Effects of Tetrandrine on Apoptosis and Radiosensitivity of Nasopharyngeal Carcinoma Cell Line CNE

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Abstract   Tetrandrine is known to exert antitumor effect, however, little is known about its effect on nasopharyngeal carcinoma cells. In this study, we tested tetrandrine-induced apoptosis and radiosensitivity in nasopharyngeal carcinoma cell line CNE and investigated the possible mechanisms. Using flow cytometry and DNA electrophoresis, we found that tetrandrine could induce cell apoptosis. Further, it was shown that the level of *Bcl-2* mRNA decreased and *Bax* mRNA increased after addition of tetrandrine by using reverse transcription-polymerase chain reaction. X-ray-induced G_2 arrest was abrogated by treatment with tetrandrine, as detected by flow cytometry and mitotic index. The accumulation of cyclinB1 protein and the suppression of Cdc2 tyrosine-15 and Cdc25C serine-216 phosphorylation were detected in irradiated cells treated with tetrandrine using Western blot analysis. Taken together, these results show that tetrandrine can induce apoptosis and abrogate radiation-induced G_2 arrest in CNE cells.

Keywords   tetrandrine; nasopharyngeal carcinoma; apoptosis; gene regulation; radiosensitivity

Nasopharyngeal carcinoma (NPC), a tumor of epithelial origin, is one of the most common malignant tumors in southern China. NPC is markedly radiosensitive, hence radiotherapy is the mainstay of treatment [1]. A principal therapeutic purpose of exposing a target volume to irradiation is to produce irreversible DNA damage in malignant cells while keeping the non-malignant tissues unaffected. Moreover, this neoplasm has a high treatment failure rate because of its rapid growth and invasive behavior [2]. The fast kinetics of cell cycling in the malignant cells weakens the treatment effect during conventional fractionated radiation therapy and requires reduction in overall treatment time to achieve destruction of the tumor cells with minimal adverse effects on the surrounding normal tissue.

Almost all NPC cases are accompanied by gene mutation and/or overexpression [3]. The genes found to be associated with NPC include oncogenes (such as *Bcl-2*, *c-myc*, *Bcl-xL*, and *caspase* genes), tumor suppressor genes (such as *p53* and *Bax* genes), growth factor receptor genes, and cyclin genes. Among these genes, the *Bcl-2* family genes seem to function as oncogenes and encode proteins that function to regulate crucial steps of the apoptotic pathway. A high level of Bcl-2 protein ensures the survival advantage to the clonal population and promotes tumorigenesis [4]. However, pro-apoptotic Bax-like proteins act as tumor suppressors [5], and a high level of Bax protein promotes tumor cell apoptosis [6].

Cell cycle progression is constantly monitored to ensure that the correct sequence of events in the process of cell division is achieved and cells with DNA damage do not replicate [7]. Irradiation often causes cell cycle arrest in tumor cells. After DNA-damaging treatment, cell cycle checkpoints stop or slow down cell cycle traverse. As a result, after repair of the damage, the correct order of cell cycle transitions can be re-established. However, if repair can not be effected, cells are committed to die [8,9]. DNA damage by ionizing radiation activates G_2 checkpoint signaling pathways that communicate through several downstream mediator

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proteins, such as tumor suppressor protein p53, cyclinB1, checkpoint kinases Chk1 and Chk2, and mitotic kinase Cdc2 [8,10–12].

Tetrandrine, a bisbenzylisoquinoline alkaloid with an interesting structure, was isolated from the root of *Stephania tetrandra* and used in traditional Chinese medicine as an antirheumatic, anti-inflammatory, and antihypertensive agent. Recently, several studies have shown that tetrandrine possesses antiproliferative activity, induces apoptosis of malignant cells, and enhances radiation sensitivity in cultured tumor cells [13]. However, the molecular mechanisms by which tetrandrine induces apoptosis and radiosensitivity remain poorly understood.

In our present study, we investigated the molecular and cellular pharmacology of tetrandrine in human nasopharyngeal carcinoma CNE cells. We found that tetrandrine induces apoptosis by down-regulating *Bcl-2* mRNA and up-regulating *Bax* mRNA. We also found that tetrandrine increases cyclinB1 protein, promotes Cdc2 tyrosine-15 [Cdc2(Tyr15)] and Cdc25C serine-216 [Cdc25C(Ser216)] dephosphorylation in irradiated CNE cells, and ultimately abrogates radiation-induced G_2 arrest.

Materials and Methods

Cell culture

Human nasopharyngeal carcinoma CNE cells were purchased from the Institute of Medicinal Biotechnology (Shanghai, China). Cells were cultured in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (15% by volume), penicillin G (50 U/ml), and streptomycin (50 μg/ml). The cell line was maintained in the exponential growth phase and provided fresh medium every 2–3 d.

Drugs and antibodies

Tetrandrine used in the experiment was obtained from YinTao Medical (Fuzhou, China). The solution of tetrandrine was easily diluted in phosphate-buffered saline (PBS) to achieve a final concentration of 104 μM as a stock solution, which was diluted to different concentrations prior to use. Rabbit monoclonal anti-cyclinB1, phospho-Cdc2-Tyr15, phospho-Cdc25C-Ser216, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-rabbit immunoglobulin G horseradish peroxidase-coupled antibodies were purchased from Sigma (St. Louis, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell proliferation

To assay the cytotoxicity of tetrandrine, cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well and allowed to adhere for 24 h at 37 °C. Cells were cultured in the presence of 10–70 μM tetrandrine for 48–72 h, then MTT (Sigma) was added to each well. Following a 4-h incubation at 37 °C, dimethylsulfoxide (Sigma) was added to each well to dissolve dark blue crystal product. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad, Hercules, USA).

DNA agarose gel electrophoresis

CNE cells were treated with 20, 30, or 40 μM tetrandrine for 72 h, and untreated cells were set as negative controls. Then 2×10^6 cells from each group were harvested and purged with PBS. Lysis buffer together with protease K was added to the pellet. The mixture was maintained in a water bath at 37 °C overnight for complete lysis. The cell lysate was then centrifuged at 12,000 *g* for 10 min at room temperature and the supernatant was transferred to another clean tube. Extraction of nucleic acid was carried out with phenol:chloroform (1:1 by volume), phenol:chloroform: isopropyl alcohol (25:24:1 by volume), and chloroform. Sodium acetate (3 M) and cold ethanol were then added to the supernatant. After overnight incubation at –20 °C, the mixture was centrifuged at 12,000 *g* for 10 min at –10 °C. The pellet was dissolved in 20 μl TE buffer. DNA extract was loaded on 1.5% agarose gel containing ethidium bromide (Sigma). Electrophoresis was carried out in 1×TAE buffer at room temperature under the conditions of 75 V, 40 mA for 1–2 h. Electrophoretic bands were analyzed using image analysis software (Kodak, Rochester, USA).

Determination of apoptosis

Apoptosis was detected by translocation of phosphatidyl serine to the cell surface with the annexin V-FLUOS kit (KeyGen, Nanjing, China). The fraction of annexin V-positive cells was measured with CellQuest software 5.0 (BD Biosciences, San Jose, USA).

Examination of *Bcl-2* and *Bax* mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Eppendorf tubes and tips used in RT-PCR were soaked in 0.1% diethyl pyrocarbonate (Sigma) for 24 h, then dried and sterilized prior to use. RNA extraction was carried out following instructions from the Trizol reagent manufacturer (TaKaRa, Dalian, China). Cells were collected and

pipetted in Trizol reagent ($5-10\times10^6$ cells/ml) for full lysis. Following 5 min of incubation at $15-30$ °C, chloroform (0.2 ml/ml) was added and mixed completely with the lysate by vibration for 15 s. The lysate was then incubated at $15-30$ °C for $2-3$ min, and subjected to centrifugation at 12,000 *g* for 15 min at $2-8$ °C. The water phase supernatant was transferred to another clean tube, incubated with isopropyl alcohol (0.5 ml/ml) for 10 min at $15-30$ °C, then centrifuged at 12,000 *g* for 10 min at $2-8$ °C. The RNA pellet was washed with 75% ethanol (1 ml/ml by centrifugation at 7500 *g* for 5 min at $2-8$ °C. After drying at room temperature, the RNA pellet was dissolved in RNase-free water. The primers for *Bcl-2*, *Bax*, and *β-actin* genes were all synthesized by Sangon (Shanghai, China). The primer sequences were: *Bcl-2*, (forward) 5′-GGACCACCGCATCTCTACAT-3′, (reverse) 5′-GCACT-TTCTTCGCAGTTTCC-3′ (350 bp); *Bax*, (forward) 5′-GAGGATGATTGCCGCCGTGGACA-3′, (reverse) 5′-GGTGGGGGTGAGGAGGCTTGAGG-3′ (275 bp); and *β-actin*, (forward) 5′-TGACGGGGTCACCACACTGTG-CCCATCTA-3′, (reverse) 5′-CTAGAAGCATTTGCGGTG-GACGATGGAGGG-3′ (661 bp). The AMV one-step RT-PCR kit was from Bao Bioengineering (Dalian China). Each pair of primers was amplified through 35 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C or 57 °C for 45 s, and extension at 72 °C for 45 s with a 7 min final extension. The amplification product was then loaded on 1.5% agarose gel containing 0.3 μg/ml ethidium bromide for electrophoresis. Image analysis software was used to capture the band images.

Clonogenic assays

Exponentially growing cells were irradiated using an X-ray source at doses of $0.5-10$ Gy at room temperature, then incubated in the presence or absence of tetrandrine (2 μM) for 24 h. Following this treatment, cells were washed in PBS and trypsinized. Cells were seeded in a 24-well plate in 5 ml medium at a density of 200 cells/well. Colonies were grown for $10-14$ d. Plates were washed in PBS and colonies were fixed with 95% ethanol. Staining was carried out with 0.1% crystal violet solution. Colonies of more than 50 cells were counted for calculating surviving fraction. Six parallel samples were scored for each treatment condition.

Flow cytometry analysis of DNA content

Cells were seeded and irradiated at a dose of 4 Gy in 25 ml culture flasks then incubated in the presence of tetrandrine (0.2 and 2.0 μM) for 24 h. Cells (1×10^6) from each group were harvested and washed with PBS. Cold

ethanol (70%) was added to the pellet for a 1 week fixation. Cells were stained with propidium iodide (PI; Sigma) and subjected to flow cytometry (BD Biosciences) for cell cycle analysis.

Mitotic index

Cells were pelleted by centrifugation (500 *g*, 5 min, 4 °C), washed with ice-cold PBS, and resuspended in 0.5 ml cold, half-strength PBS for 10 min. They were then fixed with 6 ml 2% of ethanol:acetic acid (3:1 by volume) for 30 min at 4 °C. Samples of the fixed cells were subsequently pelleted by centrifugation (500 *g*, room temperature, 5 min), resuspended in 0.5 ml ethanol:acetic acid (3:1 by volume) for 10 min, and dropped onto glass slides that were air-dried and stained with Giemsa. For each sample, at least 500 cells were randomly counted using light microscopy, and mitotic cells were scored by their lack of a nuclear membrane and evidence of chromosome condensation.

Western blot analysis

Treated and untreated cells were washed three times with ice-cold PBS before being lysed in 300 μl freshly prepared extraction buffer [1% sodium dodecyl sulfate, 1 mM Na_3VO_4, 0.1 M Tris (pH 7.4), protease inhibitor mixture (Roche, Indianapolis, USA), and phosphatase inhibitor mixture (Upstate Biotechnology, Lake Placid, USA)] at 4 °C. Protein concentration was measured using a protein assay kit according to the manufacturer's instructions (Bio-Rad). Proteins were loaded at 40 μg/lane on 5% or 10% sodium dodecyl sulfate-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, USA). Membranes were blocked for 4 h in PBS-Tween (0.1%) containing 5% non-fat dried milk and probed with primary antibodies (1:1000 by volume) overnight at 4 °C. The membranes were incubated with an appropriate horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:100 by volume) for 2 h and were visualized by ECL chemiluminescent substrate (GE Healthcare Life Sciences, Fairfield, USA) following the manufacturer's instructions. Representative data were from individual experiments repeated at least three times.

Statistical analysis

Data are expressed as mean±SD. Statistical software SPSS version 11.5 (SPSS, Chicago, USA) was used to carry out one-way ANOVA followed by a SNK test. The criterion of statistical difference was taken as $P<0.05$.

Results

Irreversible growth inhibition induced by tetrandrine

The antiproliferative effect of various concentrations of tetrandrine on CNE cells is shown in **Fig. 1**. It was observed that tetrandrine inhibited cell growth in a concentration-dependent manner in MTT assays with an IC_{50} of 46.37±0.22 and 24.83±0.12 μM for 48 and 72 h exposure, respectively ($P<0.05$).
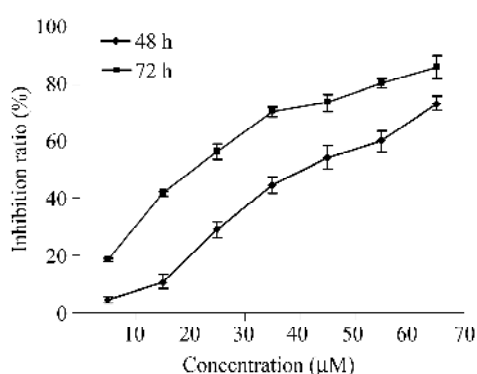

Fig. 1   Effect of tetrandrine on nasopharyngeal carcinoma CNE cell proliferation in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
Cell viability was determined by MTT assays after continuous exposure to tetrandrine for 48 or 72 h. As the drug concentration increased, the growth inhibition ratio of CNE cells increased. The image represents results from three independent experiments.

DNA fragmentation

Application of 20–40 μM tetrandrine to CNE cells for 72 h could subject the cells to apoptosis nuclear fragmentation, characterized by the appearance of a DNA ladder pattern in genomic DNA agarose gel electrophoresis (**Fig. 2**).

Apoptosis induced by tetrandrine

Induction of apoptosis was confirmed and quantified by annexin V and PI double staining. Annexin V binding revealed that the phosphatidylserine molecules had been flipped out to the outer cell surface during apoptosis, as shown in **Fig. 3**. It was observed that each plot could be divided into four regions: (1) the bottom left quadrant containing viable cells, which excluded PI and were negative for annexin V binding; (2) the bottom right quadrant containing early apoptotic cells, which were
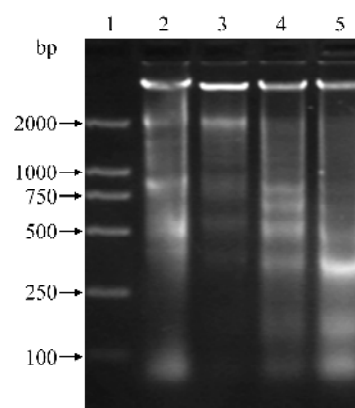

Fig. 2   DNA agarose gel electrophoresis showing ladder bands in nasopharyngeal carcinoma CNE cells
DNA from CNE cells treated with 20, 30, or 40 μM tetrandrine for 72 h was extracted for agarose gel electrophoresis, with untreated cells as the controls. The ladder pattern clearly indicates the occurrence of DNA fragmentation in groups under the action of 30 and 40 μM tetrandrine. 1, DL 2000 DNA marker; 2, negative control; 3, 20 μM tetrandrine; 4, 30 μM tetrandrine; 5, 40 μM tetrandrine.

positive for annexin V binding and excluded PI; (3) the top right quadrant containing late apoptotic cells or necrotic cells, which were positive for PI and annexin V; and (4) the top left quadrant containing impaired cells, which were positive for PI and negative for annexin V. Annexin V-positive cells increased in a concentration-dependent manner (**Fig. 3**). Apoptosis was clearly detectable with 30 and 40 μM tetrandrine for 72 h (**Fig. 3**).

Tetrandrine down-regulated expression of *Bcl-2* gene and up-regulated expression of *Bax* gene in CNE cells

To verify whether transcriptional activations of endogenous *Bcl-2* and *Bax* change after tetrandrine treatment, the expression levels of *Bcl-2* and *Bax* genes in treated and untreated cells were examined. RT-PCR analysis indicated that *Bcl-2* mRNA was clearly detectable in control cells. With increasing drug concentration, *Bcl-2* mRNA declined in a concentration-dependent manner [**Fig. 4(A)**]. Tetrandrine treatment led to an appreciable increase in cellular *Bax* mRNA after 72 h incubation with 30 and 40 μM tetrandrine [**Fig. 4(B)**].

Cytotoxicity of X-irradiation increased by treatment with tetrandrine

The effects of tetrandrine on the cytotoxicity of X-irradiation in CNE cells were investigated by monitoring cell survival using a clonogenic assay (**Fig. 5**). Cells were irradiated with different doses of X-rays then incubated with 2 μM tetrandrine for 24 h. This concentration of tetrandrine was not cytotoxic by itself with the exposure
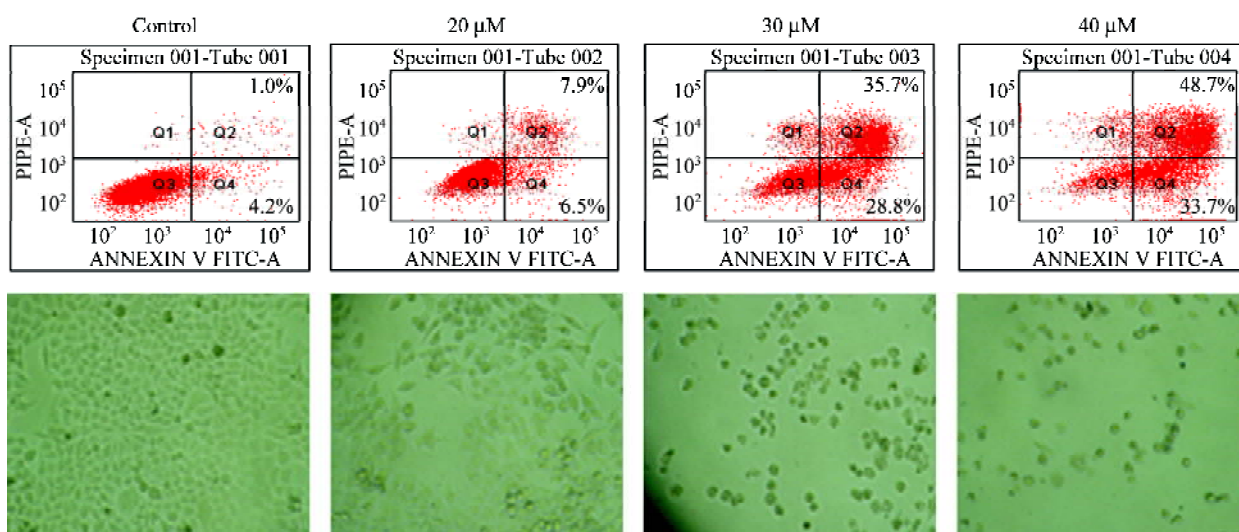
Fig. 3   Tetrandrine-induced apoptosis in nasopharyngeal carcinoma CNE cells

Apoptosis was detected by annexin V and propidium iodide (PI) staining. Cells were treated with tetrandrine for 72 h, with the concentrations of tetrandrine shown on the graph. The percentages of annexin V-positive cells in the top (PI negative) and bottom (PI positive) right quadrants (Q2 and Q4, respectively) are indicated. Annexin V-positive cells increased in a concentration-dependent manner. The top plates are fluorescence detections, and the bottom plates are light microscope images of cells. Magnification, 400×. FITC-A, fluorescein isothiocyanate-A.
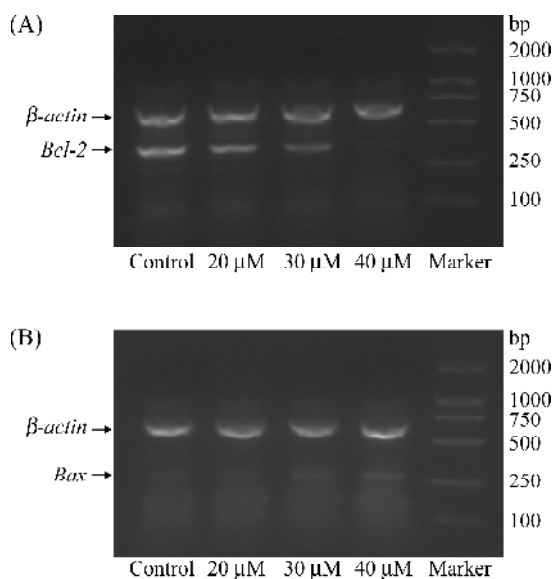

Fig. 4   Effect of tetrandrine on the expression of *Bcl-2* and *Bax* genes in nasopharyngeal carcinoma CNE cells

(A) Effect of tetrandrine on expression of *Bcl-2* mRNA. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out in CNE cells treated by 20, 30, or 40 μM tetrandrine for 72 h, with untreated cells as controls. Tetrandrine at 40 μM could significantly decrease the cellular content of *Bcl-2* mRNA. (B) Effect of tetrandrine on expression of *Bax* mRNA, with tetrandrine at 20, 30, or 40 μM applied to CNE cells for 72 h before RT-PCR, along with untreated cells as controls. With increasing drug concentration, the *Bax* mRNA level was elevated. The upper band in each image reflects amplification of the *β-actin* gene subjected to electrophoresis as an internal control. The images represent the results from one of three independent experiments.
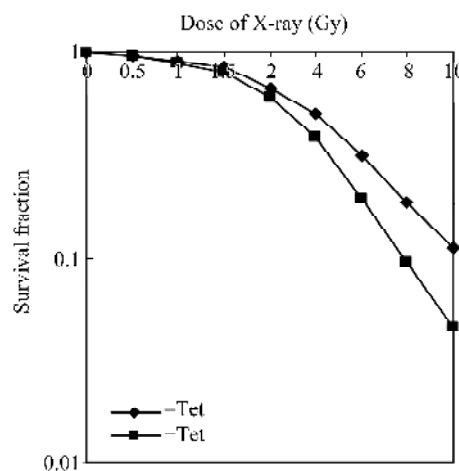

Fig. 5   Effects of tetrandrine on cytotoxicity of X-irradiation in nasopharyngeal carcinoma CNE cells measured

Exponentially growing CNE cells were plated 24 h prior to irradiation. After irradiation, the cells were incubated in the presence or absence of tetrandrine (2 μM) for 24 h, washed, and incubated for 10–14 d before colonies (>50 cells) were counted. The concentrations of tetrandrine used with cells were, by themselves, non-toxic (>90% survival; data not shown). Values shown are from one of three experiments that produced similar results representing the means and standard error of the means from three samples. The cell survival curves are shown. The parameter D_0 was used to characterize the radiosensitivity in the linear (high dose) region, and the value of parameter Dq indicated the cells' ability of repairing potentially lethal damage in the shoulder (low dose) region D_0=3.704±0.004 and Dq=0.924±0.138 for irradiated cells without tetrandrine (−Tet). D_0=2.725±0.002 and Dq=0.725±0.001 for irradiated cells treated with tetrandrine (+Tet) (ratio of D_0 values=1.359±0.002).

time used (data not shown). Cell survival curves were fitted with a single-hit multitarget model to yield values of relative parameter. The parameter D_0 was used to characterize the radiosensitivity in the linear (high dose) region, and the value of parameter Dq indicated the cells' ability to repair potentially lethal damage in the shoulder (low dose) region. For CNE cells irradiated but not treated with tetrandrine, the D_0 and Dq values were 3.704±0.004 Gy and 0.924±0.138 Gy, respectively. For cells irradiated and treated with tetrandrine, the values changed to 2.725± 0.002 Gy (for D_0) and 0.725±0.001Gy (for Dq), respectively. Therefore, tetrandrine potentiates the cyto-

toxicity of X-rays in CNE cells by approximately 1.359 folds.

Prevention of X-ray-induced G_2 arrest in CNE cells by treatment with tetrandrine

Exposure of exponentially growing CNE cells to 4 Gy of X-rays resulted in 57.243%±1.964% of the cells arresting in the G_2 phase of the cell cycle (**Fig. 6**). The addition of tetrandrine (0.2 or 2 μM) 24 h after irradiation led to a reduction in the size of the G_2-arrested population, and the percentages of cells in the G_2 phase were 47.743%± 1.372% and 30.256%±1.581%, respectively (**Fig. 6**). The
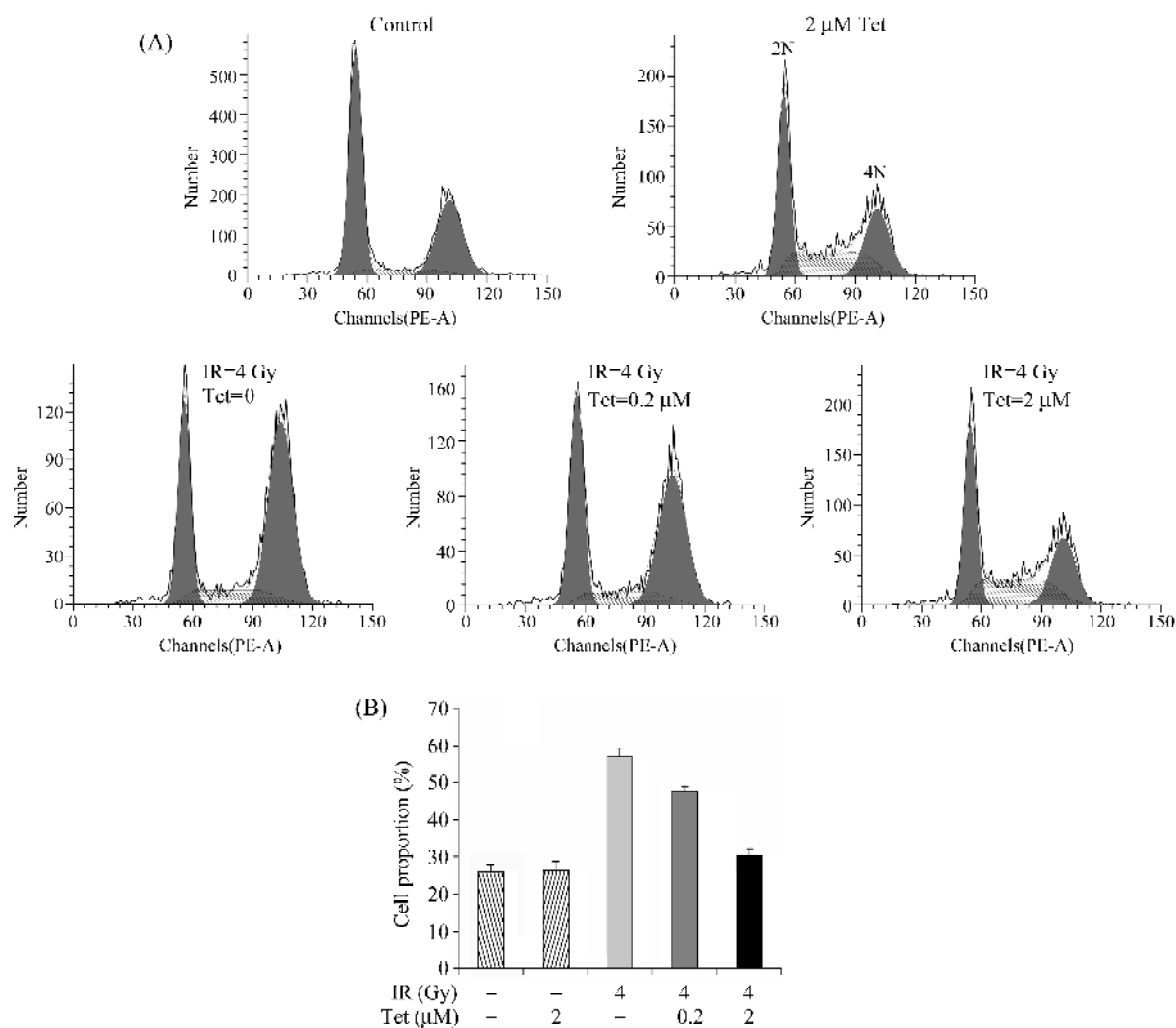

Fig. 6   Tetrandrine (Tet) abrogates radiation-induced G_2 arrest in nasopharyngeal carcinoma CNE cells
Exponentially growing CNE cells were irradiated (IR) with 4 Gy of X-rays. Following irradiation, the cells were exposed to varying concentrations of tetrandrine (0.2 or 2 μM). Twenty-four hours later they were harvested for analysis by flow cytometry. (A) Propidium iodide staining for cell cycle distribution. The flow cytometry plots shown are from an individual experiment that was conducted three times with similar results. 2N, duplum chromosome number; 4N, quadrupl chromosome number. (B) The proportions of cells in the G_2 phase (post replication stage) represent results from three independent experiments. Data were represented mean±SD. Irradiation led to accumulation in the size of the G_2-arrested population. Addition of 2 μM tetrandrine decreased the proportions of G_2 cells in irradiated cells.

effect was apparent with 2 μM tetrandrine ($P<0.05$). Hence, tetrandrine abrogates radiation-induced G_2 arrest in CNE cells.

Cell progression from G_2 phase to M phase induced by tetrandrine

Exponentially growing CNE cells were exposed to 4 Gy of X-rays then treated with 2 μM tetrandrine for 24 h. The results are shown in **Fig. 7**. Following X-irradiation, the proportion of cells in the M phase was lower than that in the control [**Fig. 7(A,B)**]. The mitotic indexes in the two groups were 1.167%±0.058% and 4.533%±0.473% [**Fig. 7(C)**], respectively. Addition of tetrandrine 24 h after irradiation led to a significant increase of the mitotic cell population (12.867%±0.666%; $P<0.01$) [**Fig. 7(C)**]. Therefore, it is clear that tetrandrine has effects on the progression of cells from the G_2 phase to the M phase.

Effects of tetrandrine and X-irradiation on cell cycle-related proteins

Results above show that tetrandrine abrogates radia-

tion-induced G_2 arrest and promotes cells from the G_2 to the M phase, and it was necessary to investigate the effect of tetrandrine on phase- associated regulatory molecules. The results showed that irradiation caused a decrease in the protein level of cyclinB1 and increase of phosphorylated Cdc2(Tyr15) and phosphorylated Cdc25C(Ser216) (**Fig. 8**). Following irradiation, the cells were then exposed to tetrandrine (0.2 or 2.0 μM) for 24 h. The results showed that an increase of cyclinB1 protein and reductions of phospho-Cdc2(Tyr15) and phospho-Cdc25C(Ser216) were observed, which were more evident when the cells were treated with 2.0 μM tetrandrine (**Fig. 8**). These results suggest that tetrandrine abrogates G_2 arrest by promoting the expression of cyclinB1 and the accumulation of Cdc2 (Tyr15) and Cdc25C(Ser216) dephosphorylation.

Fig. 8　　Tetrandrine (Tet) changes some cell cycle-related proteins in nasopharyngeal carcinoma CNE cells
Exponentially growing CNE cells were irradiated (IR) with 4 Gy of X-rays. Following irradiation, the cells were exposed to concentrations of tetrandrine (0.2 or 2.0 μM). Twenty-four hours later they were harvested for analysis by Western blotting. β-actin was used as the loading control. Data are representative of one of three independent experiments. X-rays resulted in a decrease in the protein expression of cyclinB1 and increase in the protein of phosphorylated Cdc2 tyrosine-15 [p-Cdc2(Tyr15)] and phosphorylated Cdc25C serine-216 [p-Cdc25C(Ser216)]. However, in irradiated cells, tetrandrine increased the expression of cyclinB1 and the accumulation of Cdc2(Tyr15) and Cdc25C(Ser216) dephosphorylation.
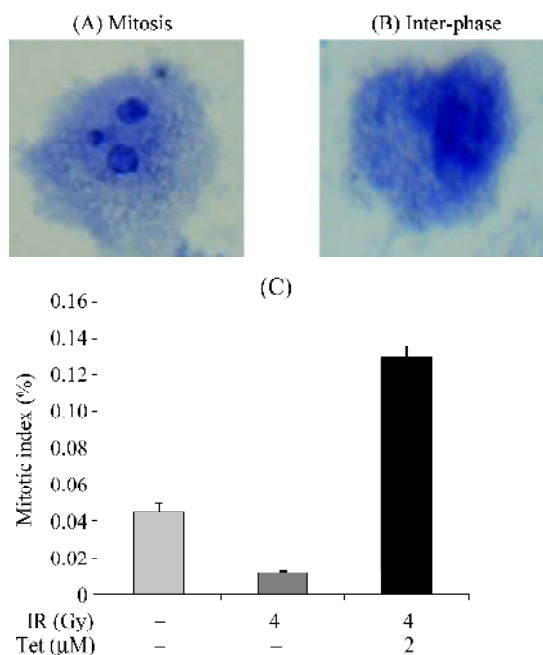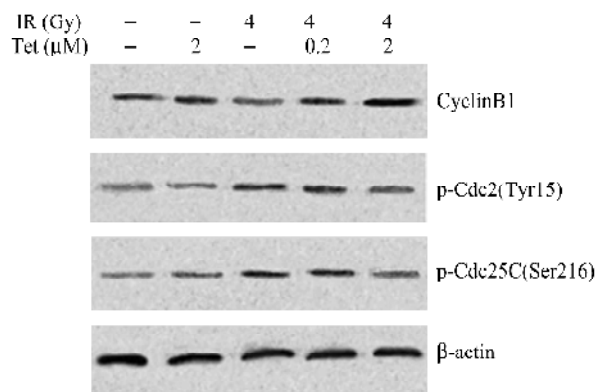

Fig. 7　　Tetrandrine (Tet) induced progression of nasopharyngeal carcinoma CNE cells from G_2 phase to M phase
Exponentially growing CNE cells were irradiated (IR) with 4 Gy of X-rays then incubated in the absence or presence of tetrandrine (2.0 μM) for 24 h. (A) Chromosomes of mitosis are condensed. (B) Chromosomes in inter-phase are un-coined. Both are light microscope images of the cells (Magnification, 1000×), stained using the Giemsa method. (C) The proportions of cells in the M phase were counted by light microscope. Error bars represent mean±SD. Tetrandrine increased the mitotic index in the irradiated cells.

Discussion

Apoptosis is a fundamental cellular death process that is essential for normal tissue homeostasis. Suppression of apoptosis in the presence of a proliferative stimulus is sufficient for tumor development. The importance of apoptosis induction in cancer therapy has been recognized and the ongoing discovery of numerous apoptosis-regulating genes provides new potential targets for a

molecular cancer therapy [14,15].

Tetrandrine possesses antiproliferative activity, induces apoptosis of malignant cells, and inhibits the proliferation of human esophageal cancer cell lines Eca-109, ECa109-C3, and human myeloid leukemic HL-60 [16,17]. Moreover, tetrandrine induces apoptosis of malignant lymphoid, myeloid cells, human monoblastic leukemic U937 cells, human T-cell lines, lung carcinoma, hepatoblastoma cells, and hepatocellular carcinoma *in vitro* [18–20]. In two-stage carcinogenesis in mouse skin, tetrandrine inhibits tumor promotion [21]. Mice treated with tetrandrine show the least metastases in a pulmonary metastatic model of colorectal cancer-bearing mice [22]. Our results were in agreement with earlier studies that some tumor types are shown to be sensitive to tetrandrine treatment. Tetrandrine had an evident inhibitory effect on the proliferation of CNE cells in a concentration-dependent manner ($P<0.05$). Furthermore, with increasing tetrandrine concentration, ladder-shaped straps in DNA gel electrophoresis and apoptosis ratios manifested gradually. These observations provide evidence for the induction of apoptotic cell death as a cause of cytotoxicity by tetrandrine treatment in CNE cells.

In order to further delineate the mechanisms underlying induction of apoptosis after tetrandrine treatment in CNE cells, we investigated the involvement of pro- and anti-apoptotic members of the *Bcl-2* family. *Bcl-2* family members are pivotal regulators of the apoptotic process that depend on the participation of mitochondria [23]. In cells protected by Bcl-2, Bax does not homodimerize or translocate to mitochondria following a death signal. Instead, the two form a minimal amount of apparent Bax/Bcl-2 heterodimers and more abundant Bax/Bcl-2 heteromultimers. For cells not protected by Bcl-2, Bax represents an inactive monomer that translocates from the cytosol to the mitochondrial membrane and then homodimerizes and induces mitochondrial dysfunction resulting in cell death [6]. Recently it has been reported that down-regulation of anti-apoptotic protein Bcl-2 might be an important target to increase the drug sensitivity of cancer cells [24]. To examine its role in the execution of apoptosis in CNE cells, we investigated the transcriptional response of the *Bcl-2* gene after tetrandrine treatment. The results showed that *Bcl-2* mRNA declined and *Bax* mRNA increased in a concentration-dependent manner. Moreover, alterations in the profile of expression of *Bcl-2* family members occurs exclusively in cells that undergo apoptosis, which is generally associated with up-regulation of *Bax*, with or without down-regulation of the anti-apoptotic gene *Bcl-2*. In the present study, it suggests that down-regulation

of the *Bcl-2* proto-oncogene and up-regulation of the *Bax* pro-apoptosis gene might be two of the mechanisms involved in DNA damage-induced apoptosis in CNE cells.

The combination of different antitumoral treatment methods is advantageous to limit unspecific toxicity often observed with an exceedingly high single treatment regimen. Results in the experiments showed that tetrandrine potentiates the response of tumor cells to irradiation *in vitro*. Tetrandrine (2.0 μM) abrogated G_2 arrest by promoting the expression of cyclinB1 and the accumulation of Cdc2(Tyr15) and Cdc25C(Ser216) dephosphorylation.

Arrest of the cell cycle in the G_2 phase following DNA damage is believed to promote cell viability by allowing time for DNA repair prior to the entry into mitosis [25, 26]. Most cancer cells have an inoperative G_1 checkpoint due to inactivation of the *p53* tumor suppressor gene but a functioning G_2 checkpoint [27]. Thus agents that abrogate G_2 arrest [28] or mutations in genes that regulate the G_2 checkpoint [29] tend to sensitize cells to DNA-damaging agents. Earlier works have reported that the sensitizing effect of caffeine and UCN-01, G_2 checkpoint abrogators, is based on their ability to release cells from the irradiation-induced G_2/M arrest and is preferentially observed in cells lacking functional p53 [25,26]. Consistent with those results, our results showed that radiation-induced G_2 phase arrests were abrogated by 2.0 μM tetrandrine in CNE cells ($P<0.05$). Furthermore, the proportion of cells in the M phase increased from 1.167%±0.058% in irradiated cells to 12.867%±0.666% in cells treated with a combination of irradiation and tetrandrine ($P<0.01$). It was also found that 2.0 μM tetrandrine preferentially sensitized CNE cells to irradiation-induced cytotoxicity, suggesting that tetrandrine might enhance the effectiveness and preference of DNA-damaging treatment to cancer cells.

Entry of all eukaryotic cells into mitosis is regulated by activation of Cdc2 kinase. Activation of Cdc2 is controlled at several steps, including cyclin binding and phosphorylation of Thr161 [30–33]. Throughout interphase and during the cellular response to DNA damage, cyclinB1/Cdc2 complexes shuttle between the nucleus and the cytoplasm because a nuclear export sequence in cyclinB1 facilitates efficient nuclear export of these complexes [34]. In the late G_2 phase, the cyclinB1-Cdc2 complex accumulates and promotes cells from the G_2 phase into the M phase [35]. DNA-damaging agents increase expression of cyclinB1 and Cdc2 proteins and further enhance the activation of the cyclinB1-Cdc2 complex [36,37]. However, the critical regulatory step in activating Cdc2 during progression into mitosis appears to be dephosphorylation of Tyr15 and Thr14 [32,38]. Cdc25C is a protein

phosphatase responsible for dephosphorylating and activating Cdc2 [39]. Cdc25C is constitutively phospho-rylated at Ser216 throughout interphase by Cdc25C-associated kinase 1, whereas phosphorylation at this site is DNA damage-dependent at the G_2/M checkpoint [40]. When phosphorylated at Ser216, Cdc25C binds to members of the 14-3-3 family of proteins, sequestering Cdc25C in the cytoplasm and preventing premature mitosis [41]. The checkpoint kinases Chk1 and Chk2 phosphorylate Cdc25C at Ser216 in response to DNA damage [42,43]. Our results showed that X-rays resulted in a decrease in the protein expression of cyclinB1 and an increase in the protein of phosphorylated Cdc2(Tyr15) and phosphorylated Cdc25C(Ser216). However, tetrandrine reversed those expressions of associated cell cycle proteins in irradiated cells.

In summary, tetrandrine might inhibit the growth of cancer cells, induce apoptosis, and efficiently enhance cytotoxity of irradiation. The anticancer mechanism of tetrandrine might be involved in up-regulation of *Bax* gene expression and down-regulation of *Bcl-2* gene expression. Furthermore, tetrandrine might abrogate radiation-induced G_2 phase arrest, which is associated with the reduction in phosphorylated Cdc2(Tyr15) and Cdc25C(Ser216) proteins and the increase in cyclinB1 protein.

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