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Tetrandrine: A Potent Abrogator of G_2 Checkpoint Function in Tumor Cells and Its Mechanism[1]

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Objective To assess the ability of tetrandrine (Tet) to enhance the sensitivity to irradiation and its mechanism in cell lines of human breast cancer p53-mutant MCF-7/ADR, p53-wild-type MCF-7 and human colon carcinoma p53-mutant HT-29 as well as in C26 colorectal carcinoma-bearing BALB/c mice. **Methods** MCF-7/ADR, HT-29 and MCF-7 cells were exposed to irradiation in the absence or presence of tetrandrine. The effect of Tet on the cytotoxicity of X-irradiation in these three cells was determined and the effect of tetrandrine on cell cycle arrest induced by irradiation in its absence or presence was studied by flow cytometry. Moreover, mitotic index measurement determined mitosis of cells to enter mitosis. Western blotting was employed to detect cyclin B1 and Cdc2 proteins in extracts from irradiated or non-irradiated cells of MCF-7/ADR, HT-29 and MCF-7 treated with tetrandrine at various concentrations. Tumor growth delay assay was conducted to determine the radio-sensitization of tetrandrine *in vivo*. **Results** Clonogenic assay showed that tetrandrine markedly enhanced the lethal effect of X-rays on p53-mutant MCF-7/ADR and HT-29 cells and the sensitization enhancement ratio (SER) of tetrandrine was 1.51 and 1.63, but its SER was only 1.1 in p53-wt MCF-7 cells. Irradiated p53-mutant MCF-7/ADR and HT-29 cells were only arrested in G2/M phase while MCF-7 cells were arrested in G1 and G2/M phases. Radiation-induced G2 phase arrests were abrogated by tetrandrine in a concentration-dependent manner in MCF-7/ADR and HT-29 cells, whereas redistribution within MCF-7 cell cycle changed slightly. The proportion of cells in M phase increased from 1.3% to 14.7% in MCF-7/ADR cells, and from 1.5% to 13.2% in HT-29 cells, but 2.4% to 7.1% in MCF-7 cells. Furthermore, the levels of cyclin B1 and Cdc2 expression decreased after X-irradiation in MCF-7/ADR and HT-29 cells, and the mitotic index was also lower. Tet could reverse the decrease and induce the irradiated cells to enter mitosis (M phase). Endosomatic experiment showed that tetrandrine caused tumor growth delay in irradiated mice. **Conclusion** Tetrandrine boosts the cell killing activity of irradiation both *in vitro* and *in vivo*. Tetrandrine is a potent abrogator for G2 checkpoint control and can sensitize the cells to radiation.

Key words: Breast cancer cell line MCF-7/ADR; Breast cancer cell line MCF-7; Colon carcinoma cell line HT-29; Colon carcinoma C26 BALB/c mice; Tetrandrine; Irradiation; Cell cycle; p53; Western blotting

INTRODUCTION

Cell cycle progression is constantly monitored to ensure that the correct sequence of events in the process of cell division is achieved and that cells with DNA damage are not replicated[1]. A common cellular response to DNA-damaging agents is the activation of cell cycle checkpoints. DNA damage induced by ionizing radiation initiates signals that can ultimately activate checkpoints to stop or slow down cell cycle traverse and re-establish the correct order of cell cycle transition. The checkpoints permit enough time for genetic repair in order to ensure hereditary stability of cells. If repair cannot be achieved, the cells would die[2-3]. DNA damaged by ionizing-radiation activates G_2 checkpoint signaling pathways, which communicate through several downstream mediator proteins, such as tumor suppressor protein p53, cyclin B, checkpoint kinases Chk1, Chk2, and miotic kinase Cdc2[2,4-6]. Cell cycle checkpoint control is one of the hot topics in recent researches. Researchers have tried to increase the lethal effect of DNA-damaging agents on tumor cells through cell cycle checkpoint control. If DNA-damaging agents could be used in combination with checkpoint abrogation, DNA damage might not be repaired completely and the therapeutic effect of radiotherapy also could not be greatly improved.

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Tetrandrine (Tet), a bis-benzylisoquinoline alkaloid, is isolated from the root of Stephania tetrandra and used in traditional Chinese medicine as an anti-rheumatic, anti-inflammatory, and antihypertensive agent[7]. Several studies have shown that tetrandrine enhances the sensitivity to radiation in cultured tumor cells and lung cancer patients[8-11]. However, the molecular mechanism by which tetrandrine enhances the sensitivity to radiation remains poorly understood. In the present study, we investigated the ability of tetrandrine to enhance the sensitivity to irradiation and its mechanism in cell lines of human breast cancer p53-mutant MCF-7/ADR, p53-wt MCF-7, and human colon carcinoma p53-mutan HT-29 and also in C26 colorectal carcinoma-bearing BALB/c mice.

MATERIALS AND METHODS

Animals and Treatment

Male BALB/c mice were obtained from Laboratory Animal Center, Chinese Academy of Medical Sciences and kept in the animal house of SPF colony. All mice used in experiments were 6-8 weeks old. Mouse colon carcinoma C26 cells were transplanted subcutaneously in the BALB/c mice for 10 days. Tumor tissues were extracted and diluted to suspension with isotonic Na chloride. The tumor cell suspension was injected into the distal lateral muscle of right thigh with 0.1 mL cell suspension (1×10^6 cells). When the perimeter of right thighs was 1 mm longer than before, the mice were randomly divided into 6 grous (10 each group): group 1:control group; group Ⅱ:10 mg/kg tetrandrine treatment group; group Ⅲ:20 mg/kg tetrandrine treatment group; group Ⅳ:12.5 Gy X-ray irradiation group; group Ⅴ:12.5 Gy X- ray + 10 mg/kg tetrandrine treatment group; group Ⅵ:12.5 Gy X-ray + 20 mg/kg tetrandrine treatment group.

Tumor-bearing right thighs were exposed to 12.5 Gy irradiation at an absorbable dose rate of 300 cGy/min at room temperature. After 2 hours, 0.2 mL Tet was injected intraperitoneally, once a day for 5 days. The tumor diameters were measured every 2 days with calipers. When the tumor diameter was longer than 20 mm, the mice were sacrifased and growth curves were plotted according to tumor diameters. Under tumor growth curves, the time (days) for the diameter of treated tumors to grow in R+5 mm (R was the diameter of tumor at the beginning of radiation) was calculated.

Cell Culture

Human breast cancer p53-mutant MCF-7/ADR, p53-wt MCF-7 cell lines and human colon carcinoma p53-mutant HT-29 cell line were prepared in our laboratory. MCF-7/ADR and MCF-7 cells were cultured at 37℃ with DMEN medium supplemented with 15% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air. HT-29 cells were grown in RPMI 1640 supplemented with 5% calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cell lines were maintained in exponential growth phase and the medium was changed every 2-3 days. The cell generation time ranged from 20 to 24 hours.

Drug and Antibodies

Tetrandrine was obtained from Kang En Bei Chemical Company (Zhejiang, China). Its purity was 98.38%. It was dissolved in 0.1 mol/L hydrochloric acid to achieve the desired concentrations in medium immediately before each experiment. Mouse monoclonal anti-cyclin B1 and Cdc2 antibodies were purchased from Santa Cruz Biotechnology Co (USA). Anti-mouse antibody and anti-mouse immunoglobulin G horseradish peroxidase-coupled antibodies were purchased from Sigma co.

Experimental Irradiation

Bottles of cultured cells were laid upside down on the therapeutic bed with an addion of 2.5 cm stuffs. All asynchronously growing cells were exposed to irradiation (linear accelerative equipment, toshy 900, Japan) at an absorbable dose rate of 300 cGy/min at room temperature.

Clonogenic Assays

Exponentially growing cells were irradiated at doses of 1, 2, 3, 4, 6, and 8 Gy. After treated with 0.1 μmol/L tetrandrine for 24 h, the cells were washed in PBS, trypsinized, and seeded in 24-well plates at the density of 200 cells/well. Colonies were grown for 10-14 days. Plates were washed in PBS, colonies were fixed with methanol and stained with 0.1% crystal violet. Colonies ≥50 cells were counted under microscope for the cloning efficiency. Six paralle samples were scored for each treatment condition.

Flow Cytometry

After irradiation at 4 Gy, the cells were treated with 0.01, 0.1, and 1 μmol/L tetrandrine for 24 h, harvested and fixed in 70% ethanol at 4℃. Before analysis, the cells were washed in PBS, digested with 500 U/mL RNase for at least 30 min at 37℃

and stained with PBS containing 50 μg/mL propidium iodide (PI) for 30 min. Analysis was performed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Cell cycle distributions were calculated on DNA plots by ModFit LT software (Verity Sofeware House, Inc., Topsham, ME).

Mitotic Index

After irradiation at 4Gy, the cells were treated with 0.1 μmol/L tetrandrine, incubated for 24 h, pelleted by centrifugation (500×g, 5 min, 4℃), washed with ice-cold PBS, and resuspended in 0.5 mL cold half-strength PBS for 10 min. The cells were fixed with 6 mL 2% ethanol: acetic acid (3:1) for 30 minutes at 4℃. 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) for 10 min, and dropped onto air-dried and Giemsa-stained glass slides. For each sample, 1000 cells were randomly counted under light microscope, and mitotic cells were scored by their lack of a nuclear membrane and evidence of chromosome condensation.

Western Blotting Analysis

After irradiation at 4 Gy, the cells were treated with 0.01, 0.1, and 1 μmol/L tetrandrine for 24 h, washed three times with ice-cold PBS before lysed in 300 μL freshly prepared extraction buffer containing 1% SDS, 1 mmol/L Na_3VO_4, 0.1 mol/L Tris (pH 7.4), and protease inhibitor mixture (Roche, Indianapolis, IN) at 4℃. Proteins were detected with a protein assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA), resolved at 50 μg/lane on 10% or 15% SDS-polyacrylamide gels, and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 5 h at 10-15 V. Membranes were blocked overnight in PBS-Tween (0.1%) containing 5% nonfat dried milk, probed for 2-3 h with primary antibodies (1:1000) including cyclin B1 and Cdc2, incubated with horseradish peoxidase (HRP)-conjugated donkey anti-mouse immunoglobulin (1:2500) for 1 h, and visualized by SuperSignal West Pico Chemo-illuminescent Substrate (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's instructions. Representative data from individual experiments repeated at least twice were shown.

Statistical Analysis

Statistical software SPSS 11.0 was employed to perform ANOVA.

RESULTS

Growth Inhibition of Colon Carcinoma 26 in Mice by Tetrandrine

We investigated the cytotoxicity of tetrandrine to BALB/c mice. The LD50 (medial lethal dose) of tetrandrine was 200 mg/kg (data not shown). We chose tetrandrine at doses (10 mg/kg and 20 mg/kg) in experiment. The time required for the diameter of treated tumors to grow ranged from R+1 mm to R+5 mm in mice treated with 20 mg/kg tetrandrine and 10 mg/kg tetrandrine in combination with radiation, which was 10.42, 7.37, 6.38, and 3.69 days, respectively, in the treatment groups (Fig. 5). The inhibition rate of tumor growth was 89%, 70%, 58%, 33%, and 22%, respectively in the groups treatment with 20 mg/kg tetrandrine and 10 mg/kg tetrandrine in combination with radiation, 12.5 Gy, 20 mg/kg tetrandrine and 10 mg/kg tetrandrine (data not show). Treatment with tetrandrine or irradiation alone resulted in a partial tumor growth delay, whereas a combination of tetrandrine and irradiation could significantly control tumor growth.

Increased Cytotoxicity of X Irradiation After Treatment With Tetrandrine

We investigated the effects of tetrandrine on the cytotoxicity of X irradiation to p53-mutant cell lines MCF-7/ADR, HT-29 and the p53-wt cell line MCF-7. In these experiments, we monitored cell survival by clonogenic assay (Fig. 1). Cells were irradiated with different doses of X-ray and incubated in the presence of 0.1 μmol/L tetrandrine for 24 h, which made MCF-7/ADR and HT-29 cells become more radiosensitive than MCF-7 cells. The Dq (quasi-threshold dose indicating the ability of cells to repair the sublethal damage) value for irradiated MCF-7/ADR and HT-29 cells not treated with tetrandrine was 2.29 Gy and 2.99 Gy, respectively. The Dq value for irradiated cells treated with tetrandrine was 1.52 Gy and 1.98 Gy, respectively. Thus the sensitization enhancement ratio (SER) of tetrandrine in MCF-7/ADR and HT-29 cells was 1.51 and 1.63, respectively. However, the Dq value for irradiated MCF-7 cells not treated with tetrandrine was 2.12 Gy, while the Dq value for irradiated MCF-7 cells treated with tetrandrine was 1.93 Gy (SER=1.10). Our results illustrate that tetrandrine could increase the cytotoxicity of X irradiation to two different cancer cell lines containing mutant p53 gene.

Abrogation of X-ray Induced G2 Arrest in Cells After Treatment With Tetrandrine

X-ray radiation only markedly induced G_2/M phase

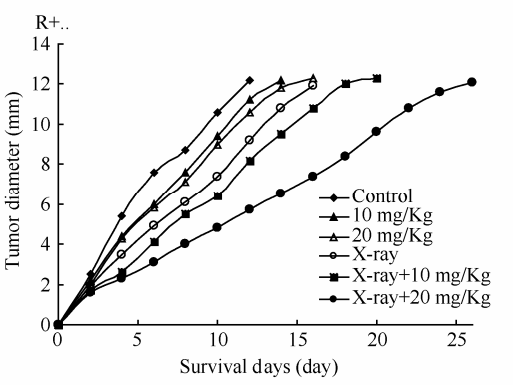

FIG. 1. Curves for tumor growth delay after treatment.

arrest in p53-mutant MCF-7/ADR and HT-29 cells, and G_1 and G_2 /M phase arrest in p53-wt MCF-7 cells. Radiation-induced G_2 phase arrest in MCF-7/ADR and HT-29 cells was abrogated after treatment with tetrandrine in a concentration-dependent manner. However, cell cycle distribution of MCF-7 cells treated with tetrandrine changed slightly, which was apparent after treatment with 0.1 μmol/L (Fig. 2).

Tetrandrine Induced Cells From G2 Phase to M Phase

Exponentially growing MCF-7/ADR, HT-29 and MCF-7cells were exposed to 4 Gy of X-ray, and

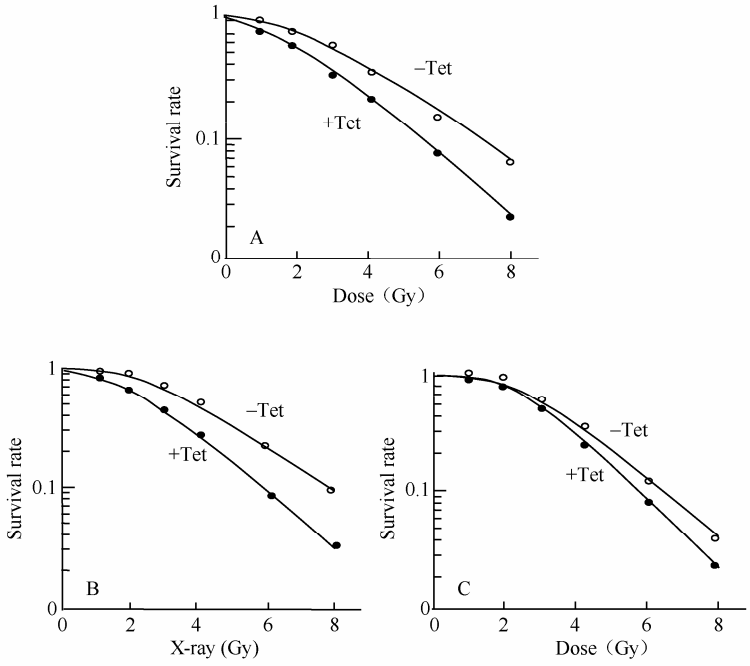

FIG. 2. Tetrandrine increases cytotoxicity of X-ray to MCF-7/ADR cells (A), human colon cancer HT-29 cells (B), and MCF-7 cells (C).

incubated in a medium containing 0.1 μmol/L tetrandrine for 24 hours. The proportion of cells in M phase increased from 1.3% to 14.7% in MCF-7/ADR cells (Fig. 3A), and from 1.5% to 13.2% in HT-29 cells (Fig. 3B), but from 2.4% to 7.1% in MCF-7 cells (Fig. 3C). These results indicate that tetrandrine might have activated some checkpoint regulatory proteins in p53-mutant MCF-7/ADR and HT-29 cells.

Effects of Tetrandrine and X Irradiation on Cyclin B1 and Cdc2 Proteins

To elucidate the mechanism of tetrandrine-abrogated G_2 arrest, we tested the effect of tetrandrine on cyclin B1 and Cdc2 proteins in p53-mutant MCF-7/ADR and HT-29 cells. As shown in Fig. 4, irradiation decreased the expression of cyclin B1 and Cdc2 proteins in MCF-7/ADR cells, whereas tetrandrine blocked their expression in the irradiated cells. The expression of Cdc2 in irradiated HT-29 cells was lower than that in cells treated with irradiation and tetrandrine. However, the expression of cyclin B1 was different in HT-29 cells treated with tetrandrine at various concentrations, and was suppressed after treatment with 1.0 μmol/L tetrandrine and nearly not changed after treatment with 0.01 μmol/L tetrandrine. These results suggest that tetrandrine could abrogate G_2 arrest by reversing the expression of cyclin B1 and Cdc2.

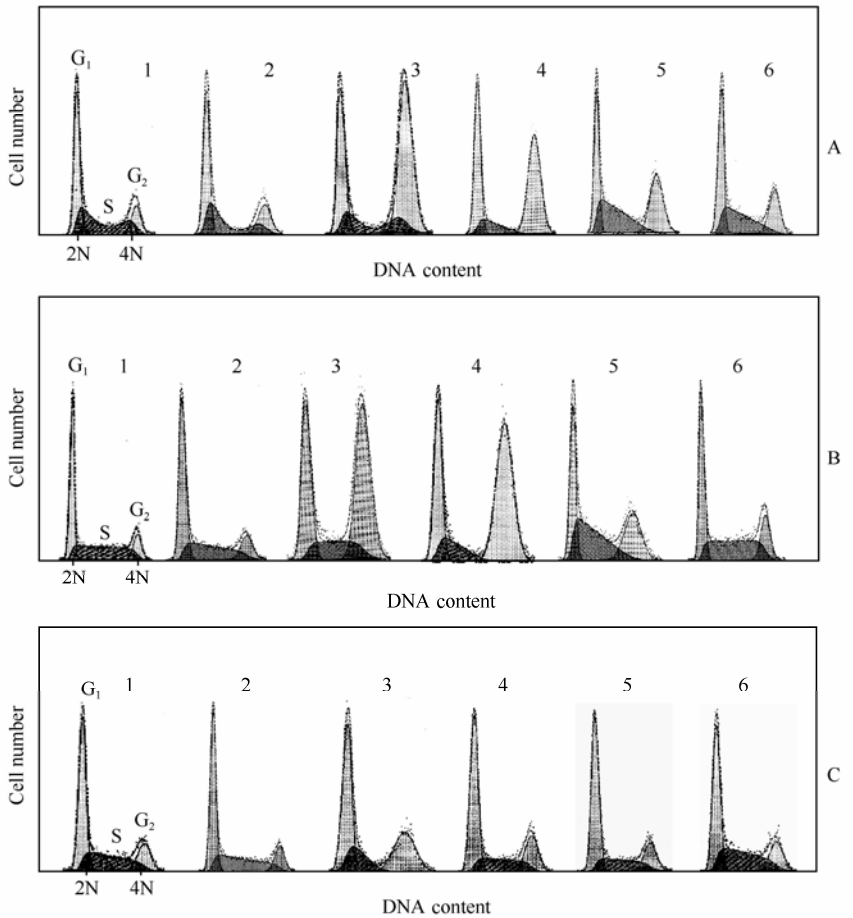

FIG. 3. Tetrandrine abrogates X-ray-induced G_2 arrest in MCF-7/ADR cells (A), in HT-29 cells (B), and in MCF-7 cells (C). 1: control group; 2: 0.1 μmol/L tetrandrine treatment group; 3: 4 Gy X-ray irradiation group; 4: 4 Gy X-ray+ 0.01 μmol/L tetrandrine treatment group; 5: 4 Gy X-ray+0.1 μmol/L tetrandrine treatment group; 6: 4 Gy X-ray+1 μmol/L tetrandrine treatment group.

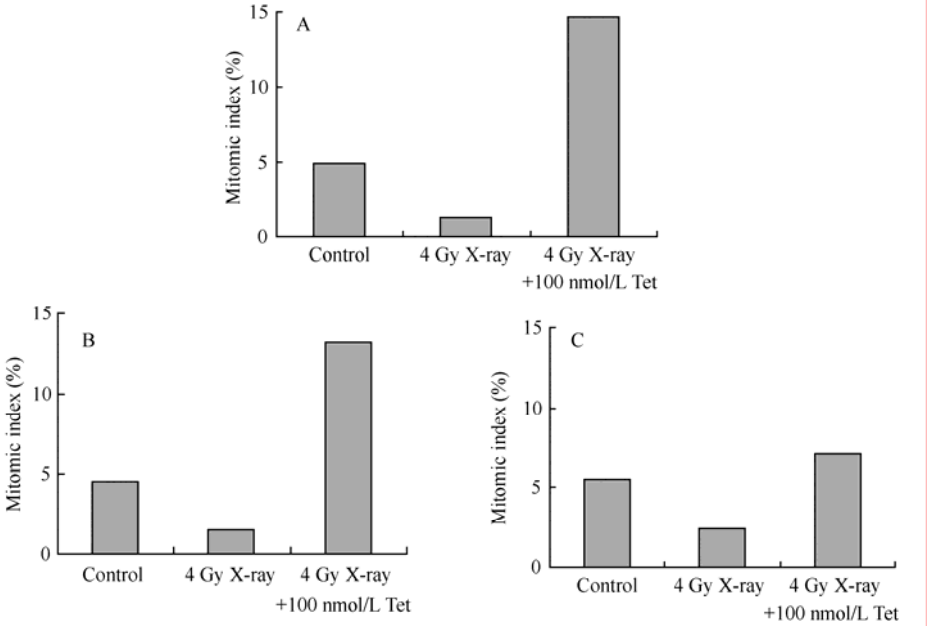

FIG. 4. Tetrandrine induces mitosis of irradiated MCF-7/ADR (A), HT-29 (B), and MCF-7 cells (C).

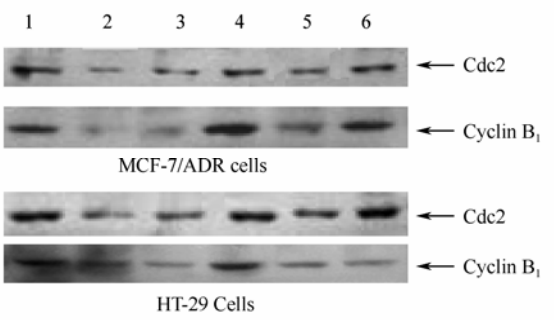

FIG. 5. Western blotting assay of cyclin B1 and Cdc2 proteins in different groups. 1: control group; 2: 4 Gy irradiation group; 3: 4 Gy X-ray+0.1 μmol/L Tet treatment group; 4: 0.1 μmol/L Tet treatment group; 5: 4 Gy X-ray +1 μmol/L Tet treatment; 6: 1 μmol/L Tet treatment group.

DISCUSSION

Radio-therapy for cancer treatment is to produce irreversible DNA damage in tumor cells by direct and indirect irradiation. Arrest of cell cycle in G_2 phase following DNA damage is believed to promote cell viability by allowing DNA repair before mitosis[12-13]. Agents abrogating G_2 arrest[14] or mutations in genes that regulate G_2 checkpoint[15] tend to sensitize cells to DNA-damaging agents. It was reported that the sensitizing effect of caffeine and UCN-01, G_2 checkpoint abrogators, is due to their ability to release cells from irradiation-induced G_2/M arrest and preferentially observed in cells lacking functional p53[16-17]. Being consistent with these results, radiation-induced G_2 phase arrest in p53-mutant MCF-7/ADR and HT-29 cells was abrogated after treatment with tetrandrine in our study. However cell cycle distribution in p53-wt MCF-7 cells treated with tetrandrine changed slightly. The proportion of cells in M phase increased from 1.3% to 14.7% in MCF-7/ADR cells, and from 1.5% to 13.2% in HT-29 cells, but from 2.4% to 7.1% in MCF-7 cells. Tetrandrine preferentially sensitized p53-mutant MCF-7/ADR and HT-29 cells to irradiation-induced cytotoxicity, suggesting that tetrandrine can enhance the cytotoxicity of irradiation *in vivo*.

Transition from G_2 to M in eukaryotic cells is regulated by the cyclin-dependent kinase, Cdc2[18]. Kinase activation of Cdc2 requires activating phosphorylation at threonine 161[19] and complex formed with either cyclin A or B1 protein[20]. Between the different phases and during the cellular response to DNA damage, cyclin B1/Cdc2 complexes shuttle between the nuclei and cytoplasm because a nuclear export sequence in cyclin B1 facilitates efficient nuclear export of these complexes[21]. In late G_2 phase, cyclin B1/Cdc2 complex accumulation promotes mitosis of cells[22]. DNA-damaging agents increase the expression of cyclin B1 and Cdc2 proteins and further activate the cyclin B1/Cdc2 complex[23-24]. In our study, irradiation decreased the expression of cyclin B1 and Cdc2 in MCF-7/ADR and HT-29 cells. Similar results have also been reported by Janss AJ *et al*.[25]. The expression of cyclin B1 and Cdc2 increased in the irradiated cells after treatment with 0.1 μmol/L tetrandrine, suggesting that tetrandrine abrogates G_2 arrest by reversing the expression of cyclin B1 and Cdc2.

The data reported in our experiments demonstrate that tetrandrine could enhance the sensitivity of tumor cells to irradiation *in vitro* and *in vivo*. Tetrandrine could abrogate irradiation-induced G_2 phase arrest by up-regulating the expression of cyclin B1 and Cdc2 proteins.

In summary, tetrandrine is a potent G_2 checkpoint abrogator and markedly enhances the cytotoxicity of irradiation *in vitro* and *vivo*. By inducing irradiated cancer cells to increase the expressionn of cyclin B1 and Cdc2 proteins, tetrandrine promotes mitosis of cells.

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