Synergistic Anti-glioma Effects in Vitro and in Vivo of Enediyne Antibiotic Neocarzinostatin and Paclitaxel via Enhanced Growth Delay and Apoptosis-Induction

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Neocarzinostatin (NCS) is a member of enediyne antibiotics with high anticancer potential. Our study was performed to explore the synergistic anti-glioma effects of NCS and paclitaxel (PTX) in vitro and in vivo. By 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the cytotoxicities of the drugs to human glioma cells U87MG and rat glioma cells C6 were evaluated. The results showed that the combinations of NCS and PTX can synergistically inhibit glioma cells survival. Cell apoptosis was detected by flow cytometry, and the results showed that the combinations of NCS and PTX synergistically enhanced apoptosis ratio of glioma cells. Western blot revealed that the cell signaling pathways of proliferation and apoptosis were synergistically regulated, in which Akt was synergistically inactivated, p53 was up-regulated with down-regulation of bel-2. Meanwhile, with the subcutaneous model of U87MG cells and intracerebral implantation model of C6 cells, the combination strategy could synergistically delay the glioma growth and significantly prolong the survival of rats bearing orthotopic glioma. This study demonstrates that the combination of NCS and PTX can potentiate the effect on survival and apoptosis of glioma cells via suppression of Akt, bel-2, and activations of p53; Meanwhile, the in vivo studies also confirmed that the combination of NCS and PTX synergistically inhibit the gliom growth. Our data about the combinational effects of NCS with PTX may provide an alternative strategy for glioma therapy.

Key words neocarzinostatin; paclitaxel; synergistic effect; cytotoxicity; apoptosis induction; glioma growth inhibition

Glioma is the most common primary cancer in central nervous system, especially in brain. Despite the low percentage of cancers, glioma is of high mortality and morbidity because of the specialty of favorable anatomical location. Especially, the glioblastoma is the most aggressive and malignant brain cancer with rapid development. The 5-years survival of glioblastoma patients are only 5%, and the median survival times of glioblastoma patients are 12–15 months.1–3 At present, the common clinic treatments of glioblastoma are surgery concomitant with radiotherapy or chemotherapy. But it is very difficult to control the progress of glioblastoma, the recurrence after first therapy is rapid for most of patients, meanwhile, the poor prognosis and survival outcome are also partially associated with races and ages, in which although the morbidity and the mortality are significantly different, the overall survival rate is not dramatically improved.4–6 The histological complex of glioma structure made the therapy outcome (survival extension) of regular postoperative radiotherapy or chemotherapy can not meet the promise of patient in different histological grade, stage and classification,7 which made it very difficult to obtain satisfactory outcome, and especially the side effects of postoperative cognitive damages seriously impaired the life quality of glioma patients with suffering aggravation.8,9 At present, there are not many choices for the postoperative chemotherapies of glioma patients. Although the malignance of glioma urgently promoted the developments of some attractive medicinal strategies, including targeting therapy, gene therapy and immunotherapy, the first-line strategies of glioma therapy are still mainly alkylating agents concomitant or after radiotherapy,10–13 nevertheless, the consequential low sensitivities or intolerance to novel therapies and the multi-drugs resistance (MDR) to the chemotherapy have badly stroke the patients’ expectation, which drove more efforts to pressingly pursue the effective and feasible clinic strategies.14–17

Enediyne antibiotic is a group of natural agents produced by microbial metabolism with potential anti-tumoral effects. At present, enediyne antibiotics are the most potent in currently known anti-tumoral agents. By the unique molecular architecture, the enediyne antibiotics are divided into 2 subgroups, 9-membered cyclic and 10-membered cyclic enediynes, in which the 2 components of 9-membered cyclic enediyne, the chromophore and apoprotein, are noncovalent binded.15–17 Neocarzinostatin (NCS) is the first naturally produced enediyne antibiotic isolated from Streptomyces carzinostaticus var. F-41, which has ever been performed preliminary observation as antitumor candidate.18–21 The NCS is a 1:1 noncovalent complex of an apoprotein and a chromophore, in which the chromophore plays the antitumor activity by DNA damaging activity.18,20 The higher efficacy of enediyne antibiotics has intrigued intensive researches on cancer inhibition. However, for most enediyne antibiotics, although the strong biological activities, especially anticancer capacity can be observed, their application are often limited by high toxicity and also low efficacy in vivo.21

To attenuate the side effects and improve the efficacy of enediyne antibiotics, combinational administration with common anticancer agent may be a feasible method. Paclitaxel (PTX), a microtubule stabilizing agent with highly potent anticancer potential, is recommended as a first-line strategy chemotherapeutic agent against many kinds of cancers, but the clinic

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implication of PTX to brain tumor is limited by the drug resistance and other side effects.\textsuperscript{22,23} Interestingly, a series of reports showed that although the blood–brain barrier (BBB) restricts the entry of many drugs into the brain, the effects of PTX on brain tumor from clinic and preclinical studies remains controversial. Heimans et al.\textsuperscript{24} reported that PTX may not cross the intact BBB, but its concentrations in the tumor tissue were in the therapeutic range, in which the BBB disruption or leakage of brain tumor may partially facilitate the PTX penetration.\textsuperscript{25–27} Many investigations revealed that the combination of PTX with other effective chemotherapeutic drugs, RNA interference or immunotherapy could improve anti-glioma efficacy \textit{in vitro} and \textit{in vivo}.\textsuperscript{28–36} The fundamental challenge to the discovery of microtubule-targeting drugs may be mainly attributed to the drug resistance.\textsuperscript{31}

It has been reported that enediyne agents (neocarzinostatin and lidamycin) and conventional chemotherapy (5-FU, gel-damycin or temozolomide) synergistically inhibited tumor cell proliferation or tumor growth,\textsuperscript{32–34} but the combination of enediyne agents and PTX has not ever been focused on. Our study intended to investigate the combinational inhibitory effects of NCS and PTX on glioma cell growth \textit{in vitro} and \textit{in vivo}. Cell proliferation and apoptosis induction of C6 and U87MG cells were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI) staining assay to estimate the synergistic effects of NCS plus PTX on glioma cells \textit{in vitro} and \textit{in vivo}. With Western blot, further researches on the corresponding signaling pathways were probed. \textit{In vivo}, with subcutaneous xenograft model of U87MG cells and cerebral orthotopic implantation model of C6 cells, the synergistic anti-glioma effects of NCS with PTX were evaluated. Our interesting report indicates that the combination of NCS with PTX might be a prospective strategy for glioma clinical therapy.

**MATERIALS AND METHODS**

**Reagents and Animals** NCS and MTT were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). PTX was purchased from Bristol-Myers Squibb Company (New York, NY, U.S.A.). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Gaithersberg, MD, U.S.A.). Annexin V-FITC/PI double staining kit and Matrigel were purchased from BD Biosciences (San Diego, CA, U.S.A.). The primary antibodies specific for β-actin, Akt, p-akt, p53, and bcl-2 were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). The human U87MG glioma cells and rat C6 glioma cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The specific-pathogen-free (SPF) male athymic mice (18–22 g) and male Wistar rats (180–220 g) were purchased from Vitalriver (Beijing, China). All the procedures of animal experiments were performed according to the protocol approved by the Animal Care and Use Committee of Shandong Institute for Drug Control.

**MTT Assay and Examination of Drug Combination** The human glioma U87MG cells and rat glioma C6 cells (2×10^5 cells/well) were respectively seeded in 96-well plates. After 24h incubation at 37°C in 5% CO₂, the media were replaced, and the cells were grouped and treated with different drugs strategies. After further 48h incubation, a standard MTT assay was performed to determine the \textit{in vitro} cytotoxicity. The culture media were displaced and the 20µL MTT were added for 4h incubation, then, 150µL dimethyl sulfoxide (DMSO) were added in each well to dissolve the formazan. After 10min oscillation, optical density (OD) values were determined at 570nm with microplate reader. All the experiments were carried out in triplicate. By the method of Chou and Talalay,\textsuperscript{35–37} the combination indexes (CIs) isobologram were calculated and plot with CalcuSyn software, and a CI value >1, CI value =1, and CI value <1 mean antagonism, additivity, or synergism, respectively.

**Apoptosis Assay** With commercial apoptosis analysis kit, Annexin V-FITC/PI double staining assay was performed as the operating manual. In brief, the cells in exponential growth phase were passaged to 6-wells plates (2×10^4 cells/well) for 24h, then the cells were treated with drugs (Control: Saline; NCS: 300nm; PTX: 6nm; Combination: NCS+PTX). After further 48h incubation, the cells were harvested and stained with Annexin V-FITC/PI for 30min. The samples were determined apoptosis by flow cytometry. The experiments were carried out in triplicate.

**Western Blot** C6 cells and U87MG cells were seeded in flasks and treated as the above dose regimens for 48h, the cells were scraped in ice-cold phosphate buffered saline (PBS) and centrifuged at 1000×g for 5min at 4°C. The pellets were dissolved in radio immunoprecipitation assay (RIPA) lysis buffer for 40min. After centrifuge, the cell supernatants were transferred to new tubes. The protein concentrations were measured with the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, U.S.A.). The proteins (20µg/lane) were loaded to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the protein was transferred to Nylon membrane. After incubation with primary antibodies (1:1000) at 4°C overnight, the membranes were probed with secondary antibodies (1:5000) and developed with enhanced chemiluminescence blot detection system (Millipore Corporation, Billerica, MA, U.S.A.). Then the density of protein was quantified with Bio-Rad Image Lab 4.1, and the relative expression levels of the proteins were normalized to the density of β-actin.

**Assessment of Anti-glioma Activity with U87MG Xenograft Model** To confirm the synergistic efficacy of NCS and PTX, the human glioma cells U87MG xenograft model was established as previous description.\textsuperscript{29,38,39} The human U87MG glioma cells (1×10^3 cells) were injected subcutaneous (s.c.) into the right flanks of athymic nude mice. After 20d, the tumors were incised into 1mm³ pieces. The pieces were implanted to the right flanks of athymic nude mice (n=24 totally). When the tumor volumes reached to 100mm³, the mice were randomly and equally divided into 4 groups and treated as the therapy strategies (1. Control group: saline via intraperitoneal (i.p.) injection; 2. NCS Group: 7.5mg/kg via tail vein injection, once every week for 2 weeks; 3. PTX Group: 10mg/kg via i.p. injection, once every 5d for 3 times; 4. Combination Group: NCS and PTX were administrated as the above). Tumor volume and body weight were recorded twice every week. The tumor volumes were calculated with the following formula: \(a×b^2/2\), in which \(a=\) the maximal length and \(b=\) the maximal width. At 28th day, all the mice were decapitated.
Establishment of Rat Brain Orthotopic Implantation Model with C6 Glioma Cell

The orthotopic implantation model of C6 cells was established to directly evaluate the efficacy. Briefly, all the rats were anesthetized and immobilized in a stereotactic frame, and then the heads were shaved and disinfected with 75% ethanol. The skull was exposed by midsagittal incision of scalp and drilled with high-speed drill at the appropriate coordinate (3 mm to the right and 1 mm anterior to the bregma). A microsyringe with 29-gauge needle was vertically inserted into the caudatum (5 mm depth), and then the rat C6 glioma cells were injected slowly over a 10-min period with automatic microinjection. After injection, the burr hole was filled with sterile bone wax and the scalp incision was closed with suture.

Assessment of Anti-glioma Activity with Glioma Orthotopic Model

For tumor size assay, the $1 \times 10^5$ rat C6 glioma cells in 10 µL for each rat were orthotopically injected. At

Table 1. The Cytotoxicities of Neocarzinostatin (NCS) and Paclitaxel (PTX) to Rat C6 Glioma Cells and Human U87 Glioma Cells Were Detected, in Which the IC₅₀ Values Were Calculated

<table>
<thead>
<tr>
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<th>IC₅₀ values (nM)</th>
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<tbody>
<tr>
<td>Rat C6 glioma cells</td>
<td>493.64±23.45</td>
</tr>
<tr>
<td>U87 glioma cells</td>
<td>462.96±16.39</td>
</tr>
<tr>
<td>NCS</td>
<td>6.53±0.32</td>
</tr>
<tr>
<td>PTX</td>
<td>5.76±0.37</td>
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Fig. 1. The Proliferations of the C6 Cells and U87MG Cells Treated with Drugs Were Determined with MTT Assay, and the Combination Indexes (CI) of NCS and PTX Were Plotted

MTT assay showed that NCS and PTX presented potently synergistic inhibitory effects on the proliferation of C6 cells (A) and U87MG cells (B). The CIs plots and normalized isobologram of NCS and PTX showed synergistic effects on the cell proliferations of C6 cells (C) and U87MG cells (D). All the data were presented as the mean±standard deviation (S.D.) (n=3).
7th day, the orthotopic implanted rats ($n=32$) were randomly and equally divided into 4 groups, every group were treated as the above strategy. After 30d, the rats were decapitated. The brains were excised and fixed in 10% formalin for 2d. The fixed brain samples were embedded in paraffin and sectioned. The routine hematoxylin and eosin (H&E) staining were performed. The stained brain slices were photographed at 40×. The maximal length and width of the tumor area were recorded. And then the tumor volumes were calculated as the above-mentioned formula. For survival analysis, the $2\times10^5$ rat C6 glioma cells in 10µL for each rat were orthotopically injected. The rats ($n=32$) were grouped and treated as the above procedure. During the treatment, the survival days of every rat were recorded for Kaplan–Meier assay.

**Statistical Analysis** All the data were presented as the mean±standard deviation (S.D.). One-way ANOVA was performed with the SPSS 13.0 for multiple comparisons. For Kaplan–Meier assay, the survival curves were established and compared by the log rank test. In all test, the statistical significance was observed when $p$ value <0.05.

**RESULTS**

**Potentiated Cell Survival Inhibition by the Combination of NCS and PTX** Firstly, the anti-proliferation effects of NCS and PTX to C6 cells and U87MG cells were determined by MTT assay. The cells were treated with various concentrations of NCS and PTX for 72h, and the IC$_{50}$ values of NCS and PTX to C6 cells and U87MG cells were $493.64\pm23.45$ nM, $462.96\pm16.39$ nM, $6.53\pm0.32$ nM, and $5.76\pm0.37$ nM, respectively (Table 1). Then, the examination of drug combination showed that the cell survival data showed highly potentiated anti-survival effects on C6 and U87MG glioma cells by combination of NCS and PTX (Figs. 1A, B). The CI values of the two drugs at different combination regimens were calculated and plotted with normalized isobologram (Figs. 1C, D), in which the values displayed synergistic anti-glioma cell survival effects of NCS and PTX in vitro.

**Enhancement of Cell Apoptosis-Induction by the Combination of NCS and PTX** By Annexin V-FITC/PI double-staining assay, the glioma cell apoptosis ratios were analyzed. The apoptosis inductions were enhanced by combination of
NCS and PTX both in C6 (Fig. 2A) and U87MG cells (Fig. 2B). Evidently, the administration of NCS plus PTX showed a higher apoptotic percentage than the single agents, with significant differences in C6 cells (Fig. 2C) and U87MG cells (Fig. 2D), indicating an in vitro synergetic apoptosis-induction effect of NCS and PTX on glioma cells.

The Regulations of Cell Signaling by the Combination of NCS and PTX  

By Western blot assay, we analyzed the regulation of apoptotic signaling pathway by the combination of NCS and PTX. As illustrated, the expressions of proliferation and apoptosis-related proteins were significantly regulated by the combinational treatment (Fig. 3). In the detection of proliferation signal pathway, the phosphorylations of Akt was significantly inactivated by the combination of NCS and PTX for both rat C6 glioma cells and human U87MG glioma cells (Fig. 3A). In apoptosis signaling detection, p53 proteins were significantly up-regulated by NCS plus PTX with markedly down-regulated expressions of bcl-2 by the combination of NCS and PTX, compared with the single administration of NCS or PTX (Fig. 3B).

The Promotion of NCS and PTX on Glioma Growth Delay and Survival in Vivo  

Firstly, we established subcutaneous xenograft model of human U87MG glioma cell in nude mice to investigate the anti-glioma effects of NCS plus
PTX. By recording of the tumor volumes and body weights, the results indicated that NCS plus PTX is more efficient than anyone single agent (Fig. 4A). After scarification of animal, the tumor weights recording also showed that NCS plus PTX is much more efficient than the single treatment with NCS or PTX in suppressing glioma growth (Fig. 4B).

Then, the rat brain orthotopic implantation model of rat glioma C6 cells was established to further investigate the efficacy on glioma growth inhibition of NCS plus PTX. For tumor volume determination, the brain tumors were sliced and H&E stained, in which the photographs depicted that NCS plus PTX exhibited significant effects on glioma growth inhibition (Fig. 5A). The tumor volumes were collected and estimated, and the statistical data showed that the combination of NCS with PTX presented more efficient anti-glioma efficacy than single treatment of NCS or PTX (Fig. 5B). Meanwhile,
the survival ratio was presented as Kaplan–Meier analysis, in which the common survival ratio of NCS plus PTX was significantly increased than NCS or PTX single treatment groups (Fig. 5C). The survival days recording of rats showed that the median survival days of the combination group was significantly prolonged, compared with NCS or PTX single treatment group (Fig. 5D).

**DISCUSSION**

In brain cancer, the glioma is the most common primary cancer with high mortality and morbidity. The control of glioma progress is far less ideal. The complexes of tissue cancer with high mortality and morbidity. The control of cell proliferation and induce apoptosis. 

Although postoperative adjuvant chemotherapy or pharmacotherapy is now being adopted greater values during the treatment period of glioma. Especially, postoperative adjuvant temozolomide combined with radiotherapy has been the standard strategies for glioma treatment. However, recurrences result from the resistances to chemotherapy and radiation has been the great challenges of glioma patients. To overcome these limitations, novel chemotherapies or combination treatment are drawing great gravitation to preclinical and clinical research. Enediyne antibiotics, a group of DNA-cleaving natural product, have been proposed for treatment of various human cancers, especially NCS, has been an effective and novel chemotherapeutic agent to nervous system cancer, therefore, our study was designed to test the combinational effects of NCS plus PTX.

Previous reports showed that NCS can efficiently inhibit cell proliferation and induce apoptosis. Our study, in consistent with previous reports, explored that NCS can efficiently decrease the survival and induced cell apoptosis of human U87MG glioma cells and rat C6 glioma cells, hence promote us to further explore the combinational effects of NCS with PTX on glioma cell. The combination of NCS and PTX excitingly induced synergistic effects on proliferation suppression and apoptosis induction of U87MG cells and C6 cells. These interesting results preliminarily suggested that the combination of NCS and PTX may be an applicable therapy to glioma treatment.

Cell proliferation and apoptosis are complicate progress, in which many signaling pathway/mechanism, including Akt, mitogen-activated protein kinase (MAPK), p53 and caspase pathway, are involved. As previous report, bcl-2 and caspase-3 are involved in NCS-induced apoptosis. In our study, we observed that PTX potentiated the cytotoxicity of NCS to glioma cell, in which Akt was synergistically inactivated by the combination of PTX and NCS; Meanwhile, NCS-induced apoptosis was also increased by PTX addition, in which up-regulation of p53 and degradation of bcl-2 were observed. The Western blot assay confirmed and explained the combinational mechanisms of NCS with PTX on glioma cell proliferation and apoptosis.

To further verify the applicability of our suggested combinational strategy, human U87MG cells subcutaneous xenograft model and rat C6 cells intracerebral orthotopic implantation model were used to detect the combinational in vivo effects on the growth delay of glioma. As indicated, the results of sub-cutaneous mouse model of U87MG cells showed that single administration of NCS or PTX can significantly inhibit the U87MG glioma growth, when combinational treated, the efficiency of combinational strategy were notably much higher than single usage on glioma growth delay. The rat intracerebral implantation of C6 cells in our research also indicated that the combinational strategy exerted much higher efficacy in glioma growth delay than single administration of NCS and PTX, moreover, the survival time of rat intracerebral implantation model was significantly prolonged by the combinational administration of NCS and PTX. The exciting in vivo data firmly validated the in vitro synergistic effect on glioma inhibition of NCS and PTX.

In conclusion, the major finding of our research presented that NCS plus PTX exerted the potentiated anti-glioma efficacy in vitro and in vivo, in which the combination of lidamycin (LDM) and temozolomide (TMZ) results in the synergistic enhancements of glioma cell proliferation inhibition and apoptosis induction in vitro, meanwhile, combination of NCS and PTX also presented higher therapeutic efficacy in subcutaneous xenograft and intracerebral orthotopic implantation of glioma cells. Therefore, our experiment clearly manifested that combinational administration of NCS and PTX may be a valuable therapeutic strategy for glioma treatment, and the combination therapy maybe deserves further evaluation in clinical trials.

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**Conflict of Interest** The authors declare no conflict of interest.

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