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Author(s)	Koon, HK; Chan, PS; Wu, ZG; Wong, RNS; Lung, ML; Chang, CK; Mak, NK
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Role of mitogen-activated protein kinas in Zn-BC-AM PDT-induced apoptosis in nasopharyngeal carcinoma cells

H. K. Koon [1], P. S. Chan [1], Z. G. Wu [2], R. N. S. Wong [1], M. L. Lung [3†], C. K. Chang [4] and N. K. Mak [1*]

[1] *Department of Biology, Hong Kong Baptist University, Hong Kong*
[2] *Department of Biochemistry, The Hong Kong University of Science and Technology, Hong Kong*
[3] *Department of Biology and Center for Cancer Research, The Hong Kong University of Science and Technology, Hong Kong*
[4] *Department of Chemistry, The Hong Kong University of Science and Technology, Hong Kong*

Photodynamic therapy (PDT) with a recently developed photosensitizer Zn-BC-AM was found to effectively induce apoptosis in a well-differentiated nasopharyngeal carcinoma (NPC) HK-1 cell line. Sustained activation of p38 mitogen-activated protein kinase (MAPK) and c-jun N-terminal kinase (JNK) as well as a transient increase in activation of extracellular-regulated kinase (ERK) were observed immediately after Zn-BC-AM PDT. A commonly used p38 MAPK/JNK pharmacological inhibitor PD169316 was found to reduce PDT-induced apoptosis of HK-1 cells. PD169316 also prevented the loss of Bcl-2 and Bcl-xL in PDT-treated HK-1 cells. However, inhibition of JNK with SP600125 had no effect on Zn-BC-AM PDT-induced apoptosis while inhibition of ERK with PD98059 or p38 MAPK with SB203580 significantly increased Zn-BC-AM PDT-induced apoptosis. Further study showed that knockdown of the p38β isoform with siRNA also increased Zn-BC-AM PDT-induced apoptosis, indicating that the anti-apoptotic effect of PD169316 in PDT-treated HK-1 cells was probably independent of p38 MAPK or JNK activation. Taken together, the results suggest that inhibition of p38β and ERK may enhance the therapeutic efficacy of Zn-BC-AM PDT on NPC cells. It should be noted that data only based on the use of PD169316 should be interpreted in caution. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS — apoptosis; MAPK; p38; isoforms; PD169316; photodynamic therapy

ABBREVIATIONS — DIC, differential interference contrast; NPC, nasopharyngeal carcinoma; Zn-BC-AM, Zinc (II) 2,3,8,8,12,13,17,18-octaethylbenzochlorin amidinium;

INTRODUCTION

Mitogen-activated protein kinases (MAPKs), a group of protein kinases activated by dual phosphorylation, are involved in controlling the growth, differentiation, movement and death of cell in response to various stimuli and stresses.[1] There are three major groups of MAPKs produced by the mammalian cells, namely p38 MAPK, c-jun N-terminal kinases (JNK) and extracellular signal regulated kinases (ERKs). ERK is generally involved in the regulation of proliferation while p38 MAPK and JNK are frequently activated and participated in stress responses and programmed cell death.[2–4] As deregulation of MAPKs signalling pathways is known to play a role in neoplastic transformation and tumour progression, chemotherapeutic agents targeting on these pathways are currently under development for the treatment of cancer or to improve response to conventional therapy.

Photodynamic therapy (PDT) is a promising therapeutic approach for cancer management. In the past decade, various types of photosensitizers were developed and evaluated for their efficacy in tumour eradication. It is now clear that the target of PDT is not restricted to a single cellular component or a single signalling pathway. Multiple intracellular components and signalling pathways have been implicated in PDT-induced cell death.[5] The role of MAPKs in PDT-induced apoptosis has not been fully studied.[6,7] Photoactivation of Pc4 was found to induce phosphorylation of p38 MAPK in CHO cells.[8] Similar findings were also observed in human adenocarcinoma HeLa cells with hypericin-PDT,[9] human keratinocytes HaCat cells and hypopharyngeal carcinoma FaDu cells treated with ALA-PDT,[10,11] murine keratinocytes Pas 212 cells treated with benzoporphyrin derivative-PDT[12] and also human leukaemia HL60 and murine mammary carcinoma 4T1 cells treated with PORF-TEG-PDT.[13] A previous study showed that Rose Bengal PDT-activated JNK was required for caspase-3 activation in the A431 carcinoma cells.[14] In contrast, TPPS2a PDT-activated JNK was found to play a

* Correspondence to: N. K. Mak, Department of Biology, Hong Kong Baptist University, 224, Waterloo Road, Hong Kong. Tel: (862) 34117059. E-mail: nkmak@hkbu.edu.hk
† Present address: Department of Clinical Oncology, University of Hong Kong, Hong Kong.

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role in providing survival signals to PDT-treated epithelial ovarian cancer cells.[15] In LFS087 and GM38A cell lines, ERK was transiently activated by Photofrin PDT,[16] whereas ERK was not affected in another PDT model.[12] Therefore, the action of PDT on the activities of MAPKs was probably cell line and photosensitizer-dependent.

We have previously demonstrated that Zn-BC-AM PDT induces apoptosis in NPC cells.[17] In the present study, we aim to study the role of MAPKs in Zn-BC-AM PDT-induced apoptosis by using specific pharmacological inhibitors and siRNA for various p38 MAPK isoforms. We found that knockdown the expression of p38β, but not p38α and p38δ isoform, would enhance Zn-BC-AM PDT-induced apoptosis. We also demonstrated the discrepancy in apoptosis enhancing effect of two commonly used inhibitors PD169316 and SB203580 in Zn-BC-AM PDT-induced apoptosis of NPC cells.

MATERIALS AND METHODS

Materials

Inhibitors of p38 MAPK (SB203580), p38 MAPK/JNK (PD169316), JNK (SP600125) and MEK/ERK (PD98059) were purchased from Calbiochem. Antibodies against p38α MAPK, p38β MAPK, p38δ MAPK, phospho-p38 MAPK (p-p38) (Thr180/Tyr182), p38 MAPK (p38), phospho-SAPK/JNK (p-JNK) (Thr183/Tyr185), SAPK/JNK (JNK), phospho-p44/42 MAPK (p-ERK) (Thr202/Tyr204), p44/42 MAPK (ERK), Bcl-xL, Bad, Bax, Bid, caspase-3, -8, -9 and β-actin were purchased from Cell Signalling Technology. Bcl-2 antibody was purchased from Dako. Stock solution of the photosensitizer Zn-BC-AM[17] was prepared in DMSO.

Cell culture

HK-1 cells[18] were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% FBS (GIBCO) and antibiotics penicillin (50 μg/ml)/streptomycin (50 μg/ml) (GIBCO). The cells were maintained and incubated in a humidified 5% CO_2 incubator at 37°C.

Zn-BC-AM PDT treatment

Except otherwise stated, HK-1 cells (3×10^5 cells/dish) were incubated overnight in 35 mm petri dish. Zn-BC-AM (1 μM) was then added and the cells were incubated at 37°C in dark for 24 h. Medium containing Zn-BC-AM was replaced with fresh medium before light irradiation. The NPC cells were irradiated at an intensity of 0.8 mW/cm^2 from a projector equipped with a 400-Watt tungsten lamp, a heat isolation filter and narrow band filter (682 ± 5 nm). After light irradiation, the cells were incubated in a 5% CO_2 incubator at 37°C until further investigation.

Propidium[Q5] *iodide exclusion assay*

Cell viability was determined by propidium iodide exclusion assay. Both adherent and floating cells were collected at 24 h post-PDT. The cells were then incubated with propidium iodide (PI, 5 μg/ml in PBS) in dark for 5 min. The cells were then immediately analysed by FACSCalibur (Becton Dickson) with the excitation wavelength at 488 nm. Fluorescent signals were collected by the FL-2 channel. Data were further analysed by the CellQuest software.

Clonogenicity assay

Viability of PDT-treated NPC cells was evaluated by the clonogenicity assay.[19] Briefly, the cells were trypsinized and collected by centrifugation (700*g*, 5 min) after PDT. 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. The stained cells were allowed to air-dry. The images and the number of colonies in each dish were captured and analysed with software Quantity-One (Bio-Rad).

Nucleus staining with Hoechst 33342

HK-1 cells were grown on coverslip placed in 35 mm petri dish and subjected to PDT as described above. At the desired time point, the cells were stained with 20 μg/ml of Hoechst 33342 (Fluka, Allentown) for 30 min. Floating cells were collected and washed twice with PBS and collected by centrifugation (700*g*, 5 min). Both adherent and floating cells were pooled and observed under the fluorescence microscope. The percentage of apoptotic cells was determined by counting at least 300 cells for each treatment group.

Western blot analysis

After PDT, total cell lysate was prepared by lysing the cells with 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 10 000 *g* at 4°C for 10 min. The protein concentrations were determined using the detergent compatible protein assay kit (Bio-Rad). Cellular proteins were then electrophoretically fractionated by SDS-PAGE. The separated proteins were electro-blotted to polyvinylidene difluoride membrane (PVDF, 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 (25 mM Tris, 140 mM NaCl, 3 mM KCl and 0.1% Tween 20). The membrane was then incubated sequentially with an appropriately diluted primary antibody for 2 h and secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Chemiluminescence was detected using the WESTSAVE Up^{TM} (Abfrontier) and visualized on an X-ray film. Quantity

One software (Bio-rad) was used to measure the densitometry of bands generated from Western blot analysis.

Transfection with plasmid DNA

HK-1 cells (3×10^5 cells) were seeded in 35 mm petri dish the day before transfection and maintained in complete medium. 3.75 μl of Lipofectamine ™ 2000 (Invitrogen) was added in 200 μl serum free medium and then incubated at room temperature for 5 min. 1.6 μg of Bcl-2 pcDNA3 plasmid (Addgene plasmid 8768) was added to another 200 μl of serum-free medium.[20] Transfection complex (400 μl) was then prepared by mixing 200 μl plasmid DNA to 200 μl Lipofectamine 2000™ and the mixture was allowed to incubate for further 30 min. During the period of incubation, the cells were replaced with 1.6 ml of fresh serum-free medium. The transfection complex was transfected to cells and incubated for 6 h. The transfection complex was then removed, replaced with fresh complete medium and incubated for overnight. Same amount of pcDNA3.1 empty vector was used as an internal control and transfected the cells as described above.

siRNA transfection

HK-1 cells (1.5×10^5) were seeded in 35 mm petri dish the day before transfection and maintained in complete medium. To prepare the transfection complex, 3.75 μl of Lipofectamine ™ 2000 (Invitrogen) was added to 200 μl serum free medium and the mixture was incubated at room temperature for 5 min. 3.3 μl of 20 μM siRNA was added to another 200 μl of serum-free medium. Transfection complex (400 μl) was prepared by adding the siRNA to the Lipofectamine 2000™ and the mixture was allowed to incubate for further 30 min. Before transfection, the cells were replaced with 1.6 ml of serum-free medium. The transfection complex was transfected to cells and the mixture was incubated for 6 h. The transfection mixture was then removed, replaced with complete medium and then incubated overnight. Same amount of non-targeting siRNA was used as an internal control and transfected to the cells as described above. The antisense sequences of siRNA were: p38α MAPK (5′- GGA AUU CAA UGA UGU GUA UUU-3′, 5′- UCU CCG AGG UCU AAA GUA UUU-3′, 5′- GUA AUC UAG CUG UGA AUG AUU-3′ and 5′- GUC CAU CAU UCA UGC GAA AUU-3′), p38β MAPK (5′- GCC CUG AGG UUC UGG CAA AUU-3′, 5′- CGA CGA GCA CGU UCA AUU CUU-3′, 5′- CCA UAG ACC UCC UUG GAA GUU-3′ and 5′- GCG CCG ACC UGA ACA ACA UUU-3′), p38δ MAPK (5′- GCU CAA AGG CCU UAA GUA CUU-3′, 5′- GGA GUG GCA UGA AGC UGU AUU-3′, 5′- GGA UUU CAC UCA GCU GUU CUU-3′ and 5′- GCC GUU UGA UGA UUC CUU AUU-3′) and non-targeting pool (5′- UGG UUU ACA UGU CGA CUA A-3′, 5′- UGG UUU ACA UGU UGU GUG A-3′, 5′- UGG UUU ACA UGU UUU CUG A-3′ and 5′- UGG UUU ACA UGU UUU CCU A-3′).

Statistical analysis

All graphs were plotted and analysed by the software Microsoft Excel, SigmaPlot and SPSS. Statistical significance ($p < 0.05$) was determined using Student's *t*-test.

RESULTS

Zn-BC-AM PDT-induced cell death and apoptosis in HK-1 cells

The viability of Zn-BC-AM PDT-treated HK-1 cells was firstly evaluated by the assessment of the integrity of cell membrane and the ability of the treated cells for colony formation. The results showed that the percentage of PI stained cells was increased in a drug and light-dose dependent manner (Figure 1A). At the concentration of 1 μM, Zn-BC-AM PDT also effectively reduced the colony formation in a light dose-dependent manner, indicating that Zn-BC-AM PDT-treated HK-1 cells underwent irreversible cell damage (Figure 1B). To determine whether Zn-BC-AM PDT induced apoptotic cell death in HK-1 cells, the morphological changes and the downstream apoptotic biomarkers were examined. Cell shrinkage and plasma membrane blebbing were observed in cells at 2 and 4 h post-PDT. Chromatin condensation (Figure 1C), one of the features of apoptotic nuclei, was also observed. In addition, proteolytic cleaved fragments of the apoptotic initiator caspase-9 (MW: 37 and 35) and apoptotic executioner caspase-3 (MW: 19 and 17) were detected at 0.5–8 h post-PDT. The appearance of the cleaved fragments of caspase-8 (MW: 43 and 41) was also observed at 2 h post-PDT (Figure 1D).

Involvement of Bcl-2 family proteins in Zn-BC-AM PDT-induced apoptosis

We further characterized the expression levels of anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic Bcl-2 family proteins (Bad, Bid and Bax) in Zn-BC-AM PDT-treated HK-1 cells. Results from the Western Blot analysis (Figure 2A) of whole-cell lysates revealed an immediate reduction (i.e. post-PDT, 0 hr) of Bcl-2, Bcl-xL and Bad after Zn-BC-AM PDT. The level of Bid started to decrease at 4 h post-PDT. However, the activated form of Bid (truncated Bid, tBid) was not detected in the PDT-treated cells. In contrast, the protein level of Bax remained unchanged between 0 and 8 h post-PDT. To further establish the role of Bcl-2 in HK-1 cells, Bcl-2 was overexpressed before PDT. Overexpression of Bcl-2 resulted in a significant reduction of apoptosis in HK-1 cells (Figure 2B). The percentage of apoptotic cells was reduced from 60 to 40% ($p < 0.05$). Overexpression of Bcl-2 also reduced the expression level of cleaved caspase-3 in PDT-treated HK-1 cells (Figure 2C). This observation clearly indicated that overexpression of Bcl-2 would reduce PDT-induced apoptosis of HK-1 cells.

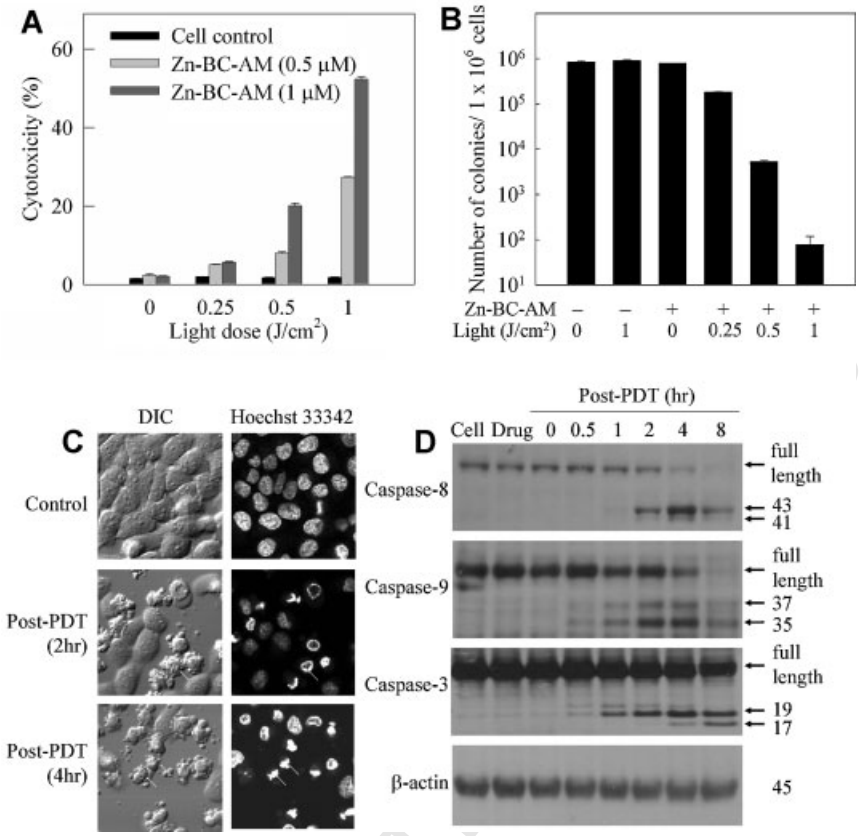

Figure 1. Zn-BC-AM PDT-induced apoptosis in HK-1 cells. HK-1 cells were incubated with Zn-BC-AM (0.5–1 μM) for 24 h, followed by light irradiation at various light doses (0.25–1 J/cm^2) as described in Materials and Methods. (A) Cell viability was determined at 24 h post-PDT by PI exclusion assay. Data were analysed by the CellQuest software and the results were expressed as mean ± SD ($n = 3$) (B) Determination of the colony formation 7 days post-PDT. The cell monolayers were fixed and stained as described in Materials and Methods. The number of colonies in each group was analysed using the software Quantity-One (Bio-Rad) and the results were expressed as the mean ± SD ($n = 3$). (C) Staining of apoptotic nuclei by Hoechst 33342. White arrows indicated the membrane blebbing (in DIC images), chromatin condensation and apoptotic nuclei. (D) Kinetics of expression of cleaved caspase-8, -9 and -3 in PDT-treated HK-1 cells. Cell, untreated HK-1 cells; Drug, Zn-BC-AM alone.

Activation of MAPKs in Zn-BC-AM PDT-treated HK-1 cells

To determine whether Zn-BC-AM PDT activated the phosphorylation of MAPKs, the states of phosphorylation of p38 MAPK, JNK and ERK were analysed (Figure 3). A very low background level of p-p38 was detected in the untreated HK-1 and Zn-BC-AM-treated HK-1 cells. An apparent increase in p-p38 was observed immediately after Zn-BC-AM PDT. The time course of increase in p-JNK was similar to p-p38. In contrast, constitutive expression of p-ERK was observed in the untreated HK-1 cells. A transient increase followed by a decrease of the p-ERK was observed after Zn-BC-AM PDT. The p-ERK signal was restored to the control level at 4 h post-PDT. The restoration of p-ERK expression might be derived from the surviving cells.

PD169316 reduced Zn-BC-AM PDT-induced apoptosis

PD169316 is a general use p38 MAPK/JNK dual inhibitor. The effect of increasing concentrations of PD169316 on the phosphorylation of p38 MAPK and JNK induced by Zn-BC-AM PDT was firstly examined. A dose dependent inhibition of phosphorylation of p38 MAPK and JNK by PD169316 was observed in PDT-treated HK-1 cells (Figure 4A). At the concentrations of 1–2.5 μM, PD169316 was found to have a greater inhibitory effect on the production of p-p38 MAPK than p-JNK. A higher concentration of PD169316 (5–10 μM) was needed to inhibit the phosphorlyation of JNK induced by Zn-BC-AM PDT. Under the same experimental condition, PD169316 (1–10 μM) also dose dependently reduced the percentage of apoptotic cells in PDT-treated HK-1 cells (Figure 4B). The anti-apoptotic effect of PD169316, as judged from the reduction of the cleaved caspase-9, -8 and -3, was also observed (Figure 4C). Previous studies have shown that MAPK phosphorylation may impair the anti-apoptotic function of Bcl-2 family proteins and result in apoptotis. As PD169316 was found to inhibit phosphorylation of p38 MAPK and JNK, we further examined the effect of PD169316 on the expression of anti-apoptotic Bcl-2 proteins (Figure 4D). PDT significantly reduced the expression level of Bcl-2 (Figure 4E) and Bcl-

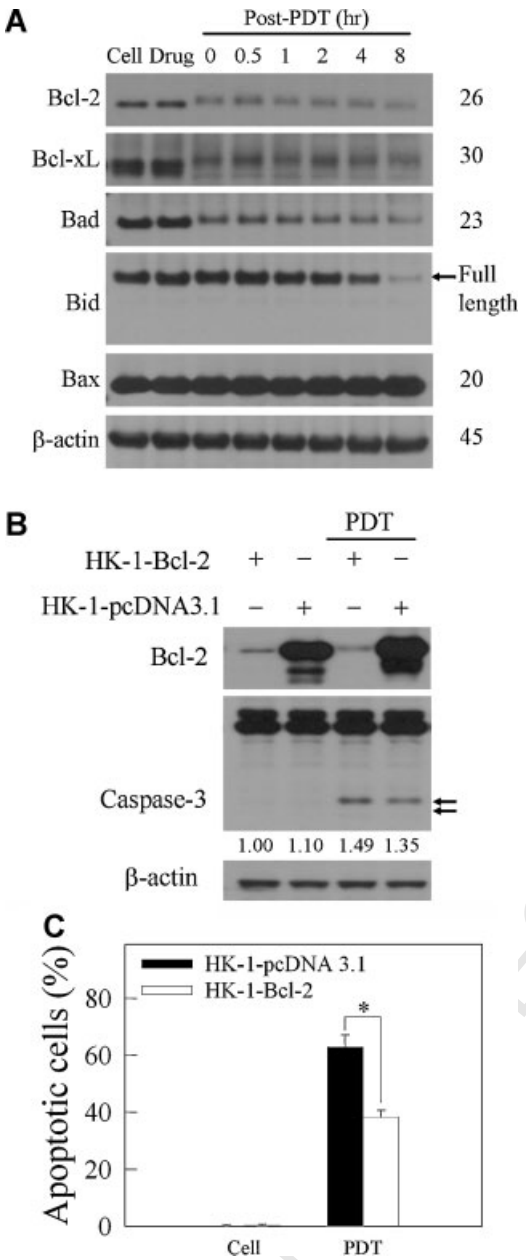

Figure 2. Expression of Bcl-2 family member proteins in Zn-BC-AM PDT-treated HK-1 cells. HK-1 cells were incubated with Zn-BC-AM (1 μM) for 24 h. The cells were then irradiated with a light dose of 1 J/cm^2 as described in Materials and Methods. (A) Kinetics of Bcl-2 family protein expressions after PDT. (B) Effect of Bcl-2 overexpression on the proteolytic cleavage of caspase-3 after PDT. Cell lysates were prepared at 4 h post-PDT. (C) Bcl-2 overexpression reduced PDT-induced apoptosis of HK-1 cells. The percentage of apoptotic cells was counted at 4 h post-PDT. At least 400 cells were counted for each group. Results were expressed as the mean ± SD ($n = 3$). * $p < 0.05$ versus PDT in HK-1-pcDNA 3.1.

xL (Figure 4F) in HK-1 cells. In the presence of PD169316, the expression level of Bcl-2 and Bcl-xL was restored. Taken together, these observations suggested that PD169316 may reduce PDT-induced apoptosis via the early inhibition of phosphorylation of p38 MAPK and JNK, reduction of PDT-induced photodamage of anti-apoptotic Bcl-2 proteins and the subsequent proteolytic cleavage and activation of downstream effector caspases.

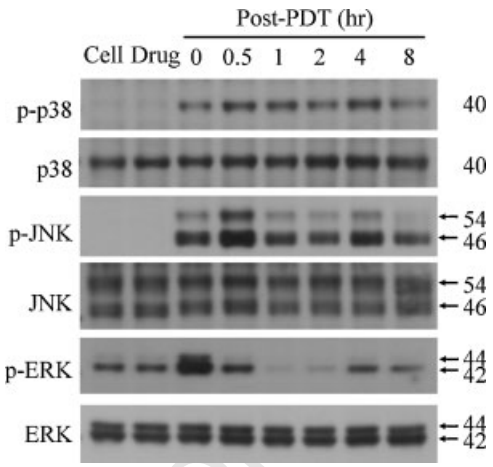

Figure 3. Kinetics of MAPKs activation induced by Zn-BC-AM PDT. HK-1 cells were incubated with Zn-BC-AM (1 μM) for 24 h, followed by irradiation with light dose of 1 J/cm^2 as described in Materials and Methods.

Contribution of p38 MAPK and JNK to Zn-BC-AM PDT-induced apoptosis in HK-1 cells

Since Zn-BC-AM PDT was found to induce the phosphorylation activation of p38 MAPK, JNK and ERK in HK-1 cells, specific kinase pharmacological inhibitors were used to evaluate the effect of p38 MAPK, JNK and ERK inhibition on the formation of apoptotic cells after Zn-BC-AM PDT. We pre-treated the HK-1 cells with different doses of PD169316 (10–40 μM), SB203580 (10–40 μM, a preferential p38 MAPK inhibitor) or SP600125 (5–20 μM, a preferential JNK inhibitor), for 1 h before light exposure (1–2 J/cm^2). The inhibitor was retained in the medium until sample harvesting. Apoptosis was then enumerated at 4 h post-PDT (Figure 5). The percentage of apoptotic cells in the control and various MAPK inhibitors-treated HK-1 cells was less than 4%. PD169316 (10 μM) was found to reduce the percentage of apoptosis at the light dose of 1–2 J/cm^2 (Figure 5A) and the inhibition was in a drug dose-dependent manner (Figure 5B). As PD169316 is a dual p38 MAPK/JNK inhibitor, it is logically to deduce that SB203580 and SP600125 may also reduce PDT-induced apoptosis of HK-1 cells to a certain extent. However, SB203580 (10 μM) was found to enhance the percentage of apoptosis of PDT-treated HK-1 cells (Figure 5C and 5D). Furthermore, apoptosis was not affected by the JNK inhibitor SP600125 (Figure 5E and 5F).

To further confirm the actions of PD169316, SB203580 and SP600125, we checked for the state of phosphorylation of p38 MAPK and JNK in PDT-treated HK-1 cells. As shown in Figure 5G, an increased in the expression level of p-p38 p-JNK was observed at 10 min post-PDT. The expression of both p-p38 and p-JNK could be reduced by PD169316. The phosphorylation of their downstream target

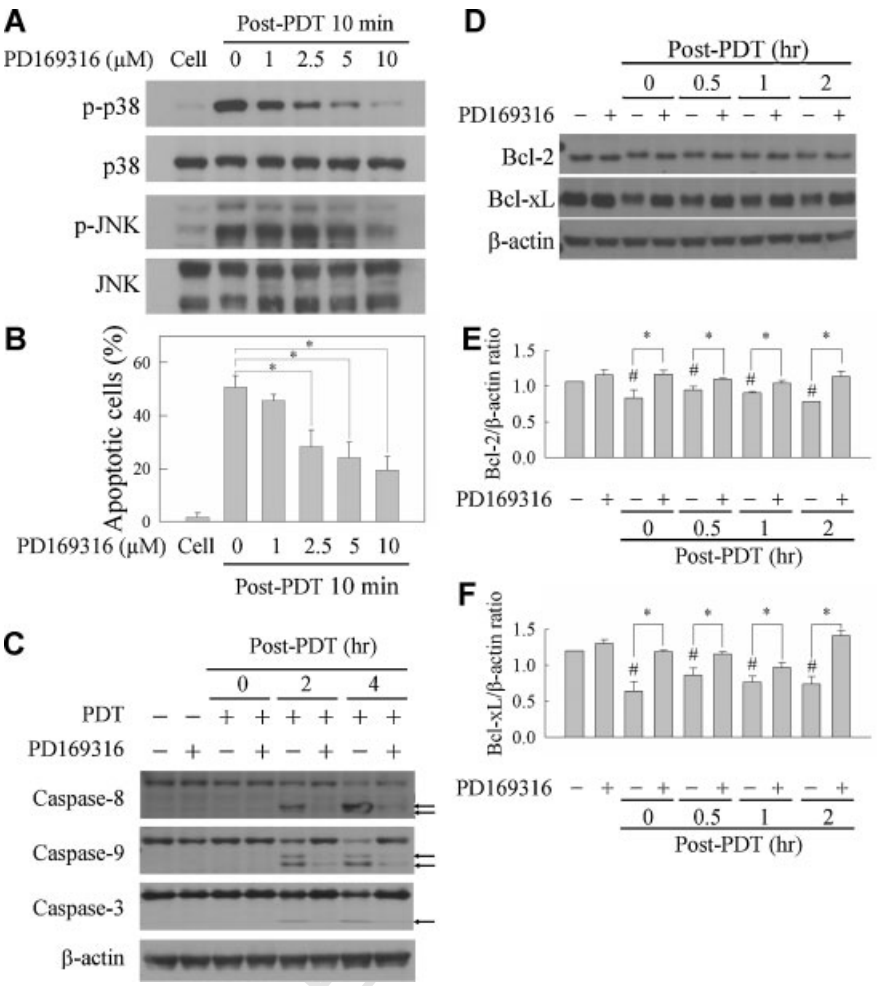

Figure 4. Reduction of Zn-BC-AM PDT-induced apoptosis by PD169316. HK-1 cells (3×10^5) in 35 mm petri dish were treated with Zn-BC-AM (1 μM) as described in Materials and Methods. Before light irradiation (1 J/cm^2), the cells were treated with various concentrations of PD169316. (A) Dose dependent inhibition of production of p-p38 and p-JNK at 10 min post-PDT. (B) Reduction of proteolytic cleavage of caspase-8, -9 and -3 by PD169316. (C) Reduction of PDT-induced formation of apoptotic cells. At least 400 cells were counted for each group. The percentage of apoptotic cells was expressed as the mean ± SD ($n = 3$). * $p < 0.05$ versus PDT alone. (D) Western blotting analysis of Bcl-2 and Bcl-xL expression. Cell lysates were prepared at 0–2 h post-PDT. (E) and (F) Statistical analysis on the ratios between Bcl-2 or Bcl-xL and β-actin in cells with or without PD169316 treatment. # $p < 0.05$ versus untreated cell control; * $p < 0.05$ versus PDT.

ATF-2 was also reduced. Similarly, the expression of p-p38 (Figure 5H) and p-JNK (Figure 5I) was reduced by their respective inhibitor SB203580 and SP600125 in HK-1 cells.

Transient knockdown of p38β isoform enhanced Zn-BC-AM PDT-induced apoptosis

Since PD169316 and SB203580 exerted an opposing effect on PDT-induced apoptosis of HK-1 cells, we applied the siRNA knockdown technology to dissect the role of p38 MAPK on Zn-BC-AM PDT-induced apoptosis. Four p38 MAPK isoforms were previously identified in mammalian cells, namely p38α,[21] p38β,[22] p38δ ,[23] and p38γ.[24] Since the expression of p38γ is restricted in skeletal muscle, heart, thymus and testis,[25,26] the role of p38α, p38β and p38δ in Zn-BC-AM PDT-induced apoptosis of HK-1 cells was examined using siRNA specifically for the expression of these three p38 MAPK isoforms. In the control experiment, siRNA specifically reduced the expression of their corresponding target (Figure 6A). The formation of apoptotic cells was then determined (Figure 6B). Only HK-1 cells transfected with siRNA of p38β enhanced the percentage of apoptotic cells (83.1%, $p < 0.05$) after PDT (Figure 6C). Knockdown of either p38α or p38δ had no significant effect on the formation of apoptotic cells induced by Zn-BC-AM PDT. The result from this study is consistent with the enhancement of apoptosis by SB203580 in PDT-treated HK-1 cells (Figure 5C and 5D).

Effects of PD98059 on Zn-BC-AM PDT-induced ERK phosphorylation and apoptosis

In addition to p38 MAPK and JNK, the expression of phosphorylated ERK (p-ERK) was also increased after Zn-

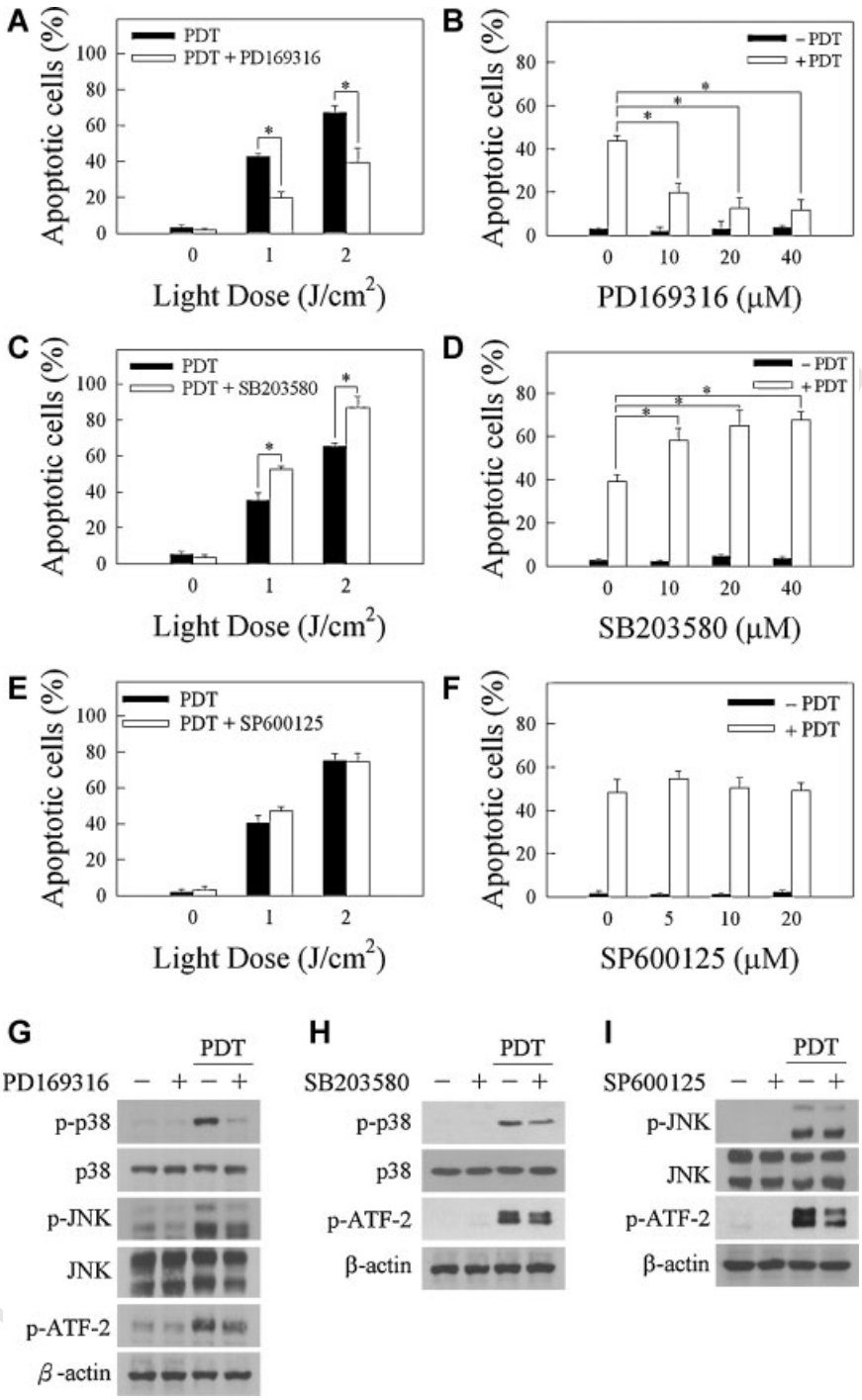

Figure 5. Effect of PD169316, SB203580 and SP600125 on Zn-BC-AM PDT-induced apoptosis. Zn-BC-AM-loaded HK-1 cells were pre-treated with 10 μM PD169316 (A), 10 μM SB203580 (C) or 5 μM SP600125 (E) for 1 h before light irradiation (1 and 2 J/cm^2). Effect of various doses of PD169316 (B), SB203580 (D) and SP600125 (F) on the production of apoptotic cells. Cells were irradiated at 1 J/cm^2. Percentage of apoptotic cells were expressed as mean ± SD ($n = 3$). $^*p < 0.05$ versus PDT control. Effect of various inhibitors on the expression p-p38, p-JNK and their downstream target ATF-2 at 10 min post-PDT (1 J/cm^2) (G, H and I). The concentrations of inhibitors used in this study were the same as A, C and E.

BC-AM PDT (Figure 3). The effect of MEK/ERK inhibitor PD98059 on Zn-BC-AM PDT-induced apoptosis was then examined. The expression of p-ERK in PDT-treated HK-1 cells was significantly reduced by PD98059 (Figure 7A). The basal expression level of p-ERK was also reduced by PD98059. In the functional study, PD98059 dose dependently enhanced PDT-induced apoptosis of HK-1 cells (Figure 7B).

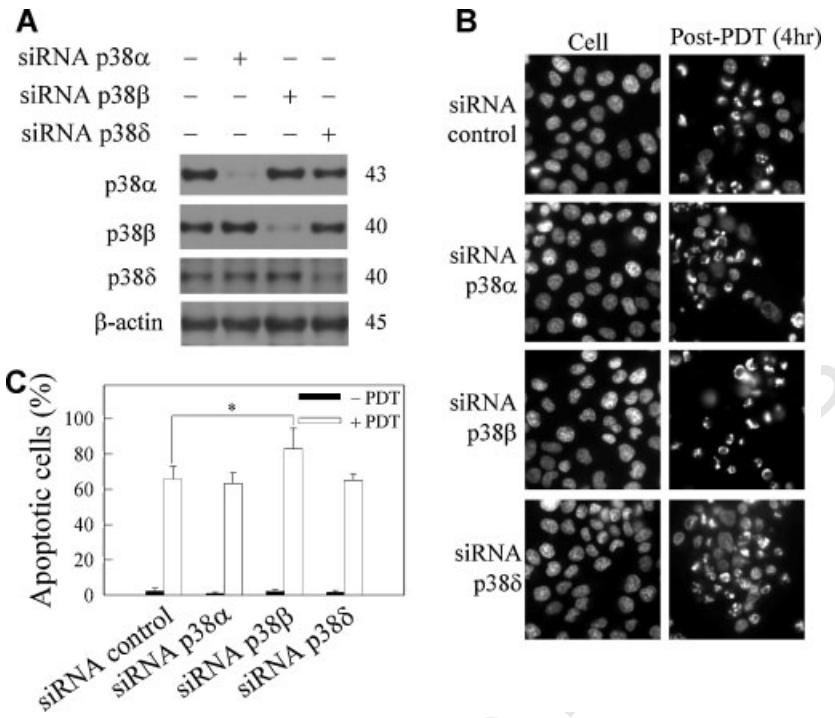

Figure 6. Effect of knockdown of the p38α, p38β and p38δ isoforms on the formation of apoptotic cells induced by Zn-BC-AM PDT. HK-1 cells (1.5×10^5) were grown overnight and then transiently transfected with siRNA-transfecting reagent complexes as described in Materials and Methods. (A) Specificity of siRNA p38 MAPK isoforms. Expression of the corresponding p38 MAPK isoforms was determined by Western Blot. (B) Transfected cells were incubated with or without Zn-BC-AM (1 μM) for 24 h. Medium was replaced with fresh medium before light irradiation. The cells were stained with Hoechst 33342 for 30 min before harvesting. Fluorescence images were captured under a fluorescence microscope at 4 h post-PDT. (C) Percentage of apoptotic cells. At least 300 cells for each treatment were counted. Results were expressed as the mean ± SD ($n = 3$). $^*p < 0.05$ versus PDT siRNA control.

DISCUSSION

MAPKs are involved in a wide range of cellular activities. These include the cell growth and proliferation, differentiation, cell movement and cell death in response to various stimuli and stresses.[1] ERK pathway is generally activated by mitogens and growth factors while p38 MAPK and JNK pathways are activated in response to chemical stimuli and environmental stresses. In addition, activation of MAPKs by different stresses such as UV[27–31] and heat[32] also leads to apoptosis. Hence, MAPKs play an important role in the apoptotic signalling pathways. Many chemotherapeutic agents are known to induce tumour cell death via the modulation of the MAPKs signalling pathways. For example, microtubule inhibitors (vinca alkaloids and paclitaxel) induce a sustained activation of JNK in many tumour cells.[33–35] Cisplatin, a DNA-damaging chemotherapeutic drug, activates p38 MAPK in ovarian carcinoma cells before the onset of apoptosis.[36] Hence, the response of the MAPK signalling pathways to PDT is complex.[37]

Therapeutic effect of PDT basically relies on the generation of reactive oxygen species by the photoactivated photosensitizers. Since MAPKs are sensitive to oxidative stress, we hypothesize that MAPKs are also involved in Zn-BC-AM PDT-induced apoptosis of NPC cells. We have previously demonstrated that PDT induces apoptosis in undifferentiated NPC cells.[38–41] In the present study, we further demonstrated the role of MAPKs in Zn-BC-AM PDT-induced apoptosis of the well-differentiated NPC cells. Zn-BC-AM PDT was found to enhance phosphorylation activation of all three major MAPKs, namely p38 MAPK, JNK and ERK, immediately after PDT. Using two different approaches, namely pharmacological inhibition with p38 MAPK inhibitor SB203580 and knockdown the expression of p38β with siRNA, we found that inhibition of phosphorylation of p38β would enhance Zn-BC-AM PDT-induced apoptosis of HK-1 cells. Differential role of p38 MAPK isoforms has previously been reported in certain studies of apoptotic cell death. Translational knockdown of p38α was found to diminish the death receptor-mediated cleavage of caspase-3 in Jurkat cells. In contrast, knockdown of p38β sensitized the cells to apoptosis in the same cell model.[42] On the other hand, inhibition of p38α would prevent the death of cardiomyocytes caused by a combination of prolonged hypoxia followed by reoxygenation, while p38β exerted a protective role in response to the hypoxic stress.[43] Although p38δ is preferentially expressed in keratinocytes[25] and p38δ is also detected in HK-1 cell, knockdown of p38δ shows no significant effect on PDT-induced apoptosis of HK-1 cells. These observations suggested that the p38β may be a potential therapeutic target to enhance the efficacy of PDT-induced apoptosis of NPC cells.

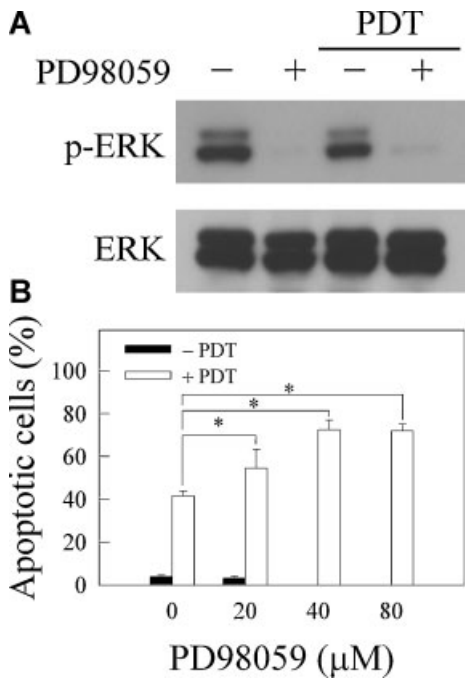

Figure 7. Effect of PD98059 on PDT-induced apoptosis. HK-1 cells (3×10^5) in 35 mm petri dish were grown overnight and then incubated with Zn-BC-AM (1 μM) for 24 hours. The cells were then treated with MEK/ERK inhibitor PD98059 (40 μM) for 1 h before light irradiation. (A) Effect of PD98059 on the expression of phosphorylated ERK in PDT-treated HK-1 cells. Cell lysates were collected at 10 min post-PDT (1 J/cm^2). (B) Dose dependent augmentation of PDT-induced production of apoptotic cells by PD98059. Apoptotic cell count was performed at 4 h post-PDT. Results were expressed as mean ± SD ($n = 3$). $^*p < 0.05$ versus PDT control.

In the functional studies, pharmacological inhibitors PD169316 and SB203580 are frequently used as p38 MAPK inhibitor.[44–46] In the present study, although PD169316 and SB203580 inhibit the phosphorylation of p38 MAPK and the downstream target ATF-2, these two inhibitors exert an opposing effect in PDT-treated HK-1 cells. The discrepancy in the action between PD169316 (anti-apoptotic) and SB203580 (apoptosis enhancing) in PDT-treated HK-1 cells is unknown. Specificity of chemical inhibitors is always a concern especially in the cellular system when the inhibitors were used at the range of micromolar concentrations. p38 MAPK inhibitors had previously been shown to inhibit many enzymes outside of the MAPK family.[47] The anti-apoptotic effect of PD169316 might be due to the off-target effect of the inhibitors in the HK-1 cells. The possible action of PD169316 in PDT-treated NPC cells is currently under investigation.

Apart from p38β, the ERK inhibitor PD98059 also enhanced the production of apoptotic cells after Zn-BC-AM PDT. ERK is generally considered as a survival kinase involved in the control of cell growth. Unlike p38 MAPKs and JNK, p-ERK is constitutively expressed in the HK-1 cells. It is interesting to note that PD98059 also inhibits the basal expression level of p-ERK in the control HK-1 cells. However, the viability of the cell was not affected. This may be explained by the fact that other signalling pathways such as PI3K/AKT and NF-κB are also involved in the regulation of cell survival. In the PDT-treated HK-1 cells, p-ERK appears to counteract PDT-induced apoptosis as inhibition of the expression of p-ERK results in augmentation of PDT-induced apoptosis of HK-1. This explanation is supported by the previous observation that sustained activation of ERK1/2 would protect cells from PDT-induced damage.[16]

Combination of p38 MAPK inhibitor and PDT in clinical application is worthy for discussion. First of all, inhibition of specific p38 isoform can directly enhance apoptosis of PDT-treated tumour cells. Secondly, p38 MAPK inhibition may indirectly control the growth of tumour cells. A recent study showed that p38 MAPK inhibitor would suppress PDT-induced VEGF production.[48] VEGF is one of the key angiogenic factors and anti-angiogenesis is a strategy currently used in cancer therapy. Combination of PDT and selective p38 MAPK inhibition might enhance the efficacy of the treatment. However, this treatment approach needs to further be tested in the animal model.

In summary, we demonstrated that Zn-BC-AM PDT-induced apoptosis in the well differentiated HK-1 NPC cells could be enhanced by the inhibition of p38β isoform and ERK. The protective effect of PD169316 in Zn-BC-AM PDT-induced apoptosis of the HK-1 cells is likely beyond the action on p38 MAPK and JNK. The use of PD169316 as the p38 MAPK/JNK inhibitor for the investigation of the apoptotic cell death should be in caution. Other methods such as siRNA are recommended to be used in parallel to the chemical inhibitors.

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