

## Cisplatin enhances the efficacy of 5-aminolevulinic acid-mediated photodynamic therapy in human head and neck squamous cell carcinoma

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**Abstract.** Photodynamic therapy (PDT) has become a promising option for the treatment of head and neck, and other forms of cancer. 5-Aminolevulinic acid (ALA) is one of the popular photosensitizers used in PDT. It is a heme precursor and is converted to a photosensitizer protoporphyrin IX. In this present study, the combination of anticancer drug cisplatin (CDDP)- and ALA-mediated PDT was used to study the cytotoxicity *in vitro* as well as *in vivo*. Human head and neck cancer cells AMC-HN3 were treated with cisplatin- and ALA-mediated PDT individually, and also in combination. Several approaches like confocal microscopic study, cytotoxicity assay, etc have been performed to study the intracellular accumulation of protoporphyrin IX in cells and its effectiveness in PDT, when treated in combination with chemotherapy drug, cisplatin (CDDP). The combination of treatments efficacy was also studied in tumor xenograft model. Compared to the individual treatments, combination of CDDP and PDT was found to be more cytotoxic in AMC-HN3, and also more effective in reducing the tumor volume in mice xenograft. Thus with the combined therapy, not only the efficacy of treatment can be enhanced, but the doses of the drugs can also be lowered. This in turn can reduce the side effects of the chemotherapy drugs. Therefore, this study may lead to a potential drug-PDT combination that may be a useful treatment modality for human head and neck cancer.

**Key words:** Cisplatin — ALA — Photodynamic therapy — Head and neck cancer

**Abbreviations:** ALA, 5-aminolevulinic acid; CDDP, cis-diamino-dichloro-platin (cisplatin); DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FBS, foetal bovine serum; PDT, photodynamic therapy; PpIX, protoporphyrin IX.

### Introduction

Photodynamic therapy (PDT) is used to treat several types of cancer by the administration of a light absorbing chemical,

known as photosensitizer and light of a specific wavelength. The photosensitizer is activated by absorbing light and produces singlet oxygen, causing damage to the tumor cells (Dougherty et al. 1998). The mechanism of the anticancer effect of photodynamic therapy has been studied where generation of singlet oxygen and free radicals were reported to promote the secondary necrosis of the cancer cells (Henderson and Dougherty 1992). Hematoporphyrin photosensitizers are one of the commonly used photosensitizers in PDT (Pujol-Lereis et al. 2010). But the main disadvantage of currently used hematoporphyrin photosensitizer in PDT is its long-lasting skin phototoxicity (Wolford et al. 1995). Therefore, several studies are being performed to reduce this skin phototoxicity. Recently, 5-aminolevulinic acid (ALA) is becoming popular

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in PDT to treat various diseases (Kennedy et al. 1992). ALA is converted to photosensitizer, protoporphyrin IX (PpIX) in the biosynthetic pathway of heme (Heinemann et al. 2008). After exogenous administration of ALA, PpIX accumulates within cells, resulting in comparatively low activity of ferrochelatase, the enzyme responsible for the conversion of PpIX to heme. The useful aspect of ALA-PDT is that protoporphyrins IX along with other intermediates are rapidly eliminated from the body and hence the risk of prolonged skin photosensitivity is limited to one to two days. This may be one of the important reasons for choosing ALA as an efficient photosensitizer in PDT (Kennedy et al. 1990, 1992). Several studies have been reported to use ALA as a photosensitizer in PDT to treat several cancers like gastrointestinal tract, skin, lung, bladder, oral cancer (Karmakar et al. 2007; Bourré et al. 2008; Gui et al. 2012).

On the other hand, cisplatin has been used as an anticancer drug for a long time (Go and Ajei 1999; Geisler et al. 2012). Cisplatin alone or in combination with other treatment modalities showed effectiveness in the treatment of malignant tumors of the head and neck (Uehara et al. 2006; Mao et al. 2009; de Carvalho Maroni et al. 2012; Minami et al. 2012). However, the administration of cisplatin alone has some limitations of severe side effects (Florea and Büsselberg 2011). Cisplatin has been reported to have adverse effects like renal toxicity, ototoxicity, etc (Ravi et al. 1995; Zhang et al. 2007; Yao et al. 2007). Combination of cisplatin with other conventional treatment modalities has been studied to reduce its side effects with the enhanced efficacy (Azmi et al. 2010, Ehrlich et al. 2010; Okusaka et al. 2010, Segawa et al. 2010).

In the present study, the efficacy of cisplatin (CDDP), ALA-PDT and CDDP+ALA-PDT combination therapy was compared to find out whether an enhanced efficacy can be obtained on human head and neck cancer cells, AMC-HN3. By this combination treatment with cisplatin and ALA-PDT, not only the efficacy of PDT can be enhanced but the adverse side effects of chemotherapy drugs can also be reduced by reducing the doses of individual drugs. Therefore, the combination CDDP and ALA-PDT can lead to a promising mode of treatment of head and neck cancer with an enhanced efficacy and lower side effects as well.

## Materials and Methods

### Chemicals

The photosensitizer ALA (5-aminolevulinic acid), was purchased from Sigma-Aldrich, Korea. A stock solution was made in DPBS and kept in aluminium foil at  $-20^{\circ}\text{C}$ . Anti-cancer drug cisplatin (CDDP: cis-diamino-dichloro-platin) purchased from United Pharm. Korea and a stock solution was prepared by diluting it in dimethyl sulphoxide (DMSO). For cell culture, all medium supplements, that is DMEM,

foetal bovine serum (FBS) and antibiotics were supplied by Hyclone (South Logan, UT, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), DMSO were purchased from Sigma (St. Louis, MO, USA).

### Cell culture

The human head and neck squamous carcinoma cell line AMC-HN3 were grown in DMEM culture medium containing 10% FBS and 1% antibiotic-antimycotic solution in a cell culture flask (Nunc, Denmark). The cells were maintained at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a humidified atmosphere. The morphology of the cells was observed by inverted microscope (Olympus CK40, Japan).

### Accumulation of intracellular photosensitizer

To check the accumulation of photosensitizer into cells,  $10^5$  cells/ml of AMC-HN3 cells, in exponential growth phase, were seeded in a 6 well plate and incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  incubator for 24 h and were allowed to adhere to the surface of the culture plate. After 24 h of incubation, the culture media was replaced with the fresh serum free medium containing 1 mM ALA. Formation of PpIX and its accumulation in the cells were observed by the confocal laser scanning microscope after 0, 3, 6 and 9 h of treatment. The images taken by confocal microscope were analyzed by Image J software (National Institutes of Health) to quantify the fluorescence of the cells.

### CDDP monotherapy

To measure the cell cytotoxicity by CDDP monotherapy, 100  $\mu\text{g}/\text{ml}$  CDDP was filtered with sterile 0.2  $\mu\text{m}$  syringe filter (Gelman, USA) and diluted with 100  $\mu\text{l}$ , pH 7.3 in DPBS (Hyclone, USA) and the cells were treated with different concentrations from 0–25  $\mu\text{g}/\text{ml}$ .

### ALA-PDT monotherapy

In a 96 well plate, cells were treated with ALA starting from 0–200  $\mu\text{g}/\text{ml}$  in a serum-free medium and incubated at  $37^{\circ}\text{C}$  for 24 h. After 24 h of incubations, the culture medium were replaced with the fresh one and the laser irradiation was performed by using a laser of 632 nm for 15 min to obtain an energy of 6.0  $\text{J}/\text{cm}^2$  using a reflector plate 10 cm above the diffuser tip.

### CDDP+ALA-PDT combination therapy

The cells were treated with 6.25  $\mu\text{g}/\text{ml}$  CDDP and ALA (25  $\mu\text{g}/\text{ml}$  and 50  $\mu\text{g}/\text{ml}$ ) in serum-free medium, and incubated for 24 h. After incubation, a laser of 6.0  $\text{J}/\text{cm}^2$  was irradiated using a reflector plate above the 10 cm diffuser tip.

### Cell cytotoxicity test

MTT assay methods (Scudiero DA et al. 1988) were used to evaluate the effect of CDDP, ALA-PDT and CDDP+ALA-PDT in combination against AMC-HN3 cells. Briefly, after the treatment with CDDP and ALA-PDT singly or in combination for 24 h, 50  $\mu$ l of MTT solution (2 mg/ml) was added to each well and incubated for 4 hours. After incubation, the medium of each well were removed and 150  $\mu$ l of DMSO was added to dissolve the violet-blue crystals of metabolized products. The absorbances of each well were measured at 540 nm using Asys UVM340 microplate Reader, Biochrom. The percentage of cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Mean optical density in test well}}{\text{Mean optical density in control well}} \times 100$$

### Cell morphology study by optical microscopy

The concurrent appearance of the cells before and after treatment of CDDP and ALA-PDT were observed through the inverted microscope (Olympus CK40, Japan). The morphology of the cells of control and treated groups were observed and the photographs were taken. Cell morphology of control and treated groups were also compared.

### Tumour xenograft

AMC-HN3 cell lines were maintained at 37°C temperature and 5% CO<sub>2</sub> in an incubator for an exponential growth. Cells were then harvested by 0.25% trypsin-EDTA and a suspension of cell with a concentration of 10<sup>8</sup> cells/ml in a medium was prepared. The cell suspension was injected subcutaneously into the back of 6 week old BALB/c/nu/nu female mice with a 30 gauge (G) 0.1 ml insulin syringe. The formation of tumors was observed from 1 week to 4 weeks and the tumor sizes were measured once a week. The animal studies were performed in accordance with the experimental protocols of the animal ethics committee of Dankook University.

### Photodynamic therapy of tumors

When the tumor reaches about 100–300 mm<sup>3</sup> in size, the experimental tumor xenograft mice ( $n = 60$ ) were divided into five experimental groups as follows:

- Laser group ( $n = 10$ ): tumors irradiated from the four directions by 632 nm diode laser (132 J/cm<sup>2</sup>, 400 mW)
- ALA group ( $n = 10$ ): ALA-administered group
- CDDP group ( $n = 10$ ): 2 mg/kg of CDDP-administered group
- ALA-PDT group ( $n = 15$ ): (ALA-PDT groups in laboratory mice), 375 mg/kg of ALA were injected intra-

peritoneally and after 6 h, laser was administered for PDT

- CDDP+ALA-PDT group ( $n = 15$ ): ALA-PDT and CDDP combined treatment group.

The cells without any treatment (neither with CDDP nor with ALA-PDT) served as a control group.

### Determination of the antitumor effect

To study the antitumor effect, tumor volume was measured twice in a week after treating the mice as mention above. Tumor volume of each mouse from each group was measured with calipers and was calculated using the following formula:

$$V = (4/3 \times A \times B \times C) \times 1/2$$

where V is volume, A is longest diameter, B is shortest diameter and C is diameter perpendicular to A and B.

### Hematoxylin and eosin staining

Frozen tissue sections on glass slides were fixed in acetone (Sigma-Aldrich) for 10 min. The tissue sections were allowed to dry for 10 min and then immediately stained with Mayer hematoxylin (Sigma-Aldrich) for another 10 min. Afterward, the slides were rinsed in tap water and then stained with eosin (Surgipath, Richmond, IL, USA) for 10 s. The slides were then dehydrated in 95% and 100% ethanol in succession for 10 s followed by 30 s incubation in xylene (Sigma-Aldrich). Finally, the slides were mounted on glass coverslips, sealed and analyzed under the microscope. Stained tumor sections were visualized at 100 $\times$  magnification and images were taken for further study.

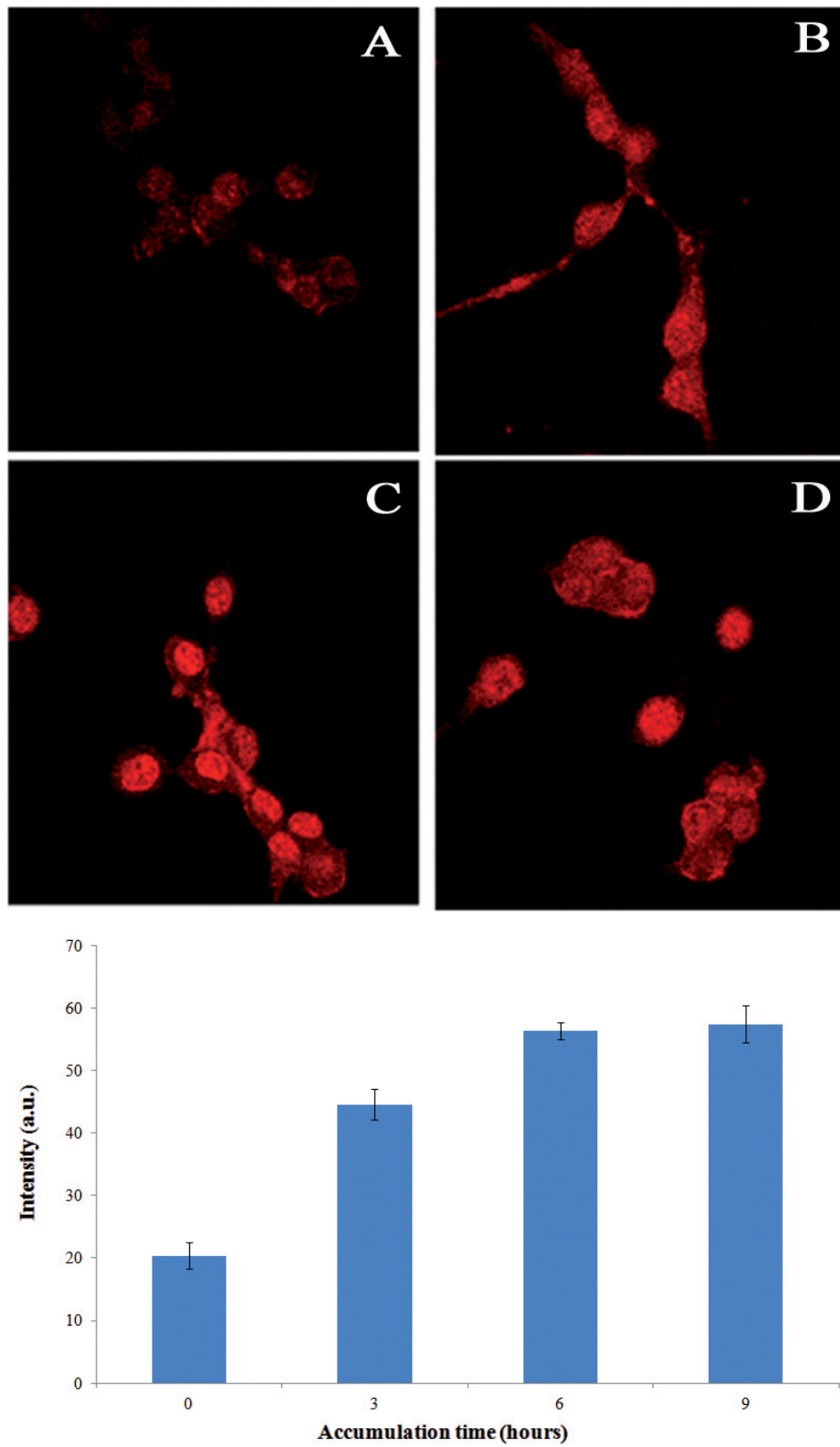
### Statistical analysis

Results are expressed as the mean  $\pm$  standard deviation (SD). The significance of differences was evaluated by one-way analysis of variance (ANOVA) followed by the least significant difference procedure (LSD). Differences were considered statistically significant at  $p < 0.05$ .

## Results

### Observed the accumulation of intracellular photosensitizer

The intracellular accumulation of PpIX was observed by confocal laser scanning microscopy according to incubation time 0, 3, 6 and 9 hours after ALA application. The fluorescence intensities of the cells were measured by Image J software. The changes in average intensities of the each well with incubation time were expressed in a histogram. From the histogram it can be stated that the PpIX fluorescence was saturated after 6 h of ALA application to AMC-HN3 cell (Fig. 1).



**Figure 1.** Intracellular accumulation of PpIX according to incubation time with ALA – 0 h (A), 3 h (B), 6 h (C), and 9 h (D) after ALA application. Graph shows that the fluorescence of PpIX was saturated at 6 h after the ALA application to AMC-HN3 cell.

### Optical microscopy

The morphologies of the cells treated with PDT only and CDDP+ PDT combination were observed through inverted microscope and compared with the control. In the control group, most of the cells were attached to the bottom of the culture flask and were in growing condition. In ALA-PDT group, some of the cells were eliminated from the bottom of the cell culture flasks and in CDDP+ALA-PDT group a large number of cells was eliminated from the bottom of the culture flask. The morphology of the treated cells in combination group was also changed compared to the control cell. In combination group, most of the cells were round shaped. Changes in the cell membrane can also be observed (Fig. 2).

### Cytotoxicity study by MTT assay

The viability of cell was measured by MTT assay and the percentage of cell viability was calculated for different concentrations of CDDP and also for different time interval from 3 to 48 h. From 1.56  $\mu\text{g/ml}$  or higher concentrations of CDDP, overall cell viability was significantly lowered. From the MTT assay, it can be stated that the viability of AMC-HN3 cell was decreased with the increasing concentration of CDDP as well as the incubation time (Fig. 3A).

From the concentration of 12.5  $\mu\text{g/ml}$ , ALA begun to show a significant difference in cell viability compared to the control. Viability of the cells was decreased significantly with the increasing concentration of ALA for PDT. Most of the cells were dead for the ALA concentration of 100  $\mu\text{g/ml}$  and above (Fig. 3B).

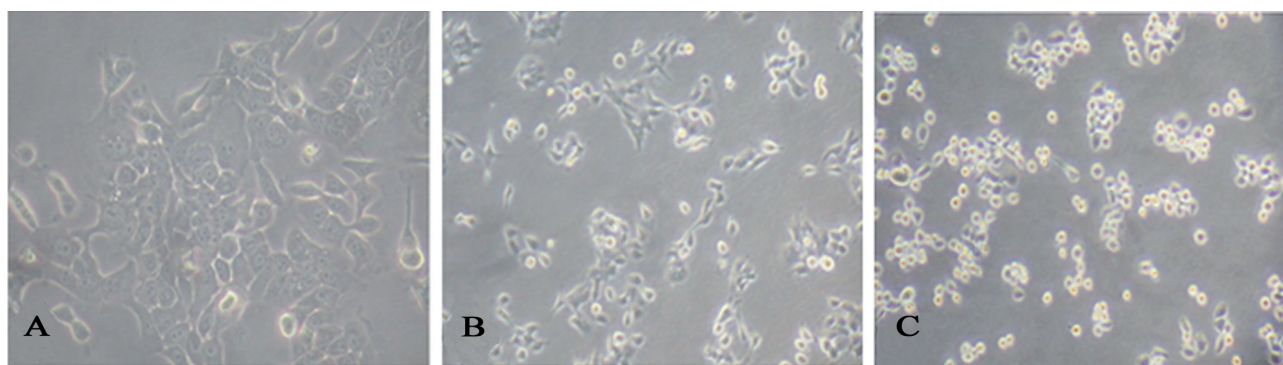
AMC-HN3 cells were treated with CDDP and ALA-PDT individually as well as in combination. Viability of cell was decreased when the cells were treated with 6.25  $\mu\text{g/ml}$  of CDDP and incubated for 24 h. Cell viability was also decreased for ALA-PDT at concentrations of 25  $\mu\text{g/ml}$  and

50  $\mu\text{g/ml}$  of ALA. However, the cytotoxic effect of ALA-PDT was much higher for higher concentration of ALA. But for the cells treated with combination of 6.25  $\mu\text{g/ml}$  of CDDP and different concentrations of ALA in PDT, the cell viabilities were dramatically decreased compared to the individual treatments of CDDP and ALA-PDT respectively. Moreover, most of the cells were dead for the highest concentration of ALA (50  $\mu\text{g/ml}$ ) for PDT in combination with CDDP (Fig. 3C).

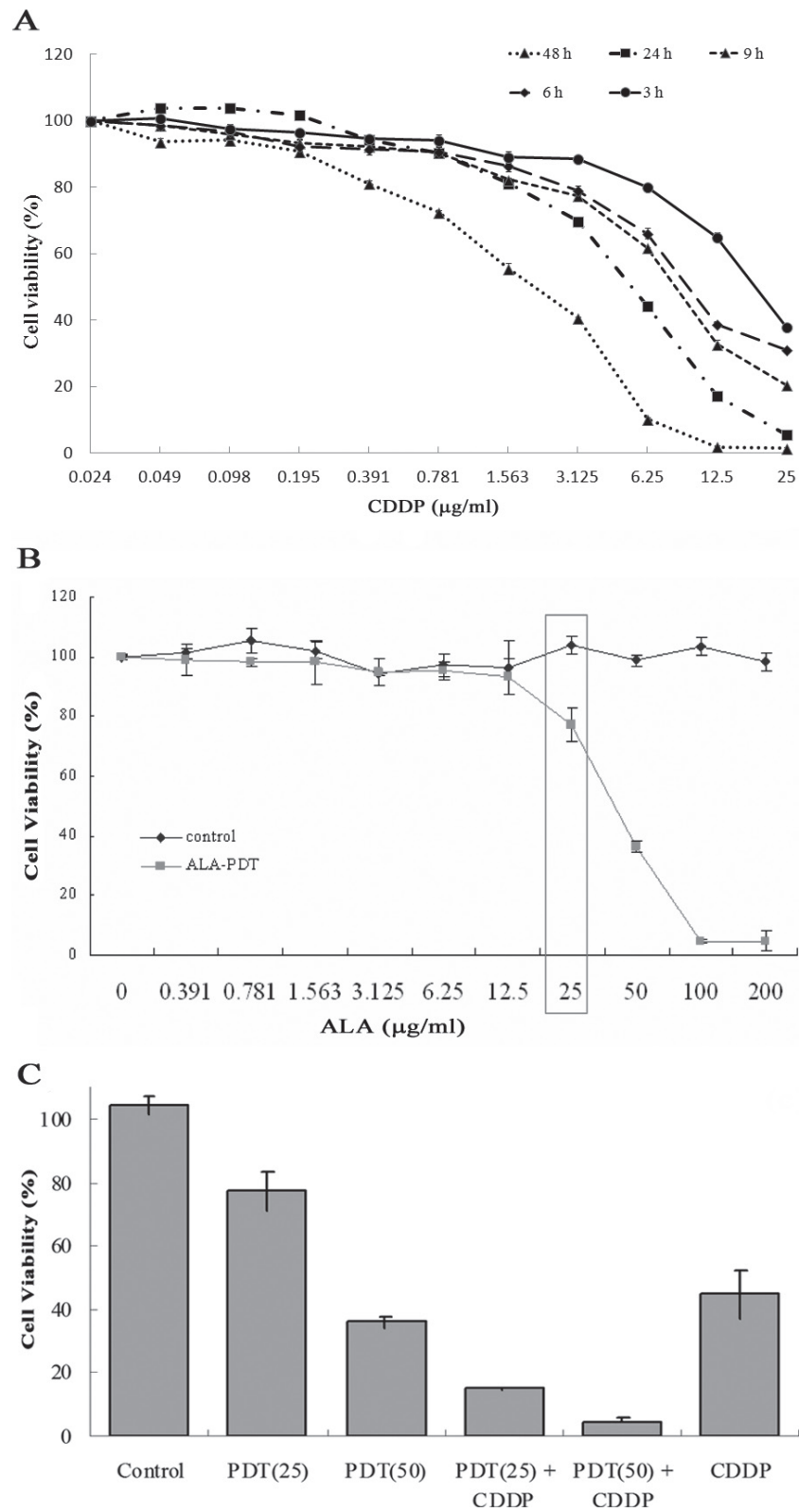
### The photodynamic therapy of xenograft tumors

After 4 weeks of treatment, Laser group, ALA group and CDDP group showed an increase in the size of the tumor in all cases. In ALA-PDT group, the tumor was not healed in 3 mice (20%), in 8 cases (53%) tumor was cured and 4 cases showed tumor recurrence (27%). On the other hand, in CDDP+ALA-PDT group, complete tumor remission was occurred in 12 cases (80%) and one case showed tumor recurrence (7%) (Fig. 4).

After 28 days of PDT treatments, size of the tumor was compared to that of prior to treatment. For Laser group, 2 weeks after the laser treatment the size of the tumor was increased compared to that of before treatment from  $532 \pm 51 \text{ mm}^3$  to  $842 \pm 167 \text{ mm}^3$  whereas 4 weeks after it was observed to be increased steadily to  $1258 \pm 370 \text{ mm}^3$ . Two weeks after the administration of ALA only (ALA group), the size of the tumor was increased from  $510 \pm 12 \text{ mm}^3$  to  $860 \pm 8.5 \text{ mm}^3$  and again after four weeks, it was increased steadily to  $1344 \pm 252 \text{ mm}^3$ . Two weeks after administration of CDDP only (CDDP group), the size of the tumor was increased from  $396 \pm 102 \text{ mm}^3$  to  $574 \pm 104 \text{ mm}^3$  and again after four weeks it was increased steadily to  $1125 \pm 94 \text{ mm}^3$ . On the other hand, two weeks after administration of ALA-PDT (ALA-PDT group), the size of the tumor was decreased from  $512 \pm 38 \text{ mm}^3$  to  $68 \pm 2.5 \text{ mm}^3$  but for four weeks, it was slightly increased to  $195 \pm 129 \text{ mm}^3$ . How-



**Figure 2.** Microscopic findings of AMC-HN3 cells in culture flask (magnification  $\times 200$ ) before and after treatment. **A.** Normal cells without any treatment. **B.** Cells treated with ALA-PDT only. **C.** AMC-HN3 cells treated with CDDP+PDT in combination.



**Figure 3.** A. Cell viability of AMC-HN3 cell by incubation time with various concentrations of cisplatin. B. Effect of various concentration of ALA-based PDT on AMC-HN3 cell viability. C. Effect of ALA-PDT+CDDP combination therapy on AMC-HN3 cells. Control, normal cells with no treatment; PDT(25), PDT with 25 µg/ml of ALA; PDT(50), PDT with 50 µg/ml of ALA; CDDP, 6.25 µg/ml of CDDP.

ever, for administration of ALA-PDT and CDDP combined therapy (CDDP+ALA-PDT group), the size of the tumor was decreased from  $445 \pm 29 \text{ mm}^3$  to  $32 \pm 40 \text{ mm}^3$  after two weeks and after four weeks it was again decreased slightly to  $27 \pm 85 \text{ mm}^3$  (Fig. 5A).

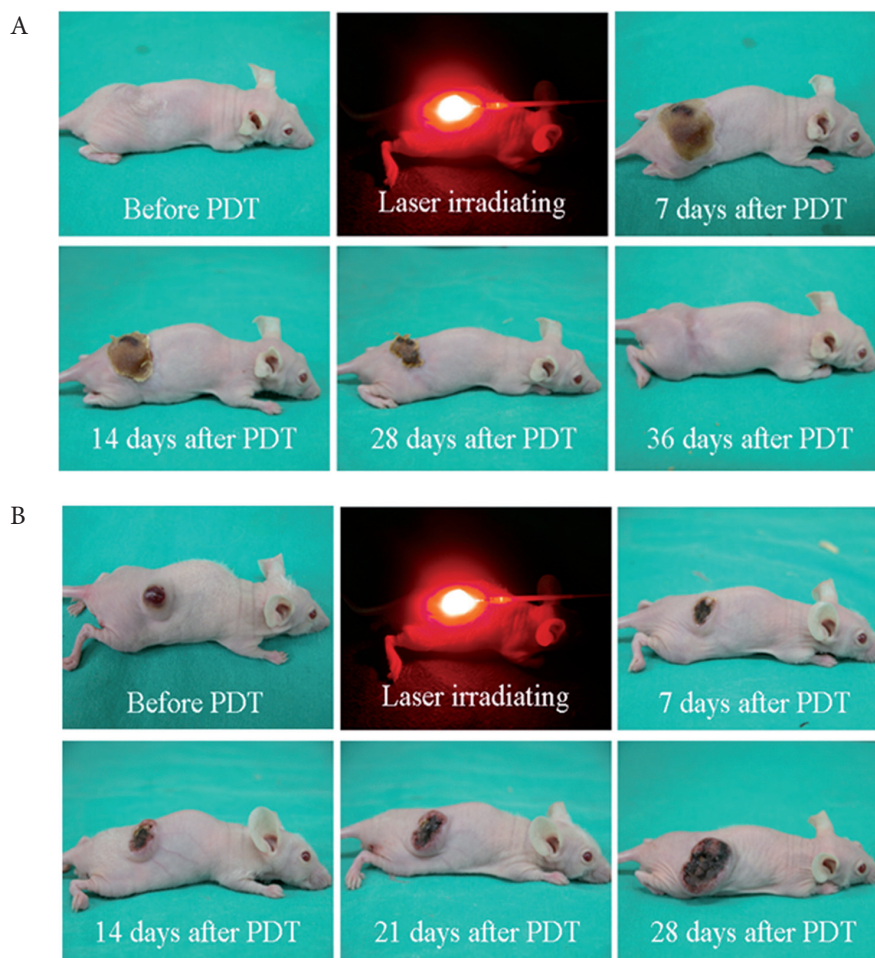
Hematoxylin-eosin staining of tumor xenograft was performed to confirm the therapeutic effect of the combination treatment with CDDP and ALA-PDT. Throughout observations were done for the control and combination treatment groups for 24 h before treatment; 24 h and 48 h after the treatment. The tumor necrosis and lymphocyte infiltration and a wide range of coagulative necrosis of the tumor were also observed for 48 h of post treatment with combination therapy (Fig. 5B).

### Discussion

The main advantage of using ALA in PDT is that it has less side effect compared to other photosensitizers (Kennedy and Pottier 1992). Generally, most of the currently used

photosensitizers are spread all over the body after administration. This may cause toxicity in different organs during PDT. However, ALA is mainly accumulated in mucous membrane, therefore a relatively lesser risk of side effects are expected. In addition, ALA can also be administered orally unlike the other photosensitizers, which are commonly administered by injection (Kennedy et al. 1990, Sharman et al. 1999).

The amount of generated PpIX in cultured cell lines can be observed by measuring the fluorescence of PpIX after ALA administration. Generally maximum fluorescence of PpIX can be observed at 1 to 6 hours after the administration of ALA (Regula et al. 1995). The accumulated PpIX is responsible for the release of serum into the medium. So the media should be serum free in the experimental groups (Bourré et al. 2008). Thus serum free media was used for our present *in vitro* study. A chemotherapy drug cisplatin was used in combination to increase the efficiency of killing tumor cells by ALA-PDT. In this combination therapy, the lowest concentration of CDDP was used  $6.25 \mu\text{g/ml}$  which was close to the IC50 value after 24 h of incubation with the drug. On the



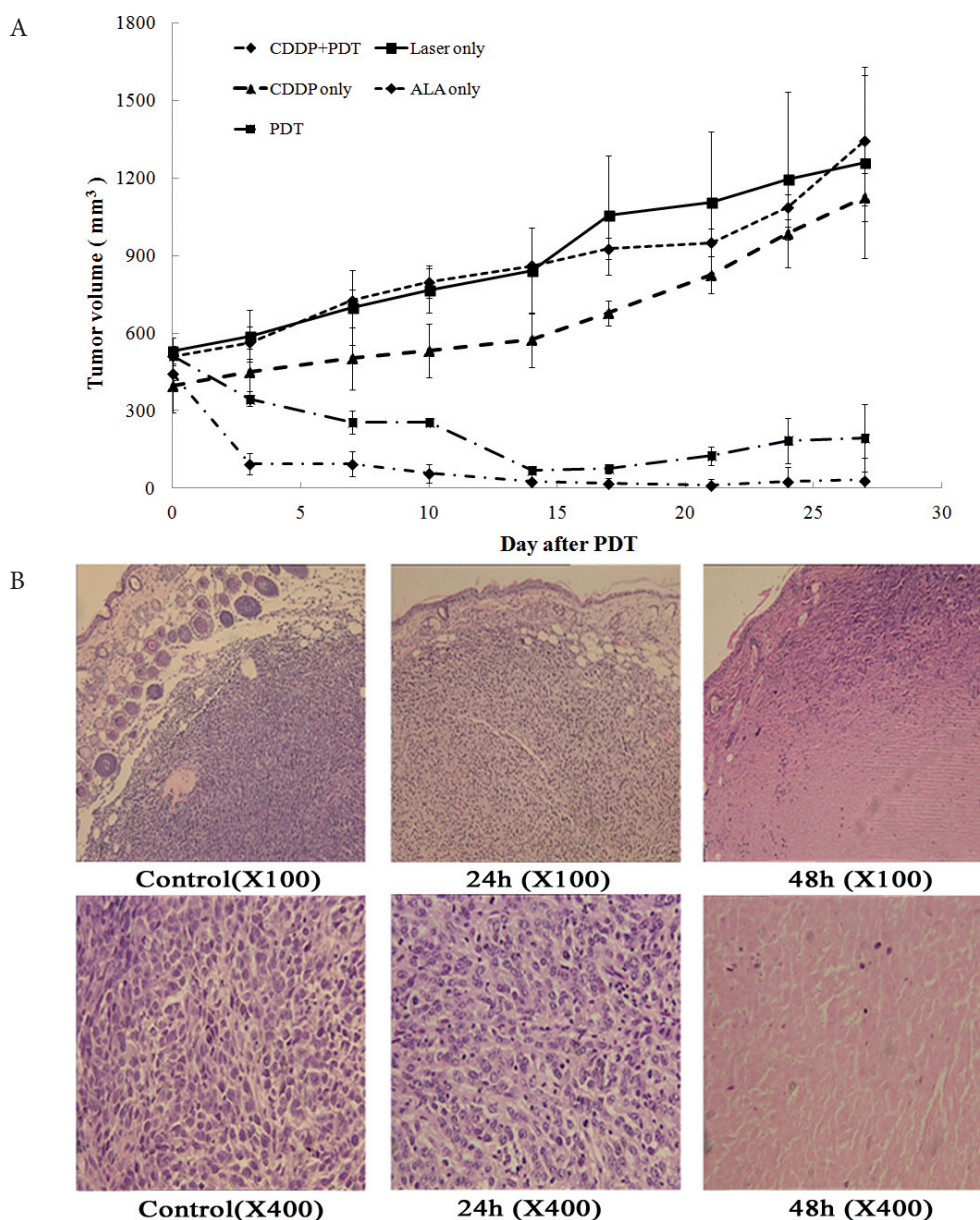
**Figure 4.** Effect of ALA-PDT+CDDP combination therapy on xenograft tumor of mice before and after 7, 14, 28, 36 days of laser irradiation. **A.** Photograph showing complete remission of tumors in combination treatment. Complete remission was observed in approximately 80% of cases. **B.** Photograph showing no remission of tumors. Approximately 7% mice showed recurrence of tumors.

other hand, 25  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$  concentrations of ALA were selected for this combination treatment.

From the cell viability study, the percentages of cell viability were decreased when the cells were treated with 25  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$  of ALA in PDT, respectively. Again, for CDDP at a concentration of 6.25  $\mu\text{g/ml}$ , the percentage of cell viability was approximately near the IC50 value compared to the control. But for the combination of CDDP with PDT at 25  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$  of ALA, the cell viabilities were

dramatically decreased. Therefore, the combination therapy was more effective than the monotherapy and showed higher efficacy even in the lower concentration of CDDP. Cell morphology was also changed in combination treatment. From microscopic study, it was found that the number of apoptotic cells with rounded shape was much higher compared to the control and even individual treatments.

For *in vivo* xenograph study, there was no significant difference in tumor size after 4 weeks of treatment with laser,



**Figure 5.** **A.** Representative graph showing changes in tumor volume for each treatment group. **B.** Histologic findings of xenografted tumor after ALA-PDT+CDDP combination therapy. Tumors collected from the mice without any treatment i.e. neither CDDP nor ALA-PDT served as a control group. After 24 h of ALA-PDT treatment, there were focal tumor necrosis and lymphocytic infiltration. Again after 48 h of ALA-PDT, there was extensive coagulative tumor necrosis.



ALA and CDDP, respectively. But in the case of combination treatment with CDDP and ALA-PDT, significant decrease in tumor volume can be observed and recurrence of tumor was also significantly lower compared to the individual treatments. This result also signifies the enhance efficiency of combination therapy than the mono-therapy. Xenographed tumours were stained with Hematoxylin-eosin before the treatment, 24 h and 48 h after the treatment. From the microscopic study of the stained slides, necroses of the tumor and lymphocyte infiltration were observed in the tissue after 24 h of treatment and on the other hand, coagulative necrosis with inflammatory cells was observed around the tumor tissue after 48 h of treatment.

Therefore, the combination of ALA-PDT and CDDP has much more cytotoxic effect on AMC-HN3 cell than the effect of ALA-PDT and CDDP alone. Thus an enhanced effect can be obtained with the same concentrations of drugs applied individually. On the other hand, lower concentrations of CDDP as well as ALA can also be applied to achieve the same efficacy of individual treatments by combining two treatment modalities. Thus, the adverse side effects of CDDP can be reduced by reducing the concentration in combination therapy. Though more extensive studies are required, this mode of treatment can be an alternative treatment modality for reducing the side effects of cisplatin with a higher efficacy against head and neck cancer and other types of cancer.

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*The authors have no conflict of interest to declare.*

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