RESEARCH ARTICLE

Synergistic Anticancer Activity of 5-Aminolevulinic Acid Photodynamic Therapy in Combination with Low-dose Cisplatin on Hela Cells

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Abstract

Objective: Photodynamic therapy (PDT) is a promising modality for the treatment of various tumors. In order to assist in optimizing treatment, we applied 5-ALA/PDT in combination with low-dose cisplatin to evaluate cytotoxicity in Hela cells. Methods: Antiproliferative effects of 5-ALA/PDT and cisplatin, alone and in combination, were assessed using MTT assay. To examine levels of apoptosis, Hela cells treated with 5-ALA/PDT, and combination treatment were assessed with Annexin-V/PI by flow cytometry. To investigate the molecular mechanisms underlying alterations in cell proliferation and apoptosis, Western blot analysis was conducted to determine the expression of p53, p21, Bax and Bcl-2 proteins. Results: MTT assays indicated that combination treatment obviously decreased the viability of Hela cells compared to individual drug treatment. In addition, it was confirmed that exposure of Hela cells to 5-ALA/PDT in combination with low-dose cisplatin resulted in more apoptosis *in vitro*. Synergistic anticancer activity was related to upregulation p53 expression and alteration in expression of p21, Bcl-2 and Bax. Conclusion: Our findings suggest that administration of 5-ALA/PDT in combination with the low-dose cisplatin may be an effective and feasible therapy for cervical cancer.

Keywords: 5-ALA/PDT - low-dose cisplatin - combination treatment - synergestic anticancer activity

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Introduction

Cervical cancer is the third most common cancer in women worldwide, with 85% of cases occurring in developing countries, where cervical cancer is the second most frequent cause of cancer death in women (Jemal et al., 2011) . The Asia Oceania region accounts for just more than 50% of all cases and deaths from the disease world-wide (Garland et al., 2012). It is reported the bimodal distribution of cervical cancer with two modals peaking at 35-39 and 60-64 years old, respectively (Jemal et al., 2003). With the recent trend toward delaying childbearing, for the younger patients fertility-sparing options have become more important for the management of this disease. In addition, an effective therapy technique is also required for those patients who are so elderly that they cannot undergo surgery or radiotherapy .

Photodynamic therapy (PDT) is a promising and effective approach which has gradually gained much attention. PDT involves the administration of a given photosensitizer (PS), its selective accumulation in malignant tissue (Gomer and Dougherty, 1979), and the subsequent irradiation by light of appropriate wavelength which activates the PS to generate reactive oxygen species (ROS) in the presence of oxygen. ROS, especially singlet oxygen radicals may trigger the targeted cells apoptosis or necroptosis (Oleinick et al., 2002). PDT could reserve complete organic structure and preserves a women's fertility function for the younger patients, and could be repeated multiple times due to low risk of side-effects in the old patients that they cannot undergo surgery or radiotherapy. However, the efficiency of PDT depends upon the PS and the limited penetration of the laser , and further study of improving the anticancer efficacy of PDT is desirable .

Cisplatin is widely prescribed in the management of various cancers. By forming adducts to DNA, cisplatin inhibits DNA replication and chain elongation, which accounts for its antineoplastic activity (Suo et al., 1999). But its application can be limited due to its side effects , in particular dose-limiting nephrotoxicity and hepatotoxicity , and also inherent and acquired resistance can exist (Rabik and Dolan, 2007). In early stage cervical cancer cisplatin has been confirmed to be effective in controlling or delaying tumor growth (Suprasert et al., 2007). Moreover cisplatin combined with docetaxel based concurrent

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chemoradiotherapy in advanced cervical cancer has more pronounced sensitizing effect for advanced cervical cancer (Ke et al., 2012).

A few preclinical studies have been performed to establish potential advantages produced through combination of cisplatin with PDT (Crescenzi et al., 2004; Crescenzi et al., 2006; Uehara et al., 2006; Compagnin et al., 2010; Ge et al., 2011; Kim et al., 2012). He et al. (2008) have explored the effects of 5-Aminolevulinic acid photodynamic therapy (5-ALA/PDT) and possible mechanisms involved in the treatment of cervical cancer in vivo an vitro . Therefore, our work attempts to examine the effects of moderately toxic doses of cisplatin and 5-ALA /PDT administered separately and together on Hela cells and evaluate combination treatment in which the dose of the toxic compound could be decreased without reducing efficacy. In this study, Hela cells are utilized as in vitro model of human cervical cancer. Initially, the effects are evaluated on cell viability of single treatment of 5-ALA/ PDT, cisplatin and combination treatment of 5-ALA/ PDT and cisplatin. Then to explore the mechanism of synergistic anticancer activity in the combination of two therapies , the changes are observed in cell apoptosis and expression of p53 signal pathway .

Materials and Methods

Cell lines and Chemicals

Hela cell lines were given by Qilu Hospital cryogenic laboratory of Shandong University (Jinan, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone* Fetal Bovine, Beijing, China), penicillin (100 units/ml) and streptomycin (100 units/ml) (Sigma-Aldrich st. Louis, MO, USA) at 37°C and in an atmosphere of 5% CO_2. The culture medium was changed on alternate days until confluence. Cisplatin was purchased from Qilu Pharm (Jinan, China) and 5-ALA from Shanghai Red-Green photosensitizer Institute (Shanghai, China). Cisplatin and 5-ALA were dissolved in phosphate-buffered saline (PBS) whose pH was adjusted to 7.4 to obtain a 20 mg/L and 10 mM stock solution respectively. The stock solutions were kept at -20 °C in the dark before use. The final concentrations of cisplatin (0.1, 1, 2.5, 5, 10, 20 mg/L) and 5-ALA (0.1, 0.25, 0.5, 1, 2, 4 mM/L) were obtained directly in the serum-free culture medium at the time of incubation. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide(MTT) were supplied by Sigma-Aldrich (St.Louis.MO, USA).

Cell viability assay of photodynamic activity

The exponentially growing Hela cells (7×10^4 cells/ml) were seeded into 96-well plates. After overnight incubation, the cells under study were incubated with 5-ALA at various concentrations (as described above) for 4 h. Then the cells were washed in PBS twice. The PDT was carried out using Aila laser generator apparatus XD-635AB (Shanghai Fudan-Zhangjiang Bio-Pharmaceutical, Shanghai, China) and the wavelength was set at 635 nm. Under PDT treatment, duration of the laser irradiation was calculated into the effective dose of light energy in J/cm^2. The cultures were subjected to laser irradiation 5 J/cm^2, and then returned to be incubated with DMEM complete culture medium for 24 hours. The cell viability was determined by MTT assay. 20 μl of MTT was added to each cell-culture well and continued to be cultured for 4 h. To achieve solubilization of the formazan crystal formed in viable cells, Dimethyl Sulfoxide DMSO (150 μl) was added to each cell-culture well, followed by gentle shaking for 10 min, and absorbance at 490 nm was recorded using a VersaMax microplate reader (Molecular Devices, California, USA). Three wells were assigned to each group, the means of their values were used as the measured wells. After the addition of PS to the cells, all procedures were carried out in minimal ambient lighting. The MTT assay was repeated three times for consistency. The percentage of cell viability was calculated by dividing the mean absorbance in each treatment group by the mean absorbance in the control group.

Cell viability assay of chemotherapy with cisplatin

For treatment with cisplatin, cell samples were prepared as described for PDT. Hela cells were preincubated for 24 hours before cisplatin was added to their plates at various concentrations (as described above) and continued to be incubated for 24 h. This procedure was carried out in triplicate. After incubation ,cells were washed twice in PBS and released into fresh complete culture medium to be incubated for 24 h. Cell viability was evaluated with MTT.

Cells Viability treated with the combination treatment of PDT and cispaltin

To determine the synergistic anticancer activity of 5-ALA/PDT and cisplatin, Hela cells were incubated in the presence of cisplatin (final concentrations were 0.1, 1, 2.5, 5, 10, 20 mg/L, respectively) for 24 h. After the exposure ,the cisplatin was removed by the medium exchange or 1 mM 5-ALA was added into the cells and incubated in the dark for additional 4 h. Then cells were washed in PBS twice times. PDT was carried out with the light dose of 5 J/cm^2 at the appropriate distance (from the IC_{50} data for 5-ALA/PDT). After irradiation, PBS was replaced with complete culture medium and cells were again retained in the incubator for 24 h. Finally, MTT was used to evaluated the cell viability of combination treatment of 5-ALA/PDT and cisplatin .

Flow cytometry analysis of apoptosis

Hela cells were incubated in the 6-well plate and incubated for 24 hours. Then the cells were treated with complete medium (controls) or medium supplemented with 5-ALA (1 mM 5-ALA, 5 J/cm^2 laser dose of PDT), or combined treatment (1mM 5-ALA ,5 J/cm^2 laser dose of PDT in combination with 0.1, 1, 2.5, 5, 10, 20 mg/L cisplatin respectively) according to the above method described. Apoptosis of 5-ALA/PDT and combination treatment on Hela cells were analyzed by flow cytometry at determined time points . The annexin V-fluorescein isothiocyanate (Annexin V-FITC)/propidium iodide (PI) apoptosis detection kit (Bestbio. Co. Ltd. Shanghai, China) was use to measure typical apoptosis and necrosis

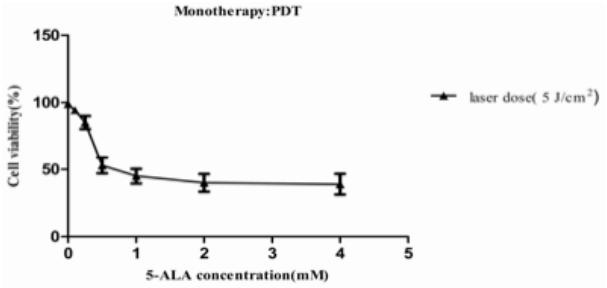

Figure 1. Cell Growth Inhibition Effects of 5-ALA/PDT. Hela cells were incubated in the dark with different concentrations of 5-ALA (0, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 mM) and with laser irradiation 5 J/cm2. Values are expressed as mean percentage ±SD. MTT assay showed cytotoxicity of 5-ALA/PDT was increasing in a dose-dependent manner along with the rise of the photosensitizer(PS) concentrations, and there were significant differences between 5-ALA/PDT treated groups and control group (P<0.05)

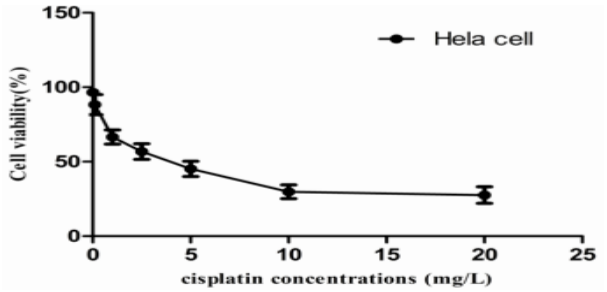

Figure 2. Cytotoxicity of Cisplatin on Hela Cells in Vitro. Hela cells were exposed to increasing doses of cisplatin (0-20 mg/L) for 24 h .Cell viability was measured by MTT assay. Cytotoxic effects with various concentrations of cisplatin were induced in a dose dependent manner compared with cells without cisplatin (P<0.01)

according to the manufacturer's instructions. Briefly, cell suspensions were washed twice and adjusted to a concentration of 1× 10^6 cells/mL with ice-cold PBS. Then the cells were resuspended in 400 μL of binding buffer and stained with 5 μL of Annexin V-FITC and 10μL PI for at least 20 min at 4 °C in the dark. Flow cytometry was carried out with a 488-nm laser coupled to a cell sorter (FACSCalibur; BD Biosciences, San Jose, USA). The forward- and side-scatter gates were set to exclude any dead cells from the analysis and at least 10,000 events were collected for each sample. The apoptosis rates of all treated groups were expressed by calculating the mean values of three experiments.

Western blot analysis for p53, p21, Bcl-2 and Bax

To test whether 5-ALA/PDT ,cisplatin alone and combined treatment altered p53 signal pathway, we measured the expression of p53, p21, Bcl-2 and Bax by Western blot analysis. Hela cells treated with 5-ALA/PDT (1mM 5-ALA, 5 J/cm^2 laser dose of PDT), cisplatin (5 mg/L) or combination treatment (1mM 5-ALA, 5 J/cm^2 laser dose of PDT in combination with 5 mg/L cisplatin) were harvested and washed in PBS. Cell lysates were prepared by using whole cellular protein extraction kits (Beyotime Biotech. CO., China). Protein concentration was routinely measured with BCA Protein Assay Kit(Beyotime Biotech. CO., China). Protein was mixed with 2 × sodium dodecyl sulfate (SDS) sample buffer and separated on 10% SDS-polyacrylamide gel (SDS-PAGE), then it was transferred to nitrocellulose filters .Filters were blocked for 1 h in 5% nonfat dry milk in TBST and probed with primary antibodies: anti-p53, anti-p21, anti-Bcl-2, anti-Bax and anti-β-actin specific rabbit polyclonal Ig G (Santa Cruz Bio., Santa Cruz, CA , USA, Dilution 1:500) overnight at 4 °C. Then filters were washed with TBST 15 min × 3 times and incubated for 1 h with horse-radish peroxidase(HRP)-labeled secondary antibody (dilution 1:10,000) in TBST. The proteins in the filters were detected using an electrochemiluminescent Western blotting detection reagent (Amersham, Buckinghamshire, UK).

Statistical analysis

All assays were set up in triplicate, and the results were expressed as the mean ± standard deviation (SD). Statistical significance was determined by ANOVA and an unpaired Student's t-test. The Values for the different groups were compared. P calues of less than 0.05 were considered statistically signigicantly.

Results

Cytotoxicity of single or combination treatment

To explore whether cisplatin and 5-ALA/PDT have positive interactions with mortality of Hela cells when given in combination, it is necessary to determine their own cytotoxicity. Our results showed that cytotoxicity was induced in a dose-dependent manner by 5-ALA/PDT. As shown in Figure 1, cytotoxicity of 5-ALA/PDT was increasing in a dose-dependent manner along with the rise of the PS concentrations, and there were significant differences between PDT treated groups and control group in which the the concentrations of 5-ALA were zero (P<0.05). The dose-response curve obtained after MTT assay showed that when 5-ALA concentrations rose from 0.1 mM to as much as 2 mM, cell viabilities decreased remarkably along with the rising concentrations of 5-ALA. Whereas increase of 5-ALA concentration from 2 mM to 4 mM only induced further 2%-7% cell death in Hela cells.

In Figure 2, cytotoxic effects with various concentrations of cisplatin were induced in a dose dependent manner compared with cells without cisplatin (P<0.01). Cisplatin concentrations of as much as 10 mg/L killed up to (70.166±4.658)% of cells, where as the remaining fraction appeared to be more resistant to treatment, and increase of cisplatin concentration to 20 mg/L only induced further 5% cell death. That is to say, when concentrations of cisplatin increased from 10 mg/L to 20 mg/L, its toxic effects on Hela cells did not correspondingly rise 2-fold, but only mildly elevated.

To ascertain the effect of combination treatment ,we administered PDT at sublethal doses using an 5-ALA concentration of 1 mM, 5 J/cm^2 laser dose of PDT (The 5-ALA concentration and laser dose were adopted in agreement with the optimal protocol obtained previously experiment.) and different concentrations of cisplatin (0-20 mg/L). Combination treatment showed dramatically increased cytotoxicity on Hela cells compared with

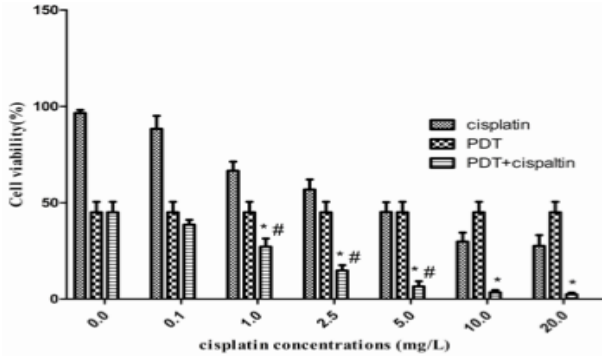

Figure 3. The Cytotoxicity of Single Versus Combined 5-ALA-PDT and Various Concentrations of Cisplatin (0, 0.1, 1, 2.5, 5, 10, 20 mg/L) on Hela Cell. Cell viability was determined by MTT assay 24 h after laser irradiation. The combination treatment group showed dramatically increased cytotoxicity on Hela compared with PDT and cisplatin alone in the groups .Columns, average of three determinations, bars, SD.* : significantly different (P<0.05) from the single cisplatin or PDT and #: significantly different (P<0.05) from combined treatment with lower concentrations of cisplatin by the student's t-test

PDT and cisplatin alone (P<0.05) (Figure 3). This is in agreement with other experiments, in which cytotoxic drug such as cisplatin, gemcitabine were found to act synergistically with PDT in vitro (Crescenzi et al., 2004; Crescenzi et al., 2006). In Hela cells, cell viability induced by PDT + 5 mg/L cisplatin was only 6.607±2.656% and which was significantly lower than combination treatment with 0.1, 1.0, 2.5 mg/L cisplatin. When the concentration of cisplatin continued to rise to 10 and 20 mg/L in combination treatment groups, in spite of the decreased cell viabilities, there were no significant differences in the three combination treatment groups with 5, 10, and 20 mg/L cisplatin. The results of this experiment indicated that the combination treatment of 5-ALA/PDT and low-dose cisplatin effectively inhibits cell proliferation.

Apoptosis induced by 5-ALA/PDT and combination treatment

To verify that 5-ALA/PDT and combination treatment inhibit cell proliferation by inducing cell apoptosis, we investigated apoptotic cells by applying Annexin-V and PI double staining method. As shown in Figures 4, apoptosis were both induced in Hela cells when they were treated with 5-ALA/PDT and combination treatment. But significantly higher rate of apoptosis (early apoptosis and late apoptosis) were observed in combination treatment compared with 5-ALA/PDT (P<0.05). Moreover, in the combination treatment groups, 5-ALA/PDT combined with 5,10, and 20mg/L cisplatin caused more apoptotic cells than the groups of 5-ALA/PDT combined with 0.1, 1, and 2.5 mg/L cisplatin (P<0.05). But there were no significant differences in apoptotic rate of of the three combination treatment groups 5-ALA/PDT in combination with 5, 10, and 20 mg/L cisplatin. These results suggested 5-ALA/PDT in combination with relative low-dose cisplatin could lead to greater apoptosis inducing potential in Hela cells and raising the concentration of cisplatin in the combination treatment group did not improve the apoptotic rate.

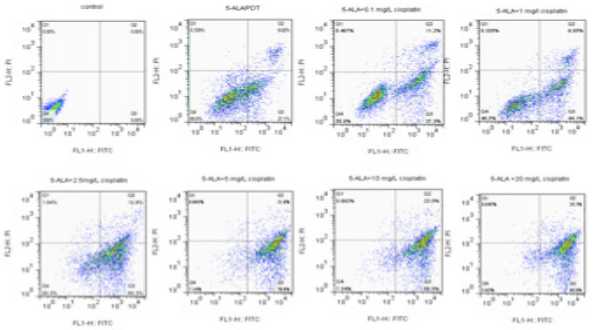

Figure 4. Apoptosis of 5-ALA/PDT Treatment and Combination Treatment of 5-ALA/PDT + Cisplatin (0.1, 1, 2.5, 5, 10, 20 mg/L) Mediated on Hela Cells by Flow Cytometry. After treatment the apoptotic cells were stained with Annexin V/PI .The apoptotic cells in combination treatment were significantly higher than in 5-ALA/PDT.The apoptotic cells in combinaton treatment groups of 5-ALA/PDT and 5, 10, 20 mg/L cisplatin were higher than 5-ALA/PDT and 0.1, 1, 2.5 mg/L cispaltin.But no significant differences were obtained in apoptotic cells of the three combination treatment groups of 5-ALA/PDT and 5, 10, 20mg/L cisplatin

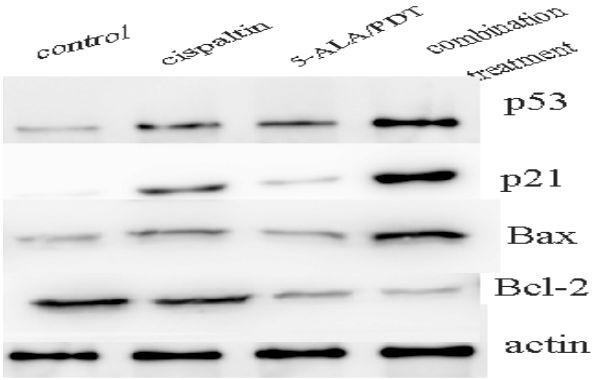

Figure 5. Western Blot Analysis for p53, p21, Bcl-2 and Bax in Control and Treated Cells. SDS-PAGE were carried out on cell lysates of control cells and treated cells 24 h after the end of treatment with 5-ALA/PDT alone (1 mM 5-ALA 5 J/cm^2 laser doses of PDT, cisplatin alone (5 mg/L) and their combination treatment

The expression of p53, p21, Bcl-2 and Bax on Hela cells afer 5-ALA/PDT, cisplatin and combination treatment

Although there is no mutation of the p53 gene in Hela cells, its product p53 protein is virally inactivated by HPV E6-activated ubiquitin-dependent protease degradation (Scheffner et al., 1990). However, it has been previously reported that p53 can be reactived upon treatment with cispaltin (Wesierska-Gadek et al., 2002; Schloffer et al., 2003; Liu et al., 2008). In our study, we also observed that expression of p53 protein and its down-stream targets cyclin p21 was upregulated after 5 mg/L cisplatin treatment. Meanwhile, Bax (a pro-apoptotic member of the Bcl-2 family) was upregulated and anti-apoptotic Bcl-2 protein did not change obviously. Then we examined the effect of 5-ALA/PDT (1 mM 5-ALA, 5 J/cm^2 laser dose of PDT) on the cellular expression of p53 pathway in Hela cells. As shown in Figure 5, p53 expression increased in Hela cells after irradiation 24 h, while slightly elevation were observed in the expression of p21. Similarly, 5-ALA/PDT induced slightly increased Bax expression and Bcl-2 expression noticeable decreased. Then in combination

treatment p53 expression increased significantly compared with single treatment, and p21 expression had increased obviously compared with cisplatin treatment. This illustrates that PDT in combination with low-dose cisplatin can induce cell apoptosis through activation of apoptosis-related protein p53 to a more significant level, as compared with each single treatment. The expression pattern of Bax and Bcl-2 subsequent to combination treatment showed the same tendency but more stronger effect compared with 5-ALA/PDT alone. These results suggested that combination treatment significantly enhanced molecular functions changes of p53 signaling pathway induced by cisplatin treatment in Hela cells .

Discussion

In the present study, we investigated 5-ALA/PDT for their demonstrated inhibiting effects along with low-dose cisplatin on human cervical cancer cells consisting HPV-18 subtypes (Hela cells). Our initial experiments investigating the effects of combination treatment on cell viability showed enhanced cytotoxic effects on Hela cells with relatively low doses of 5-ALA/PDT in combination with cisplatin. Subsequently, we scrutinized the molecular pathway involved in combination treatment-induced apoptosis in the anticipation that it will provide an experimental proof for the clinical application of this combination. We found that 5-ALA/PDT in combination with low-dose cisplatin could synergistically enhance anticancer activity through activated p53 signal pathway.

5-ALA is one of the second-generation PS and it has been approved to be used in clinical trial by the USA FDA since 2000. 5-ALA itself does not serve as PS but a natural biological precursor in the heme biosynthetic pathway, which produces protoporphyrin IX (Pp IX), an intrinsic and safe PS (Peng et al., 1997; Davila, 2011; Ishizuka et al., 2011). 5-ALA induces effective accumulation of PS Pp IX in tumor cell and has high clearance rate in vivo without phototoxicities. Some studies have demonstrated that 5-ALA/PDT can effectively inhibit the growth of cancer cells in vitro (Peng et al., 1997; He et al., 2008; Chen et al., 2011a; Chen et al., 2011b; Gui et al., 2012). The same PDT result in our study also demonstrated that 5-ALA/PDT induced cytotoxicity depending on PS concentrations. But when the 5-ALA concentration was > 2 mM, the cell survival rate reached a plateau . The results suggested a consistence with reports (He et al., 2009; Chen et al., 2011b) that as PpIX is the product in the heme biosynthetic pathway and biosynthetic capacity of cells is limited, when 5-ALA reached a higher concentration, the cellular PpIX concentration becomes saturated and obviously could not be increased.

It has been reported that PDT with mitochondria-localizing PS, such as 5-ALA, can induce rapid cell death via apoptosis (Tsai et al., 2005; Tsai et al., 2009). Our study confirmed that cytotoxicity induced by 5-ALA/PDT and combination treatment was caused by induction of apoptosis. Initially, in combination treatment the apoptotic rate increased with the rising concentrations of cisplatin (48.5% ~ 91.9%). However, when the concentration of cisplatin elevated up to > 5 mg/L, the apoptotic rate no longer increased remarkably (91.9%-93.7%), suggesting that cisplatin might play a sensitized role in combination treatment.

There are several reports linking induced increased expression of p53 and apoptosis after 5-ALA/PDT (Yow et al., 2007; He et al., 2009). In our study, we found that 5-ALA/PDT might exert their synergistic anticancer activity with low-dose cisplatin in Hela cells through enhancement of p53 signal pathway. The p53 ptotein is a sequence-specific DNA-binding protein, and it is presumed to be involved in cellular response to DNA damage, producing arrest in the G1 phase of the cell cycle through induction of p21 to allow efficient repair of the DNA before entry to S phase, or promote apoptosis via transcriptional activation of pro-apoptotic(Bax) or repression of anti-apoptotic gene if damage is too large to be repaired (Maclaine and Hupp, 2009) .

The cisplatin is an effective DNA crosslinking agent and it has been suggested that low-dose cisplatin could make the tumor cell radiosensitive and inhibit DNA repair processes (Fu et al., 1988; Lagrange et al., 1996). The accumulation of p53 is a key event in cisplatin-associated chemotherapy . Similar to these observations, in our study we also found a significant increased expression and transcriptional activity of p53, as evidenced by substantial up-regulation of p53-downstream major targets ,up-regulated p21 and Bax in Hela cells treated with cisplatin alone therapy. Up-regulated p21 can prevent the activation of cyclin E/A-dependent kinase 2 complex and induce cell-cycle G1 phase arrest (Massague, 2004). Bax/Bcl-2 is known to be a key regulator of apoptosis and crucial determinant of cellular fate (Danial and Korsmeyer, 2004). The ratio of Bax/Bcl-2 sets the threshold of susceptibility to apoptosis for the intrinsic pathway. 5-ALA/PDT also up-regulated the p53 levels in Hela cells, and then low-dose cisplatin and 5-ALA/PDT exerted an overlapping effect on up-regulation of p53 and its down-stream targets. Therefore, the synergistic activated to up-regulation of p53, p21 and Bax/Bcl-2 ration contributes greatly to enhance growth suppression in combination treatment of 5-ALA/PDT and low-dose cisplatin .

These results thus offered a mechanistic understanding of the observed enhancement synergistic anticancer activity in our study after 5-ALA/PDT in combination with low-dose cisplatin. These findings can lead to new treatment strategies and could also pave the way in the reduction of the amount of anticancer agents required as therapeutic dose. A reduction in the amount of cisplatin and PS can eventually lead to reduction in the toxicity and side effects caused to the patients. Therefore, our finding suggests that administration of 5-ALA/PDT in combination with low-dose cisplatin may be an effective and feasible therapy for cervical cancer.

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