

# Targeted<sup>01</sup> Inhibition of the EGFR Pathways Enhances Zn-BC-AM PDT-Induced Apoptosis in Well-Differentiated Nasopharyngeal Carcinoma Cells

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## ABSTRACT

Epidermal growth factor receptor (EGFR), a receptor often expressed in nasopharyngeal carcinoma (NPC) cells, is one of the recently identified molecular targets in cancer treatment. In the present study, the effects of combined treatment of Zn-BC-AM PDT with an EGFR inhibitor AG1478 were investigated. Well-differentiated NPC HK-1 cells were subjected to PDT with 1  $\mu$ M of Zn-BC-AM and were irradiated at a light dose of 1 J/cm<sup>2</sup> in the presence or absence of EGFR inhibitor AG1478. Specific protein kinase inhibitors of downstream EGFR targets were also used in the investigation. EGFR, Akt, and ERK were found constitutively activated in HK-1 cells and the activities could be inhibited by the EGFR inhibitor AG1478. A sub-lethal concentration of AG1478 was found to further enhance the irreversible cell damage induced by Zn-BC-AM PDT in HK-1 cells. Pre-incubation of the cells with specific inhibitors of EGFR (AG1478), PI3k/Akt (LY294002), or MEK/ERK (PD98059) before light irradiation were found to enhance Zn-BC-AM PDT-induced formation of apoptotic cells. The efficacy of Zn-BC-AM PDT can be increased through the inhibition of EGFR/PI3K/Akt and EGFR/MEK/ERK signaling pathways in NPC cells. Combination therapy with Zn-BC-AM PDT and EGFR inhibitors may further be developed for the treatment of advanced NPC. *J. Cell. Biochem.* 9999: 1–8, 2009. © 2009 Wiley-Liss, Inc.

**KEY WORDS:** EGFR; NPC; PHOTODYNAMIC THERAPY

Epidermal growth factor receptor (EGFR) is a member of ErbB family of tyrosine kinase receptors (RTKs). The transmembrane protein receptor is activated by specific ligands, including epidermal growth factor (EGF) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ) [Yarden, 2001]. EGFR is frequently overexpressed and found in many types of human cancers such as lung, breast, prostate, colorectal, and head and neck cancer [Sunnerhagen et al., 1993; Salomon et al., 1995; Mendelsohn, 2002]. Hyper-activation of EGFR pathway is often seen in cancer cells overexpressing EGFR [Hirsch et al., 2003] or overproducing the ligands [Prenzel et al., 1999]. This result in the promotion of malignant transformation and tumor growth by inhibiting the apoptotic mechanism, regulating the cell cycle, angiogenesis and metastasis [Baselga, 2001, 2002; El Rayes et al., 2004].

Nasopharyngeal carcinoma (NPC) has a high prevalence in Hong Kong, southern China, and Southeast Asia. Recent studies indicate

that EGFR may be a target for NPC treatment. Inhibition of EGFR signaling pathway by specific pharmacological inhibitors or down-regulation of EGFR using monoclonal antibodies was found to effectively induce cell cycle arrest and suppress cell proliferation in different NPC cell lines [Zhu et al., 2001; Sung et al., 2005, 2006; Huang et al., 2007]. In addition, the use of monoclonal antibody to inhibit EGFR appears to enhance the sensitivity to chemotherapy in late stage NPC patients [Chan et al., 2005]. However, the apoptosis-enhancing effect through the EGFR inhibition in NPC cells was not previously demonstrated.

Due to the complexity of the disease, new treatment methods for locoregionally advanced or metastatic NPC are currently under development. Photodynamic therapy (PDT), a recently developed method for effective cancer treatment, may be used as an alternative to treat the recurrent and advanced NPC with fewer side effects. It is now clear that PDT can be applied as a single therapy or in

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combination with chemotherapeutic agents for cancer treatments. We have previously studied the mechanisms of PDT-induced cell death of NPC cells [Yow et al., 2000a,b; Mak et al., 2003, 2004; Koon et al., 2006; Li et al., 2006]. We found that PDT induces apoptosis of NPC cells. Here, we show that Zn-BC-AM PDT-induced apoptosis of the HK-1 NPC cells would be enhanced by inhibition of EGFR/MEK/ERK and EGFR/PI3K/AKT signaling pathways.

## MATERIALS AND METHODS

### MATERIALS

Photosensitizer Zn-BC-AM was used in this study [Mak et al., 2004]. The inhibitors of EGFR (AG1478) and MEK/ERK (PD98059) were purchased from Calbiochem. PI3k/Akt inhibitor (LY294002) was purchased from Sigma. Antibodies specific for EGFR, phospho-EGFR Tyr1068 (p-EGFR), Akt, phospho-Akt Ser473 (p-Akt), p44/22 MAPK (ERK1 and ERK2), phospho-p44/42 MAPK Thr202/Tyr204 (p-ERK1 and p-ERK2), caspase-3, Bcl-xL, Bax, and  $\beta$ -actin were purchased from Cell Signaling Technology. Bcl-2 antibody was purchased from Dako.

### CELL CULTURE

HK-1 cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% FBS (GIBCO) and antibiotics penicillin (50 units/ml) and streptomycin (50  $\mu$ g/ml) (GIBCO). The cells were maintained and incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C.

### Zn-BC-AM PDT TREATMENT

A stock solution of Zn-BC-AM (5 mM) was prepared in dimethyl sulfoxide (DMSO), and the stock solution was stored at -20°C in dark. HK-1 cells ( $3 \times 10^5$  cells/dish) in 35 mm Petri dish were incubated overnight. The cells were then incubated with Zn-BC-AM (1  $\mu$ M) at 37°C in the dark for 24 h. Medium containing Zn-BC-AM was replaced with fresh medium before light irradiation. NPC cells were irradiated by light at an intensity of 0.7–0.8 mW/cm<sup>2</sup> from a projector equipped with a 400-W tungsten lamp and a heat isolation filter and narrow band filter ( $682 \pm 5$  nm) [Mak et al., 2004]. After light irradiation, the cells were incubated in a 5% CO<sub>2</sub> incubator at 37°C until further investigation.

### PROPIDIUM IODIDE EXCLUSION ASSAY

Cell viability was determined as previously described [Do et al., 2003]. Both adherent and floating cells were collected at 24 h post-PDT. The cells were then incubated with propidium iodide (PI, 5  $\mu$ g/ml in PBS) in dark for 5 min. The cells were then immediately analyzed by FACSCalibur (Becton Dickinson) with the excitation wavelength at 488 nm. Fluorescent signals were collected by the FL-2 channel. Data were further analyzed by the CellQuest software.

### CLONOGENICITY ASSAY

Clonogenicity was performed as previously described [Tsai et al., 2004]. Briefly, PDT-treated HK-1 cells were trypsinized and collected by centrifugation (700g, 5 min). Various concentrations of cells were seeded onto 35 mm Petri dishes and colony formation was

determined 7 days after incubation. The cell monolayers were then washed twice with PBS and fixed with absolute methanol for 10 min. The cells were stained with 0.5% of crystal violet for 10 min and rinsed four times with tap water. After air drying, the images and the number of colonies in each dish were captured and analyzed with software Quantity-One (Bio-Rad).

### NUCLEAR STAINING WITH HOECHST 33342

Cells were grown on coverslip and subjected to PDT as described above. Cells were treated with 20  $\mu$ g/ml of Hoechst 33342 for 30 min before harvesting. Both adherent and floating cells were collected for analysis. Fluorescence image of the stained nucleus was observed under the fluorescence microscope.

### WESTERN BLOTTING ANALYSIS

Total cell lysates were prepared by lysing the cells with the lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40 (USB), 1% phosphatase inhibitors cocktail (Calbiochem), and 0.25% protease inhibitors cocktail (Sigma). Cell debris was removed by centrifuging the cell lysates at 10,000g at 4°C for 10 min. Protein concentrations were determined using the detergent compatible protein assay kit (Bio-Rad). Cellular proteins were then separated by SDS-PAGE, and the separated proteins were electro-blotted to polyvinylidene difluoride (PVDF) membrane (Perkin Elmer) in a transfer buffer containing 50 mM Tris, 150 mM glycine, 0.05% SDS, and 20% methanol. The membrane was blocked in 5% non-fat dry milk in TBST containing 25 mM Tris, 140 mM NaCl, 3 mM KCl, and 0.1% Tween-20. The membrane was then incubated with primary antibody for 2 h at room temperature. After washing with TBST, the membrane was incubated with an appropriately diluted secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Chemiluminescence was detected using the Western blotting substrate (Lab Frontier) and visualized on an X-ray film. ImageJ software was used to measure the densitometry of bands generated from Western blot analysis.

### STATISTICAL ANALYSIS

Student's *t*-test was used to analyze the level of significance in this study.

## RESULTS

### EFFECTS OF EGFR INHIBITOR AG1478 ON HK-1 CELLS

Expression of EGFR is common in both NPC tissues and NPC cell lines. Recent studies show that HK-1 cell line expresses a higher level of EGFR than the other NPC cell lines [Sung et al., 2005]. However, the effect of inhibition of EGFR signaling pathway in PDT-induced HK-1 cell death is unknown. In this study, we first evaluated the cytotoxicity of the EGFR inhibitor AG1478 on HK-1 cells. HK-1 cells were incubated with various concentrations of AG1478 (0–10  $\mu$ M) for 24 h and the cytotoxicity of AG1478 was evaluated by PI exclusion assay. Results in Figure 1A showed that the percentage of PI stained cells was increased in a dose-dependent manner. At the

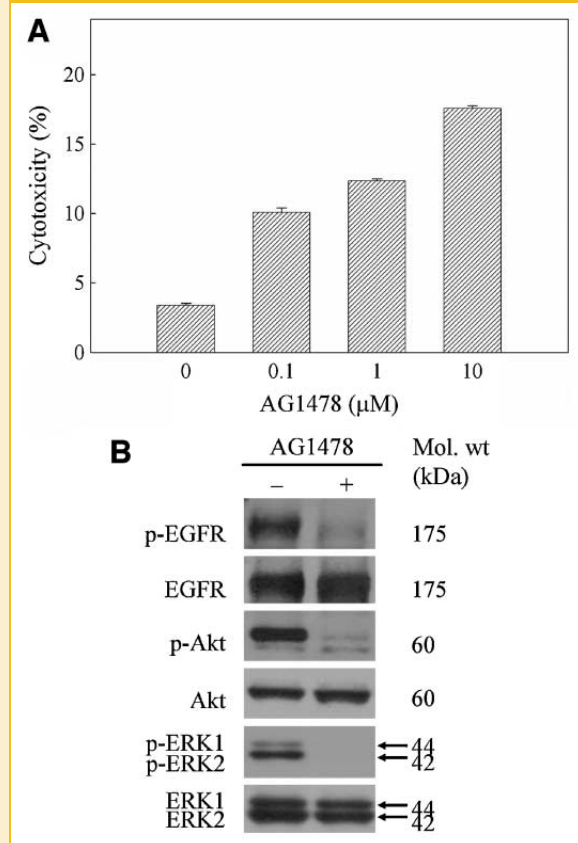


Fig. 1. Inhibition of EGFR induces cell death and inactivates Akt and ERK in HK-1 cells. A: HK-1 cells were treated with AG1478 (0–10  $\mu$ M) as described in the Materials and Methods Section. Cell viability was examined 24 h after treatment. Each data point represents the mean  $\pm$  SD ( $n = 3$ ). B: Western blotting analysis of p-EGFR, p-Akt, and p-ERK1/2 in HK-1 cells following 2 h treatment with AG1478 (10  $\mu$ M).

concentration of 10  $\mu$ M, the percentage of cytotoxicity of AG1478 on HK-1 cells was 17.5%. At this concentration, AG1478 was found to exert a low level of cytotoxicity on HK-1 cells. It is well documented that EGFR activates survival signaling pathways namely PI3K/Akt [Chan et al., 1999; Vivanco and Sawyers, 2002] and ERKs [Lewis et al., 1998]. In order to elucidate the downstream pathways of EGFR in HK-1 cells, the cells were incubated with 10  $\mu$ M of AG1478 for 2 h. Total cell lysates were then collected and the state of phosphorylation of EGFR, Akt, and ERKs were determined by Western blotting (Fig. 1B). p-EGFR, p-Akt, and p-ERK1/2 were all found to be expressed in the untreated HK-1 cells. After incubation with a sub-lethal concentration of AG1478 (10  $\mu$ M), the expression level of p-EGFR, p-Akt, and p-ERK1/2 was greatly decreased. This result suggested that Akt and ERKs are constitutively activated by EGFR in HK-1 cells and the activation can be inhibited by a sub-lethal concentration of AG1478.

#### EFFECTS OF AG1478 ON Zn-BC-AM PDT-INDUCED CELL DEATH

As EGFR constitutively activates the survival signaling pathways in HK-1 cells, we further examined whether inhibition of the EGFR signaling pathways would enhance the cytotoxicity of Zn-BC-AM

PDT. In this study, Zn-BC-AM-treated HK-1 cells were pre-incubated with AG1478 (10  $\mu$ M) for 1 h before light irradiation (0.25–1 J/cm<sup>2</sup>). Samples were collected at 24 h post-Zn-BC-AM PDT and the cell survival was determined by flow cytometry. One hour of AG1478 pre-treatment exerted only a low level of cytotoxicity on the HK-1 cells (Fig. 2A). At the light dose of 1 J/cm<sup>2</sup>, pre-treatment of HK-1 cells with AG1478 was found to significantly increase the cytotoxicity of Zn-BC-AM PDT from 37.7% to 66.0% ( $P < 0.05$ ).

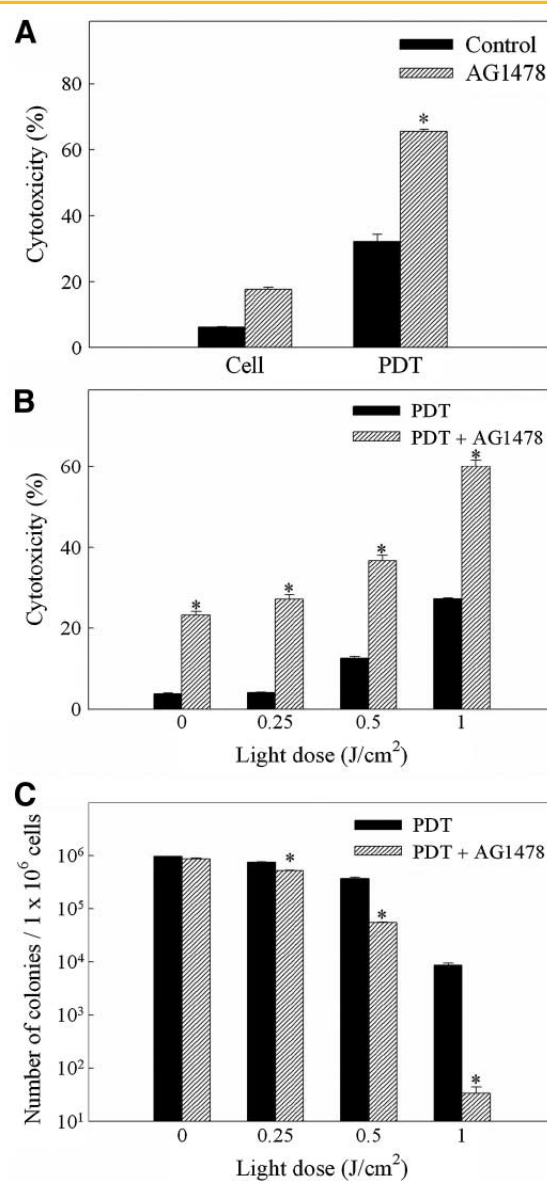


Fig. 2. Inhibition of EGFR enhanced the sensitivity of HK-1 cells to Zn-BC-AM PDT. HK-1 cells were incubated with Zn-BC-AM (1  $\mu$ M) for 24 h. Before light irradiation, the cells were pre-treated with AG1478 (10  $\mu$ M) for 1 h. A: Effect of AG1478 alone, PDT (1 J/cm<sup>2</sup>) and combined treatment on cell viability at 24 h post-treatment. B: Determination of cell viability at 24 h post-PDT with different light doses (0.25–1 J/cm<sup>2</sup>). C: Evaluation of colony forming ability at 7 days after PDT. Each data point represents the mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  versus corresponding PDT treatment.

AG1478 also increased the percentage of cell death within the light dose of 0.25–1 J/cm<sup>2</sup> (Fig. 2B). Clonogenicity assay was used to further confirm the enhancing effect of AG1478 on Zn-BC-AM PDT-induced cell death. Within the light dose of 0.25–1 J/cm<sup>2</sup>, a light dose-dependent reduction of colony formation was observed (Fig. 2C). AG1478 pre-treatment further reduces the number of colony formation in PDT-treated HK-1 cells ( $P < 0.05$ ). Taken together, inhibition of EGFR signaling pathways can enhance the cytotoxicity of Zn-BC-AM PDT in HK-1 cells.

#### ENHANCEMENT OF Zn-BC-AM PDT-INDUCED APOPTOSIS BY AG1478

It has been previously demonstrated that Zn-BC-AM PDT induces apoptosis in NPC cells [Mak et al., 2004]. In this study, we further examined whether AG1478 would enhance Zn-BC-AM PDT-induced apoptosis of HK-1 cells. Experiment was conducted to determine the formation of apoptotic cells after PDT (Fig. 3A). Formation of apoptotic cells was not observed in untreated or

AG1478-treated HK-1 cells. After PDT, the percentage of apoptotic cells was increased to 25.3% (Fig. 3B). Pre-treatment of HK-1 cells with AG1478 further increased the percentage of apoptotic cell to 46.4% ( $P < 0.05$ ). Proteolytic cleavage of the executioner caspase-3 is one of the hallmarks of apoptosis [Ali et al., 2001; Takahashi et al., 2003]. We then examined the effects of AG1478 on the proteolytic cleavage of caspase-3. Compared with the PDT group, proteolytic cleavage product (p19) was clearly detected in cells treated with AG1478 and Zn-BC-AM PDT-treated HK-1 cells at 2 h after treatment (Fig. 3C,D). These observations suggested that pharmacological inhibition of the EGFR would enhance the formation of apoptotic cells.

#### EFFECT OF PHARMACOLOGICAL INHIBITION OF EGFR DOWNSTREAM PATHWAYS ON Zn-BC-AM PDT-INDUCED APOPTOSIS IN HK-1 CELLS

Since the endogenously activated Akt and ERKs would be inhibited by AG1478, we examined the effect of EGFR inhibition on the

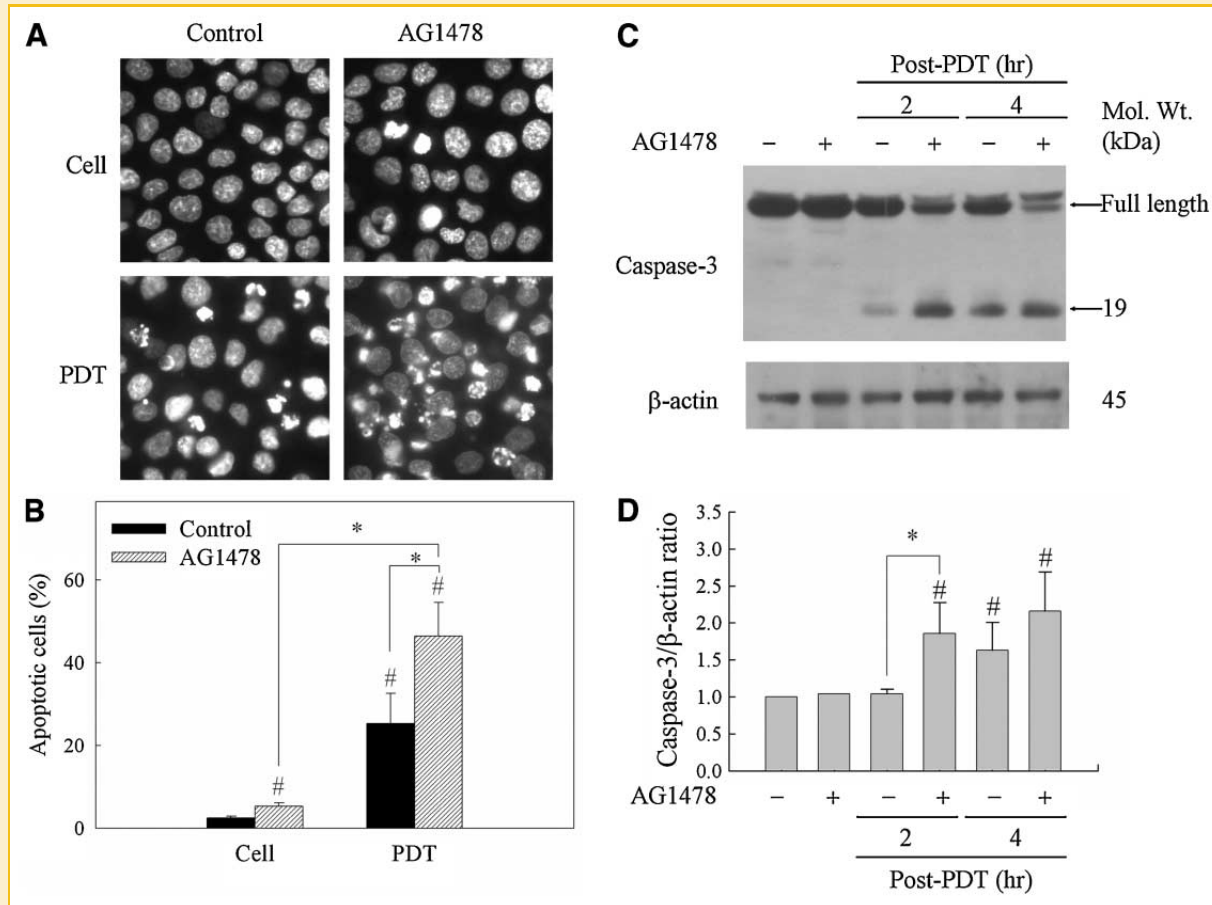


Fig. 3. Zn-BC-AM PDT synergizes with AG1478 to induce apoptosis in HK-1 cells. HK-1 cells were incubated with Zn-BC-AM (1  $\mu$ M) for 24 h. Before light irradiation (1 J/cm<sup>2</sup>), the cells were pre-treated with AG1478 (10  $\mu$ M) for 1 h. A: Staining of apoptotic cells. Both floating and adherent PDT-treated HK-1 cells were stained with Hoechst 33342 at 4 h post-Zn-BC-AM PDT. Samples were visualized under a Zeiss fluorescence microscope (40 $\times$ ). B: Percentage of apoptotic cells. At least 400 cells were counted for each condition. Each data point represents the mean  $\pm$  SD ( $n = 3$ ). C: Western blotting analysis of proteolytic cleavage of caspase-3 in Zn-BC-AM PDT-treated HK-1 cells. Cells were harvested at 2 and 4 h after light irradiation. D: Band intensity profile of caspase-3. Each bar represents the mean  $\pm$  SD ( $n = 2$ ). # $<0.05$  versus untreated cell control, \* $P < 0.05$  versus PDT.



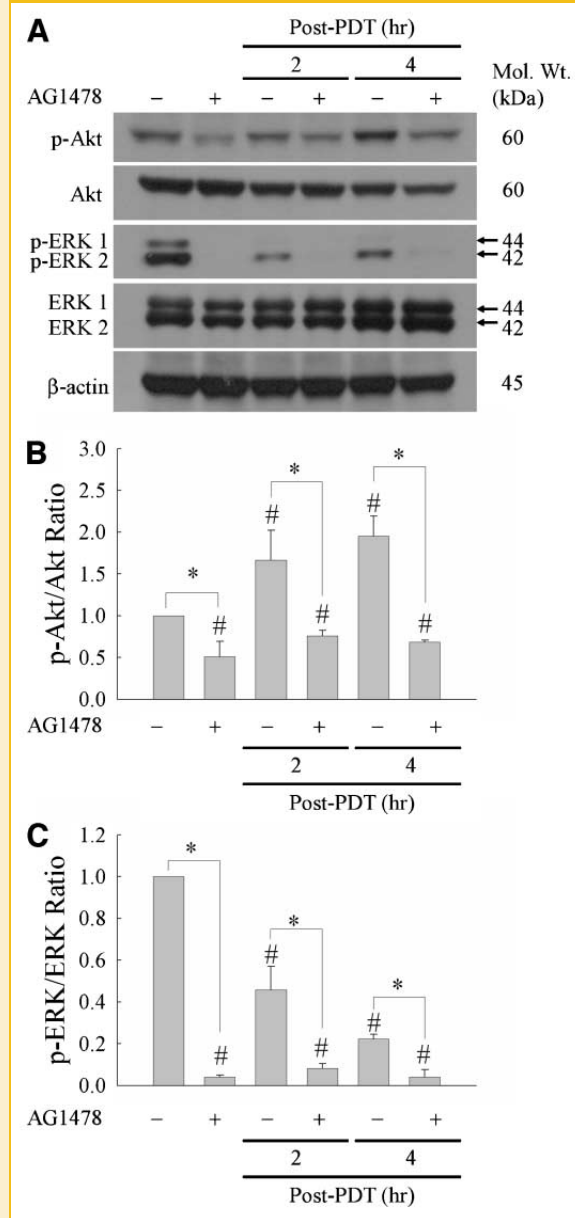


Fig. 4. Effect of AG1478 on the expression of p-Akt and p-ERK1/2. HK-1 cells were incubated with Zn-BC-AM (1  $\mu$ M) for 24 h. Before light irradiation (1 J/cm<sup>2</sup>), the cells were pre-treated with AG1478 (10  $\mu$ M) for 1 h. A: Cells were harvested at 2 and 4 h post-PDT. Expression of p-Akt and p-ERK1/2 was analyzed by Western blotting method. B,C: Band intensity profiles of p-Akt/Akt and p-ERK/ERK. Results were expressed as the mean  $\pm$  SD (n = 3). # < 0.05 versus untreated cell control, \*P < 0.05 versus PDT.

expression of p-Akt and p-ERK1/2 in PDT-treated HK-1 cells (Fig. 4). First of all, AG1478 alone was found to reduce the expression of p-Akt in untreated HK-1 cell. An increase in p-Akt was observed at 2–4 h post-PDT. Combined treatment of cells with AG1478 and PDT resulted in the reduced expression of p-Akt. It is worthy to note that the absolute amount of total Akt in AG1478 + PDT-treated HK-1 cells was lower than the untreated HK-1 cells. For the ERK1/2 pathway, AG1478 alone was found to totally suppress the

expression of p-ERK1/2 in the untreated HK-1 cells. The expression level of p-ERK1/2 was decreased at 2–4 h post-PDT. The expression level of p-ERK1/2 was remaining low after combination treatment of cells with AG1478 and PDT. To further substantiate the role of Akt and ERKs in PDT-induced HK-1 apoptosis, pharmacological inhibitors for Akt and MEK/ERKs were used. The formation of apoptotic cells was also determined at 4 h post-PDT (Fig. 5A). No appreciable formation of apoptotic cells was found in the untreated

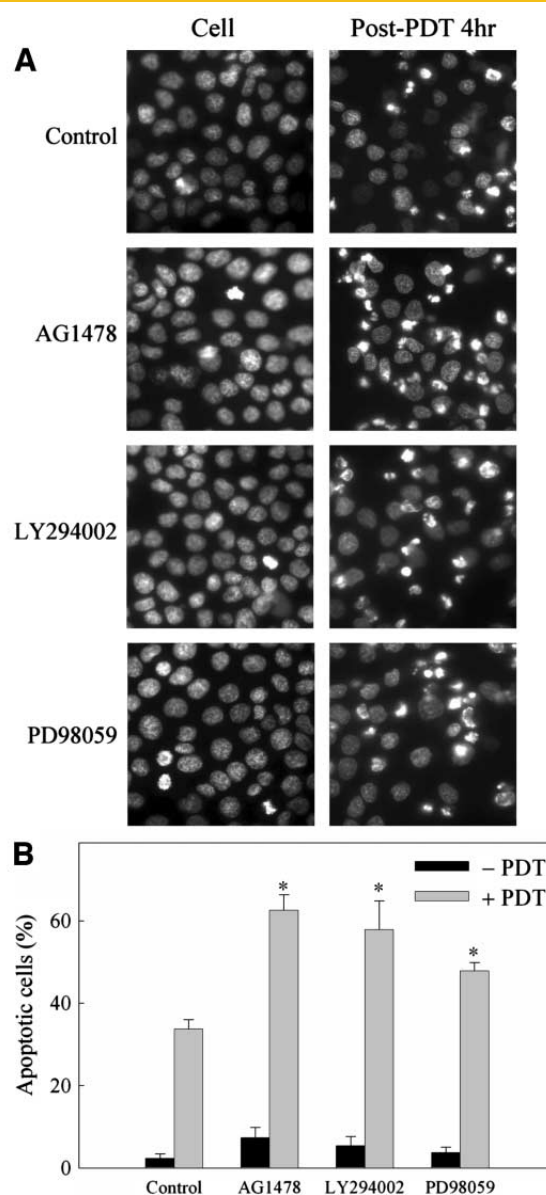


Fig. 5. Effect of AG1478, LY294002, and PD98059 on Zn-BC-AM PDT-induced apoptosis of HK-1 cells. HK-1 cells were incubated with Zn-BC-AM (1  $\mu$ M) for 24 h. Before light irradiation (1 J/cm<sup>2</sup>), the cells were pre-treated with AG1478 (10  $\mu$ M), LY294002 (10  $\mu$ M), or PD98059 (40  $\mu$ M) for 1 h. A: Cell viability was determined at 24 h post-PDT. B: Nuclear staining of apoptotic cells at 4 h post-PDT. C: Percentage of apoptotic cells. At least 400 cells were counted for each condition. Each data point represents the mean  $\pm$  SD (n = 3). \*P < 0.05 versus PDT alone.

HK-1, LY294002- or PD98059-treated HK-1 cells. Results in Figure 5B showed that pre-treatment of HK-1 cells with inhibitor significantly increased the percentage of apoptotic cells from 33.7% in the PDT group to 62.5% (PDT + LY294002) and 57.8% (PDT + PD98059) ( $P < 0.05$ ). Results from these studies indicated that PDT-induced apoptosis could be enhanced through the pharmacological inhibition of EGFR/PI3K/Akt or EGFR/MEK/ERK pathways.

#### ROLE OF Bcl-2 FAMILY PROTEINS IN COMBINED AG1478 AND PDT-INDUCED APOPTOSIS

The impact of combined EGFR pathway inhibition and PDT on the expression of Bcl-2 family proteins was then examined. HK-1 cells were incubated with Zn-BC-AM for 24 h and then subjected to 1 h pre-treatment of AG1478, LY294002 or PD98059 before light irradiation. The expression of anti-apoptotic (Bcl-2 and Bcl-xL) and pro-apoptotic (Bax) proteins were examined at 4 h post-PDT. Results showed that AG1478, LY294002, and PD98059 alone slightly increased the ratio of Bax/Bcl-xL in HK-1 cells (Fig. 6). A sevenfold increase in Bax/Bcl-xL ratio was observed in cells after PDT. The ratio was further increased after the combined treatment with inhibitors and PDT. Similarly, the Bax/Bcl-2 ratio was also increased twofold in HK-1 cells receiving the combined treatment. These observations clearly indicated that the enhanced apoptotic effect of combined Zn-BC-AM PDT and inhibition of EGFR pathways might be due to the increase in Bax/Bcl-2 and Bax/Bcl-xL ratios.

## DISCUSSION

Overexpression of EGFR in various cancer cells, including the NPC cells, is often resulted in the promotion of malignant transformation and tumor growth by inhibiting the apoptotic mechanism [Baselga, 2001, 2002; El Rayes<sup>94</sup> and LoRusso, 2004]. Previous study showed that reduction of EGFR protein expression was correlated with the anti-proliferative effects of silicon phthalocyanine (Pc4)-PDT on A431 cells [Ahmad et al., 2001]. In the in vivo study, a regimen of combined cetuximab and benzoporphyrin derivative monoacid A (BPD)-PDT was found to improve the survival of tumor bearing mice [del Carmen et al., 2005]. Despite inhibition of EGFR signaling pathway by pharmacological specific inhibitors was found to effectively induce cell cycle arrest and suppress cell proliferation in different NPC cell lines [Zhu et al., 2001; Sung et al., 2005; Huang et al., 2007], the effect of targeting EGFR in PDT-induced cell death had not been fully elucidated. Results presented in the present study represent the first experimental study to evaluate the efficacy of combination of EGFR inhibitor and PDT on the death of NPC cells. We showed that constitutively expressed phosphorylation of EGFR could be inhibited by a sub-lethal concentration of AG1478. Augmentation of cell death was found when the cells were treated with a combination of AG1478 and Zn-BC-AM PDT. Our results further demonstrated that the inhibition of EGFR could also enhance apoptosis of the HK-1 cells, as judged from the increased in apoptotic cells as well as the early appearance of the cleaved form of executioner caspase-3. The implication of early appearance of the cleaved caspase-3 and the increased in apoptosis is worthy to

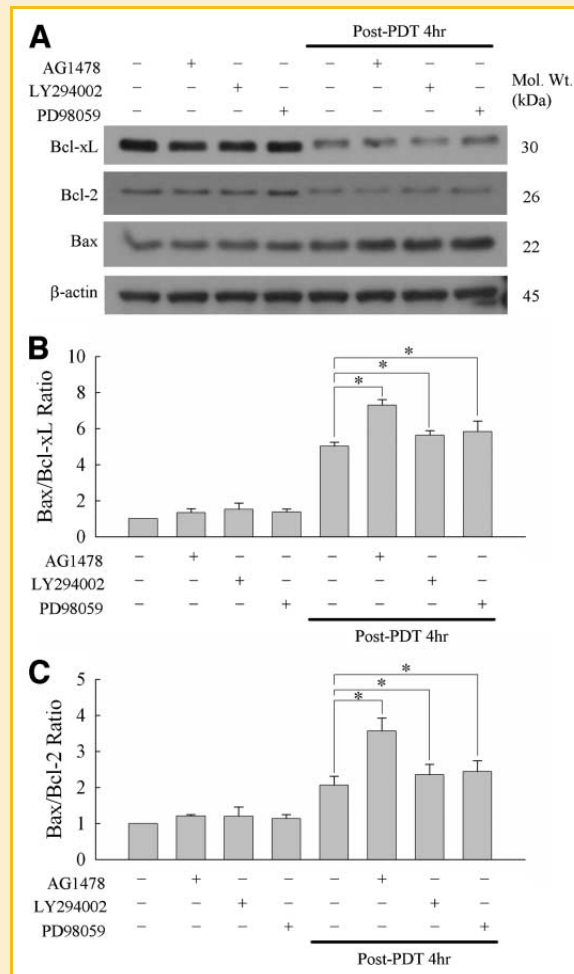


Fig. 6. Effects of inhibition of the EGFR signaling pathways on the expression of Bcl-2 family proteins. HK-1 cells were PDT-treated as described in Figure 5. Cells were harvested at 4 h post-PDT. A: Expression of the anti-apoptotic (Bcl-xL and Bcl-2) and the pro-apoptotic (Bax) proteins. The ratio of Bax/Bcl-xL (B) and Bax/Bcl-2 (C) were determined by densitometric analysis of bands from Western blot. \* $P < 0.05$  versus PDT alone.

discuss. In general, apoptosis is an irreversible process. However, chemotherapeutic drug-treated tumor cells may recover and even survive after the initiation of apoptosis [Tang et al., 2009]. It is well known that oxidative stress (e.g., singlet oxygen) is generated during photoactivation of the photosensitizer. In AG1478 + PDT-treated HK-1 cells, it is probably that all the cell death signals, namely the oxidative stress caused by PDT and inhibition of the survival pathways caused by AG1478, are delivered to the cell and initiate the process of apoptosis (including the activation of caspase-3) shortly after the treatment. As a result of this, the damaged cells may not be able to repair the damage and recover within a short period of time.

PI3K/Akt is one of the major survival pathways regulated by EGFR [Yarden, 2001; Henson<sup>95</sup> et al., 2006]. Akt regulates cell survival through phosphorylation of the downstream targets including apoptotic proteins, transcription factors and protein kinases [Datta et al., 1999; Henson and Gibson, 2006]. Activation of

Akt has been demonstrated in human breast cancer cell treated with photofrin-PDT [Bozkulak et al., 2007]. In the present study, increase in p-Akt was observed in HK-1 cells after PDT. The presence of activated Akt in cells undergoing cell death is worthy to discuss. The increased in p-Akt was previously explained as a survival response counteracting the cell death caused by oxidative stress [Zhuang and Kochevar, 2003]. Maddika et al. [2008] recently found that the anticancer drugs methotrexate and docetaxel would activate Akt and enhance the cell death process. The pro-apoptotic effect of p-Akt was attributed to the constitutively phosphorylation of CDK2 and the subsequent disruption of cell cycle control through the cytoplasmic sequestration of CDK2 to the cytoplasm. In another study, activation of Akt was also demonstrated in cells undergoing apoptosis [van Gorp et al., 2006]. All these studies indicate that Akt plays a dual role in cells and activated p-Akt can be detected in cells undergoing apoptosis. In the present study, an increased in p-Akt was observed at 4 h after PDT. Further functional study showed that PDT-induced apoptosis of HK-1 cells was significantly increased by the Akt inhibitor LY294002. Based on this observation, the increased expression of p-Akt in PDT-treated HK-1 cells may be explained as a survival response counteracting the cell death process.

In addition to the Akt pathway, EGFR signaling is also known to activate ERKs through the cascades of Ras/MEK/ERK [Henson and Gibson, 2006]. ERKs, one of the members of mitogen-activated protein kinase (MAPKs), are highly sensitive to different stimuli. In the present study, pharmacological inhibition of ERKs with PD98059 also enhanced the PDT-induced cell death as well as apoptosis of HK-1 cells. All these results suggested that both Akt and ERKs were involved in AG1478 mediated augmentation of PDT-induced cell death process.

It had been previously demonstrated that ionizing radiation would suppress the expression of Bcl-2 and inhibit EGFR signaling in various cancer cell lines [Bianco et al., 2002]. Inhibition of EGFR also promoted paclitaxel-induced Bcl-2 down-regulation via the EGFR/ERK-dependent but Akt-independent pathways in renal cell carcinoma, resulting in the enhancement of apoptosis [Sumitomo et al., 2004]. In the present study, Zn-BC-AM PDT was found to reduce the expression of Bcl-xL and Bcl-2 in PDT-treated HK-1 cells. In contrast, the expression of pro-apoptotic Bax protein was further increased after the combined treatment with both PDT and the EGFR inhibitor. The balance between the expression of anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL) and pro-apoptotic proteins (e.g., Bax) is one of the important indicators to determine the cell fate. In the steady state, Bcl-2 and/or Bcl-xL antagonizes Bax from triggering apoptotic signals by oligomerization on the outer membrane of mitochondria [Hsu and Youle, 1997; Mikhailov et al., 2001]. Therefore, the increase in the ratios of Bax/Bcl-xL and Bax/Bcl-2 would facilitate Bax to undergo active conformational change and subsequently induce mitochondrial membrane permeabilization [Gross et al., 1999]. The increase in Bax/Bcl-xL and Bax/Bcl-2 ratios might explain the apoptosis-enhancing effect of EGFR inhibitor in PDT-treated HK-1 cells. These observations suggest that the efficacy of PDT on NPC cells can further be improved by chemotherapeutic agents targeting the EGFR and the associated downstream pathways.

In summary, the present study evaluated the efficacy and the underlying mechanisms of combined EGFR inhibitor and Zn-BC-

AM PDT on the HK-1 NPC cells. The efficacy of Zn-BC-AM PDT was increased through the inhibition of EGFR/PI3K/Akt and EGFR/MEK/ERK signaling pathways in HK-1 cells. Combination therapy of Zn-BC-AM PDT with EGFR inhibitors may further be developed for the treatment of NPC.

## ACKNOWLEDGMENTS

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