacting material 197 (CRM197), cisplatin combined with CRM197 plus LY294002, and cisplatin combined with CRM197 plus wortmannin, respectively. Data are given as mean ± standard deviation of $n=4$ individual experiments. $^{**}P<0.01$ versus control; $^{\#\#}P<0.01$ versus cisplatin combined with CRM197; $^{\Delta}P<0.05$ versus LY294002; $^{+}P<0.05$; $^{++}P<0.01$ versus wortmannin.

MTT. The growth inhibition rate induced by CRM197 at 1 μg/ml was approximately 7% of H295R cells and approximately 10% of SW-13 cells [13]. The administration of 1 μg/ml CRM197 to five gastric cancer cell lines for 72 h significantly augmented the number of apoptotic cells. The maximal and minimal percentages of apoptotic cells were approximately 18% in NUGC3 cells and 6% in MKN74 cells, respectively [14]. Five breast cancer cell lines were treated with 100 nmol/l CRM197 for 48 h. The percentage of apoptotic cells was 58.2% in BT549 cells and approximately 20% in the other cell lines [12]. The percentages of apoptotic cells induced by CRM197 in our research are different from others because of the various cell lines with different sensitivities to CRM197 as well as the

Fig. 7



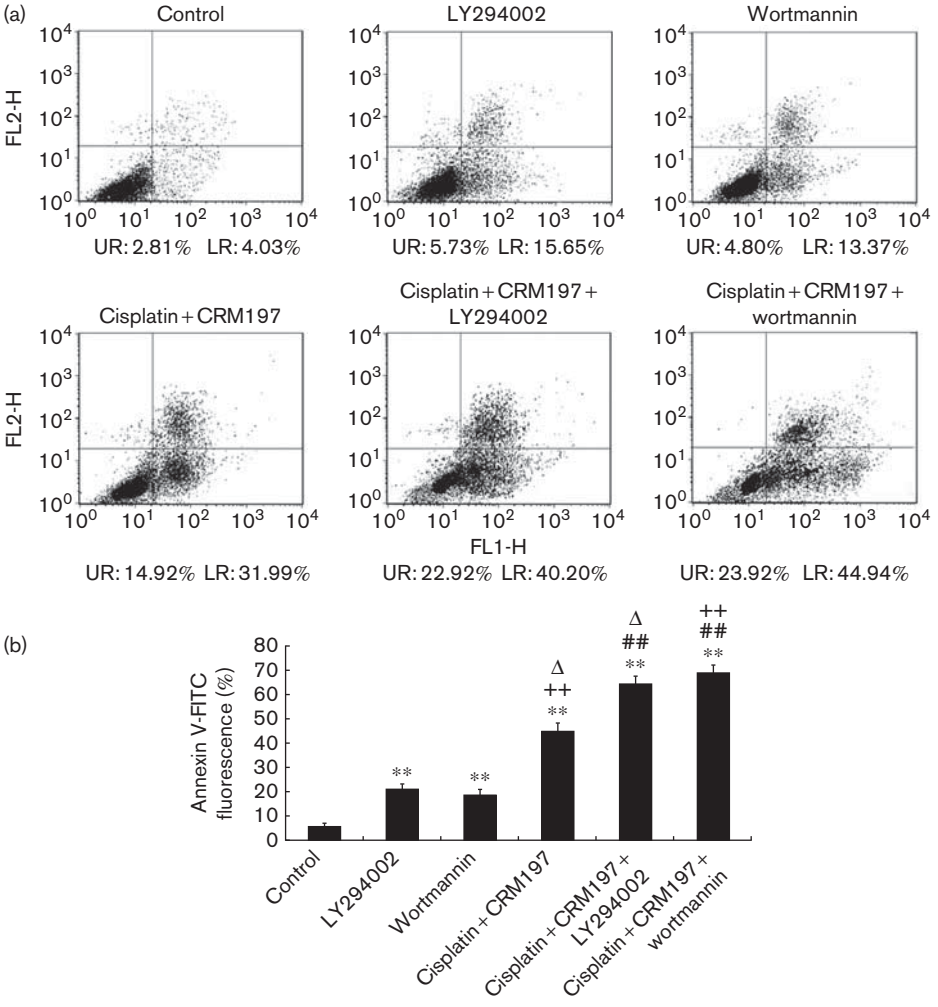
Effects of inhibition of the PI3K/Akt pathway on U251 cell growth. Cells were treated with different treatments for 24 h and then growth-inhibitory effects were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data are given as mean ± standard deviation from four independent experiments ($n=5$ duplicate wells per group). $^{**}P<0.01$ versus cisplatin combined with CRM197; $^{\#\#}P<0.01$ versus LY294002; $^{\Delta}P<0.01$ versus wortmannin.

different administration times. Moreover, we investigated the effects of a combination of cisplatin with CRM197 on U251 cell growth and apoptosis. Compared with the treatment of cisplatin or CRM197 alone, the combination of cisplatin with CRM197 for 24 h significantly inhibited U251 cell growth (Fig. 2) and induced apoptosis (Figs 3 and 4), which showed that CRM197 could enhance the anticancer effects of cisplatin against U251 cells.

The PI3K/Akt signaling pathway has been implicated in glioma progression, invasion, and angiogenesis [25–27]. Activation of Akt involves phosphorylation of threonine 308 and serine 473. Previous research showed that cisplatin could induce Akt activation in ovarian, pancreatic, breast, and small cell lung cancer cell lines as well as in Rat-1 fibroblasts, as measured by threonine 308 phosphorylation. In this study, cisplatin activated Akt phosphorylation in U251 cells, as measured by serine 473 phosphorylation (Fig. 5). Inhibition of Akt activity decreased the survival of the cells exposed to cisplatin, suggesting that cisplatin-induced Akt activation leads to cisplatin resistance [23,24]. CRM197 has previously been shown to inhibit PI3K/Akt signaling by blocking the cleavage of diphtheria toxin receptor to a soluble form HB-EGF (sHB-EGF) [9,12,14]. sHB-EGF is a potent mitogen and chemoattractant for a number of different cell types. Binding of sHB-EGF to epidermal growth factor receptor can activate multiple signaling pathways, including the PI3K/Akt pathway [11]. Our data showed that CRM197 inhibited Akt phosphorylation in U251 cells (Fig. 5). Meanwhile, the combination of cisplatin with CRM197 significantly inhibited cisplatin-induced Akt phosphorylation. The specific PI3K inhibitor LY294002 or wortmannin could inhibit human glioma cell growth associated with reduced p-Akt [28,29]. These two inhibitors may have limited clinical use, however, because they have potential adverse side effects, poor pharmacologic properties, low stability, and poor solubility [30]. Based on previous reports and the data from our preliminary experiment, we demonstrated that LY294002 and wortmannin decreased p-Akt expression in a dose-dependent manner. LY294002 at 10 μmol/l or wortmannin at 1 μmol/l was the optimal dose that could inhibit the p-Akt expression in U251 cells maximally (data not show) [31,32]. To further verify that the inhibition of the PI3K/Akt pathway might contribute to the depressed growth and increased apoptosis in U251 cells induced by a combination of cisplatin with CRM197, LY294002 and wortmannin were added. Our study found that the combination of cisplatin with CRM197 plus LY294002 or wortmannin could significantly inhibit Akt phosphorylation (Fig. 6), growth and apoptosis of U251 cells compared with groups without LY294002 or wortmannin (Figs 7 and 8), suggesting that the inhibition of the PI3K/Akt pathway could augment the sensitivity of tumor cells to cisplatin-induced cytotoxicity and promote apoptosis. However, no significant difference was found between the groups that had LY294002 and wortmannin.

In conclusion, CRM197 could induce growth inhibition and apoptosis of U251 cells. Cisplatin combined with CRM197 could inhibit the PI3K/Akt pathway induced by cisplatin to enhance the growth inhibition and apoptosis of U251 cells. The treatment of CRM197 in conjunction with cisplatin may lead to improved clinical outcomes in glioma patients.

Fig. 8



Effects of inhibition of the PI3K/Akt pathway on U251 cell apoptosis were assayed by fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (Annexin V and PI) staining for flow cytometry for 24 h. (a) Quadrant dot plot by flow cytometry following Annexin V and PI staining. (b) Quantitative analysis of the early and late apoptosis rate. Data are given as mean ± standard deviation of $n = 3$ individual experiments. $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus cisplatin combined with CRM197; $^{\Delta}P < 0.01$ versus LY294002; $^{++}P < 0.01$ versus wortmannin. LR, lower right quadrant; UR, upper right quadrant.

Acknowledgements

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Conflicts of Interest

There are no conflicts of interest.

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