Inactivation of Epidermal Growth Factor Receptor and Downstream Pathways in Oral Squamous Cell Carcinoma Ca9-22 Cells by Cardiotoxin III from *Naja naja atra*

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Cardiotoxin III (**1**), a basic polypeptide with 60 amino acid residues isolated from *Naja naja atra* venom, has potential therapeutic activity in cancer. Treatment with **1** reduced phosphorylation of EGFR and Akt, as well as ERK in Ca9-22 cells. Moreover, **1**-treatment inhibited constitutive activation of STAT3 and STAT5 in a time-dependent manner. Up-regulation of Bax and down-regulation of anti-apoptotic proteins including Bcl-2, Bcl-X_L, and myeloid cell leukemia-1(Mcl-1) were also found in cells treated with **1**. In addition, **1**-treatment disrupted mitochondrial membrane potential ($\Delta\Psi$m) and resulted in release of mitochondrial cytochrome *c* and activation of both caspases-9 and -3. AG1478, a specific pharmacological inhibitor of EGFR activation, mimics the cytotoxic effects of **1**. Taken together, these results showed that **1** causes significant induction of apoptosis in Ca9-22 cells via abolition of the EGFR-mediated survival pathway of these cells. Thus, cardiotoxin III appears to be a potential therapeutic agent for killing oral squamous carcinoma Ca9-22 cells.

The survival rate for patients with squamous cell carcinoma (SCC) of the oral cavity (OSCC) remains low despite advances in diagnosis and treatment.[1] OSCC usually develops in areas of the epithelium exposed to carcinogens and likely results from an accumulation of cellular and genetic alterations, which lead to the aberrant expression of many proteins involved in cell growth regulation.[2,3] The epidermal growth factor receptor (EGFR) plays an important role in the regulation of cell proliferation, differentiation, development, and oncogenesis.[4−6] Binding of EGF to EGFR on the surface of tumor cells facilitates its heterodimerization and homodimerization with itself or other members of the EGFR family, providing docking sites for a variety of adaptor proteins and enzymes involved in the recruitment and activation of downstream intracellular-signaling cascade, including mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and signal transducer and activator of transcription (STAT) proteins.[7,8] Abnormalities in the expression and signaling pathways downstream of the EGFR contribute to the progression, invasion, and maintenance of the malignant phenotype in OSCC. Therefore, the known oncogenic potential mediated by EGFR and its high-level expression in tumor cells make this oncoprotein an ideal target for antitumor therapeutic approaches.[9,10]

EGFR inhibitor-induced cell death has been reported to be associated with mitochondrial dysfunction.[11,12] Previous reports have demonstrated that some Bcl-2 family members located on the mitochondrial membrane can alter the permeability of the mitochondrial membrane and trigger the release of cytochrome *c*[13,14] or caspases,[15] thus activating the post-mitochondrial caspase cascade, leading to apoptotic cell death.

Cardiotoxins, a group of highly basic polypeptides of 60 amino acid residues, are present abundantly in the elapid family of snakes and show very diverse pharmacological functions including hemolysis, cytotoxicity, and depolarization of the muscles.[16] Moreover, these toxins show preferential cytotoxicity toward cancer cells, probably mediated by inhibiting protein kinase C activity or through a membrane fusion effect.[17−19] In our previous studies, cardiotoxin III (**1**) has been shown to induce dose- and time-dependent apoptosis in K562 cells via the intrinsic pathway involving the mitochondrial membrane permeability, the release of cytochrome *c*, and subsequent caspase activation.[20,21] In the present investigation, **1** was shown to be involved as an apoptotic-inducing factor against the solid tumor cell Ca9-22, mediated through EGFR and downstream signaling pathways.

Results and Discussion

Effects of Cardiotoxin III (1) on Antiproliferation and Apoptosis in Ca9-22 Cells. To verify the effect of **1** on cell growth, Ca9-22 cells were treated with 4 μg/mL **1** for 24 h, and cell survival was assessed by a MTT assay. A significant loss of viability was detected at 4 μg/mL **1** in a time-dependent manner (Figure 1A).

To determine whether **1** decreases cell survival by the induction of apoptosis, the amount of sub-G1 DNA was analyzed by flow cytometry. As shown in Figure 1B, **1**-treated Ca9-22 cells resulted in a markedly increased accumulation of cells in the sub-G1 phase in a time-dependent manner. In addition, Ca9-22 cells were cultured with **1** for 24 h and then stained with annexin V to detect the externalization of PS on the cell membrane. The percentage of annexin V-positive cells, indicative of early apoptosis, increased from 2.1% in the control cells to 9.6, 36.7, and 10.2% in cells treated with 4 μg/mL **1** for 6, 12, and 24 h, respectively. Moreover, **1**-treatment increased the percentage of cells positive for both annexin V and propidium iodide (hallmarks of late apoptosis) from 0.9% in the controls to 5.4, 5.7, and 39.9%. Taken together, it was concluded that **1** reduces cell viability of Ca9-22 cells through the induction of apoptosis.

Effects of Cardiotoxin III (1) on the Expression of Bcl-2 Proteins by Western Blot Analysis. To gain further insight into the molecular events associated with **1**-induced apoptosis, the effects of **1** on the expression levels of Bcl-2 members by western blot analysis were examined. Treatment with **1** resulted in a time-dependent decrease in the expression of Bcl-2, Bcl-X_L, and Mcl-1 and an increase in the expression of Bax (Figure S1, Supporting Information). However, the expression of Bid was not appreciably affected by **1**-treatment.

Effects of Cardiotoxin III (1) on Release of Cytochrome *c* and Change of Mitochondrial Membrane Potential ($\Delta\Psi$m). The release of cytochrome *c* from the mitochondria into the cytosol

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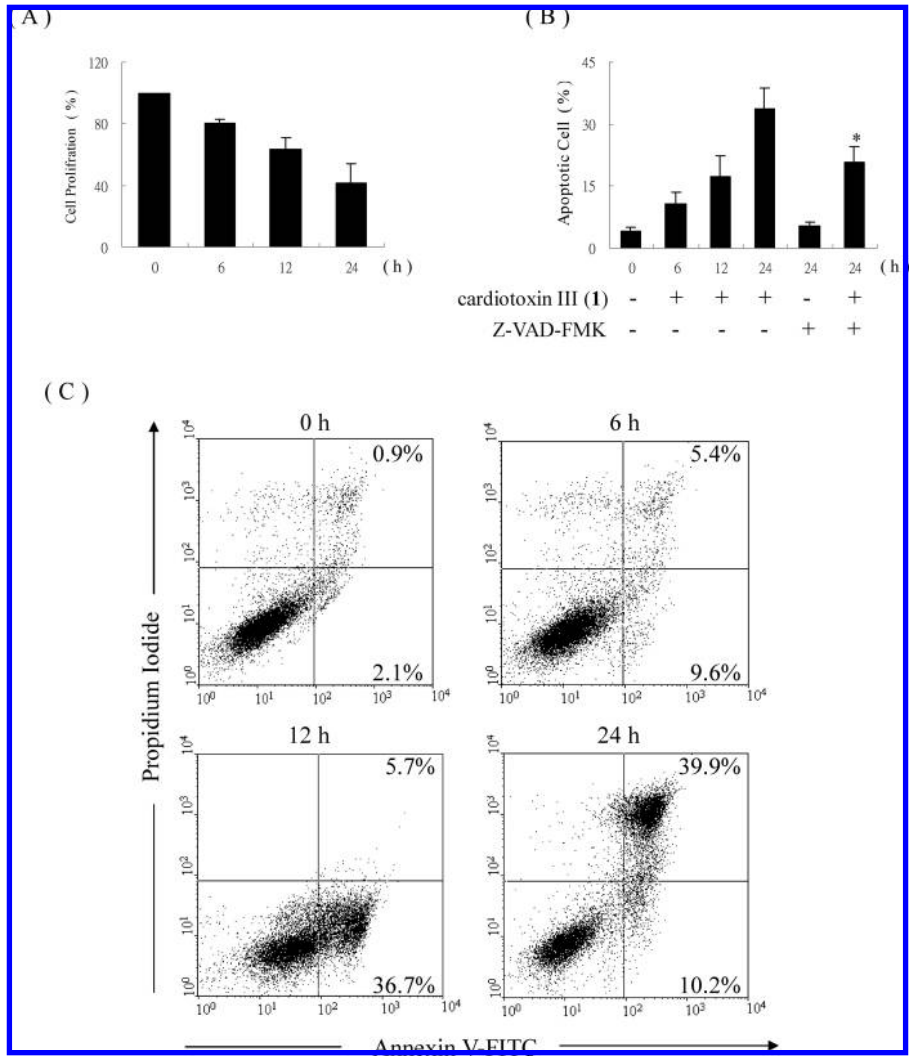

Figure 1. Mediation by cardiotoxin III (**1**) as time-dependent growth inhibition and apoptosis in Ca9-22 cells. (A) Suppression of the growth of Ca9-22 cells by **1**. Cells were incubated with 4 μg/mL **1** for 6, 12, and 24 h. Cell proliferation assays were performed using MTT as described in the Experimental Section. The graph displays the mean ± SD (standard deviation) of three independent experiments. (B) Increase of sub-G1 (apoptotic cells) populations in CA9-22 cells by treatment with **1**. Cells were treated with 4 μg/mL **1** for 6, 12, and 24 h or in combination with 100 μM Z-VAD-FMK at 24 h. Then, the cells were washed, fixed, and stained with propidium iodide and analyzed for DNA content by flow cytometry as described in the Experimental Section. *$p < 0.05$, when compared with the **1**-treated group. (C) Flow cytometric analysis of phosphatidylserine externalization (annexin V binding) and cell membrane integrity (propidium iodide staining). Cells were also treated with 4 μg/mL **1** for 6, 12, and 24 h and then analyzed for apoptosis by flow cytometry for annexin V and propidium iodide staining.

is one of the major apoptotic pathways.[22] To determine whether cytochrome *c* is released in **1**-induced apoptosis, cytosolic fractions from **1**-treated cells were prepared and cytochrome *c* was detected by western blotting analysis. A time-dependent accumulation of cytochrome *c* in the cytosol was observed in **1**-treated cells (Figure S2A, Supporting Information). Since cytochrome *c* release is linked to the loss of $\Delta\Psi$m,[23] the effect of **1** on $\Delta\Psi$m was examined by using the mitochondria-specific dye rhodamine 123.[24] As indicated in Figure S2B (Supporting Information), compared to untreated control cells (2.3%), approximately 59.9% of the cells shifted toward the left, thereby indicating that the treatment with **1** induced a dissipation of $\Delta\Psi$m. A hallmark of the apoptotic process is the activation of cysteine proteases, which represent both initiators and inhibitors of cell death. Upstream caspase-9 activity increased significantly, as shown by the observation in **1**-treated cells (Figure S2C, Supporting Information). In addition, **1** also increased the activation of effector caspase-3 in Ca9-22 cells (Figure S2C, Supporting Information). To confirm that apoptosis due to **1** occurs via the caspase-dependent pathway, cells were preincubated with 100 μM Z-VAD-FMK, a pan-caspase inhibitor, for 1 h before treatment with 4 μg/mL **1** for 24 h. Z-VAD-FMK significantly reduced the population of apoptotic cells induced by **1** on comparing with untreated control cells (Figure 1B). These results suggest that **1**-induced apoptosis is mediated through a mitochondrial-dependent pathway.

Effects of Cardiotoxin III (1) on Inhibition of Phosphorylation of EGFR, STAT3, STAT5, Akt, and ERK1/2. To examine whether the activities of EGFR, STAT3, STAT5, Akt, and ERK1/2 are down-regulated by **1**, we analyzed phosphorylation of EGFR, STAT3, STAT5, Akt, and ERK1/2 in Ca9-22 cells after treatment with **1** (4 μg/mL) for 24 h. As shown in Figure 2, **1** attenuated the levels of phosphorylated EGFR, STAT3, STAT5, Akt, and ERK1/2. However, **1** did not affect protein expression of EGFR, STAT3, STAT5, Akt, and ERK1/2 (Figure 2).

Effects of Cardiotoxin III (1) on Inhibition of EGF-Induced Autophosphorylation of EGFR and Phosphorylation of STAT3, STAT5, Akt, and ERK1/2 in Ca9-22 Cells. Using western blot analysis, the effect of **1** on EGFR autophosphorylation by its ligand, EGF, was assessed. Treatment with **1**

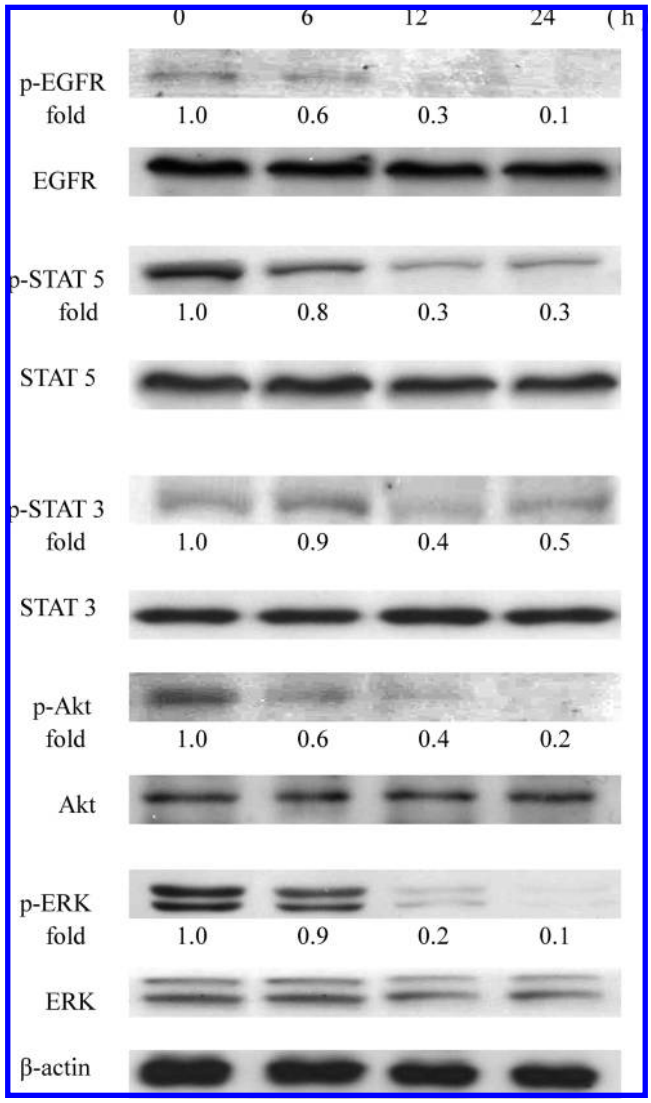

Figure 2. Inhibition of the phosphorylated (p-) EGFR, STAT5, STAT3, Akt, and ERK in Ca9-22 cells by **1**. Cells were treated with 4 μg/mL **1** for different time periods. Equal amounts of whole cell lysates (50 μg) were subjected to western blot analysis. The amount of β-actin was measured as an internal control. Signal intensities of the band were determined by using a densitometer. Similar patterns of protein expression were obtained from three independent experiments.

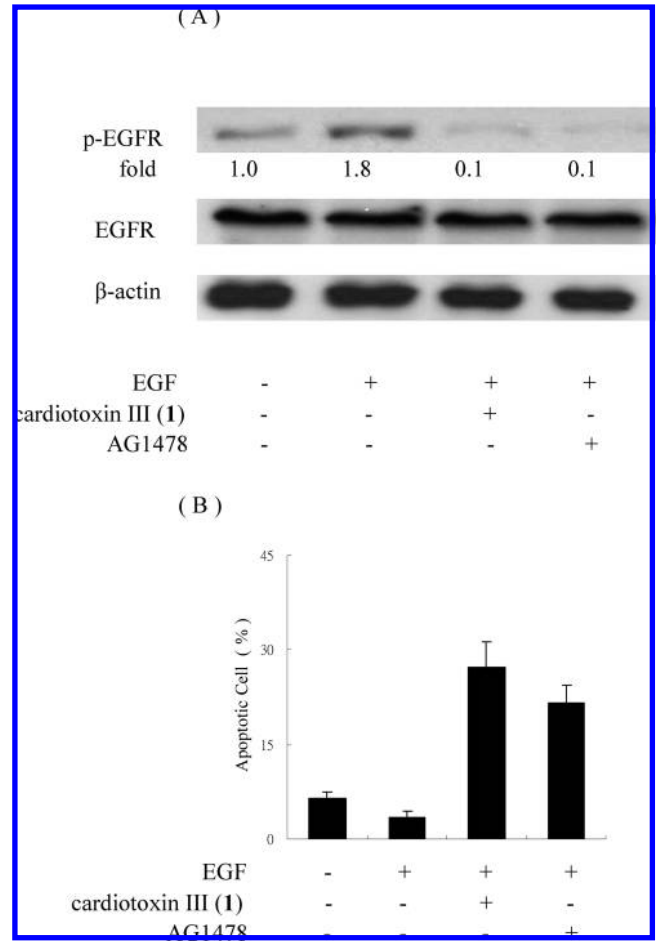

Figure 3. Effect of **1** and EGFR-specific inhibitor AG1478 on EGF-induced autophosphorylation of EGF and apoptosis of Ca9-22 cells. (A) Cells were treated with 4 μg/mL **1** or 20 μM AG1478 for 12 h and then incubated without or with 50 ng/mL EGF and harvested 15 min after EGF treatment. Total cell lysates were prepared for western blot analysis. (B) Cells were treated with 4 μg/mL **1** or 20 μM AG1478 for 24 h and then incubated without or with 50 ng/mL EGF and harvested 15 min after EGF treatment. Then, the cells were washed, fixed, and stained with propidium iodide and analyzed for DNA content by flow cytometry.

inhibited EGF-induced autophosphorylation of EGFR. The protein levels of EGFR remained unchanged. These results verified that **1** is able to inhibit the activation of EGFR. On the basis of its ability to inhibit EGF-induced activation of EGFR, the effect of treatment with **1** on the signaling cascades triggered by this receptor was then evaluated. Phosphorylation of EGFR by EGF has been reported to lead to activation of the STAT3, STAT5, Akt, and ERK1/2 pathways, which play an important role in cell survival and proliferation.[7,8] It was found that treatment with **1** inhibited EGF-induced activation of STAT3, STAT5, Akt, and ERK1/2 (Figure 3A) as well as the cytotoxic effect of **1** in Ca9-22 cells (Figure 3B).

Evidence of Dependency on EGFR of the Cardiotoxin III (1)-Mediated STAT3, STAT5, Akt, and ERK1/2 Activation. It was next determined whether cardiotoxin III-induced phosphorylation/activation of cellular signal transducing elements is downstream of events followed by EGFR activation. AG1478, a specific pharmacological inhibitor of EGFR activation, was used as a positive control.[25,26] As seen in Figure 4, AG1478 reduced the EGF-induced tyrosine phosphorylation of EGFR, STAT3, STAT5, Akt, and ERK1/2 in **1**-treated cells. These results suggest that the major pathway of **1**-mediated signals for phosphorylation and activation of signal transducing molecules starts at the level of EGFR activation.

To assess whether **1**-mediated up-regulation of Bax and down-regulation of Bcl-2, Bcl-X_L, and Mcl-1 in Ca9-22 cells occur by inhibition of the EGFR-mediated pathway, AG1478 was used as a positive control. Inhibition of EGFR activation by AG1478, mimicking the action of **1**, showed the capacity to up-regulate the protein expression levels of Bax and down-regulate the expression levels of Bcl-2, Bcl-X_L, and Mcl-1 in Ca9-22 cells (Figure 5). The present data indicate that the cytotoxic effect of **1** is mediated through EGFR.

Ca9-22 cells treated with **1** for 24 h underwent apoptosis as demonstrated by FACS analysis (Figure 1B), annexin V-binding (Figure 1C), and the intrinsic pathway involving the modulation of Bcl-2 family proteins, cytochrome *c* release, and subsequent activation of caspase-9 and caspase-3 (Figures S1 and S2, Supporting Information). In most cancer cells, apoptosis is dependent upon the mitochondrial "intrinsic" pathway, in which both caspase activation and activity are tightly controlled.[13,14] This process is regulated by pro- and anti-apoptotic proteins, such as members of

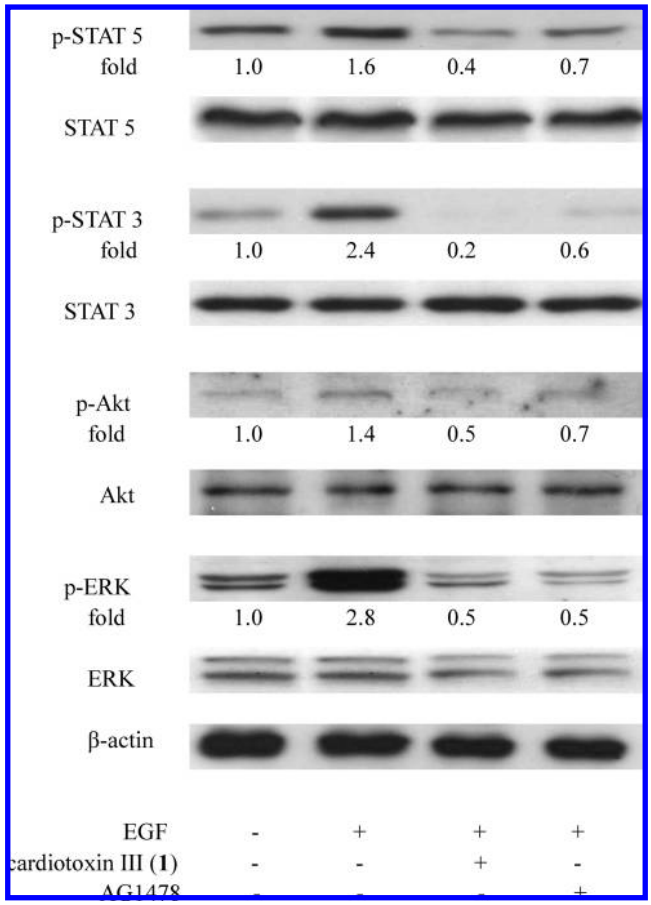

Figure 4. Effect of **1** and EGFR-specific inhibitor AG1478 on EGF-induced phosphorylation of STAT5, STAT3, Akt, and ERK. Cells were treated with 4 μg/mL **1** or 20 μM AG1478 for 12 h and then incubated without or with 50 ng/mL EGF and harvested 15 min after EGF treatment. Total cell lysates were prepared for western blot analysis.

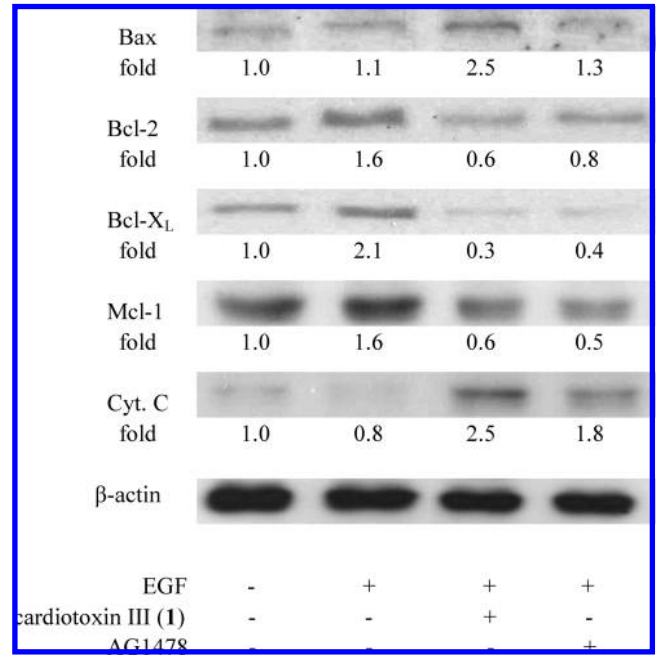

Figure 5. Effect of **1** and EGFR-specific inhibitor AG1478 on EGF-induced protein expression levels of Bax, Bcl-2, Bcl-X_L, Mcl-1, and cytochrome *c*. Cells were treated with 4 μg/mL **1** or 20 μM AG1478 for 24 h and then incubated without or with 50 ng/mL EGF and harvested 15 min after EGF treatment. Total cell lysates were prepared for western blot analysis.

the Bcl-2 family, regulating the mitochondrial pathway prior to caspase activation.[15−17] For example, caspase-9 activation is inhibited by the anti-apoptotic Bcl-2 family of proteins such as Bcl-2 and Bcl-X_L and facilitated by the pro-apoptotic Bcl-2 family of proteins such as Bax and Bad.[13,14] In the current study, treatment with cardiotoxin III reduced the levels of Bcl-2 and Bcl-X_L and increased those of Bax. This agrees with the findings of a previous study showing a concomitant increase in Bax expression and a decrease in Bcl-2 expression following exposure of SCC cells to C225.[27] In various cancer cells, AG1478 and gefitinib have been shown to induce expression of pro-apoptotic members of the Bcl-2 family such as Bak, Bax, and Bim and to inhibit anti-apoptotic members such as Bcl-2 and Bcl-X_L.[28] Recent studies suggest that Mcl-1 cooperates with Bcl-X_L to tether the pro-apoptotic proteins such as Bax, a multidomain and BH-3-exclusive domain protein, and further prevent their activation.[29] The findings that Bcl-2, Bcl-X_L, and Mcl-1 expression are significantly reduced in **1**-treated cells is consistent with these suggestions.

EGFR is expressed at high levels in a number of tumor types and in most squamous cell carcinoma and is associated with lower rates of survival.[5,7,8] In the current study, Ca9-22 cells constitutively expressed phosphorylated EGFR, which may contribute to the resistance of these cells to apoptosis. The data presented here suggest that treatment with **1** resulted in a time-dependent decrease in the phosphorylation of EGFR in Ca9-22 cells (Figure 2). Furthermore, it was also found that **1** inhibited EGF-induced autophosphorylation of EGFR (Figure 3A). The results from this study on the time−effect relationship showed that **1** inhibited tyrosine phosphorylation of EGFR. A decrease in protein expression of EGFR following treatment with AG1478 (Figure 3A) was also detected. However, the protein levels of EGFR did not change in Ca9-22 cells treated with **1** or AG1478 (Figures 2 and 3A). This indicates that inhibition of phosphorylation of EGFR by **1** is at a post-translational level.

The signaling pathways induced by activated EGFR include the PI3K/Akt and MAPK, both of which play a significant role in mitogenic and cell survival.[7,8] EGF exposure resulted in receptor autophosphorylation and activation of PI3K/Akt and MAPK pathways. PI3K is a dimeric enzyme composed of an inhibitory/regulatory (p85) and a catalytic (p110) subunit. The p85 subunit is anchored to the erbB receptor docking sites, and the p110 subunit is responsible for the phosphorylation and activation of the protein serine/threonine kinase Akt.[30] MAPK encompasses a large number of serine/threonine kinases involved in regulating a wide array of cellular processes including proliferation, differentiation, stress adaptation, and apoptosis.[31] Therefore, inhibition of the PI3K and MAPK pathways can synergize with or overcome resistance to chemotherapy, radiation therapy, and targeted agents in cancer.[32] The present observations suggest that treatment with **1** inhibited phosphorylation of Akt and ERK1/2 (Figure 2) and showed that cardiotoxin III treatment inhibited EGF-induced phosphorylation of Akt and ERK1/2 (Figure 4).

The signal transducers and activators of transcription (STAT) factors function as downstream effectors of cytokine and growth factor signaling.[33] Compared with normal cells and tissues, constitutively activated STATs have been detected in a wide variety of human cancer cell lines and primary tumors. Persistent signaling of specific STATs, in particular STAT3 and STAT5, has been demonstrated to directly contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis. STATs participate in oncogenesis through up-regulation of genes encoding apoptosis inhibitors and cell-cycle regulators such as Bcl-X_L, Mcl-1, cyclin D1/D2, and c-Myc.[34] It was found also that treatment with **1** inhibited the activation of phosphorylation of STAT3 and STAT5 (Figure 4).

Together with the findings that AG1478 attenuates the EGF-induced tyrosine phosphorylation of EGFR, STAT3, STAT5, Akt, and ERK1/2 in Ca9-22 cells (Figure 4), the present results suggest that the major pathway of cardiotoxin III-mediated signals for phosphorylation and activation of signal transducing molecules starts at the EGFR activation. Consistent with the suggestion that **1**-mediated up-regulation of Bax and down-regulation of Bcl-2, Bcl-X_L, and Mcl-1 in Ca9-22 cells were via the EGFR-dependent pathway, AG1478 treatment also led to up-regulation of Bax and down-regulation of Bcl-2, Bcl-X_L, and Mcl-1 in Ca9-22 cells (Figure 5). Collectively, the present data indicate that effects of cardiotoxin III are mediated through the EGFR pathway.

In conclusion, the results of the present study have shown that cardiotoxin III treatment abrogated activation of EGFR and downstream events including phosphorylation of STAT3, STAT5, Akt, and ERK1/2. Moreover, up-regulation of Bax expression and down-regulation of Bcl-2, Bcl-X_L, and Mcl-1 expression were observed with cells treated with **1**. Finally, caspase-9 and caspase-3 activation induced apoptotic death.

Experimental Section

Isolation of Cardiotoxin III (1). Cardiotoxin III, with a molecular weight of about 6758 and a purity greater than 98%, was isolated from the venom of *Naja naja atra* (Elapidae; Taiwan cobra) by chromatogaraphy on Sephadex G-50 and SP-Sephadex C-25 as previously described by Lin et al.[35] Solutions of **1** were prepared in phosphate-buffered saline (PBS) and sterilized by filtration.

Chemicals. RPMI 1640 medium, fetal bovine serum (FBS), trypan blue, penicillin G, and streptomycin were obtained from Gibco BRL (Gaithersburg, MD). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), ribonuclease (RNase), rhodamine 123, and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO). The following antibodies were used for western blotting: anti-phospho-ERK, ERK, Akt, STAT3, STAT5, EGFR, phospho-EGFR, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-Bcl-2, Bcl-X_L, Mcl-1, and Bax were purchased from BD Biosciences, and anti-phospho-Akt was from Upstate (Temecula, CA). Antiphospho-STAT5 and anti-phospho-STAT3 antibodies were obtained from Cell Signaling Technology (Beverly, MA), and antibody against cytochrome *c* was obtained from PharMingen (San Diego, CA). The colorigenic synthetic peptide substrates, Ac-DEVD-pNA and Ac-LEHD-pNA, and the protease inhibitor for Z-VAD-FMK as well as the EGFR-specific inhibitor AG1478 were purchased from Calbiochem (San Diego, CA). Annexin V and PI double staining kits were from PharMingen (San Diego, CA). Anti-mouse and anti-rabbit 1g G peroxidase-conjugated secondary antibodies were purchased from Pierce (Rockford, IL). Hybond ECL transfer membrane and ECL western blotting detection kit were obtained from Amersham Life Science (Amersham, UK).

Cell Culture. Oral squamous cell carcinoma Ca9-22 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

Cell Viability Assay. The viability of cells was determined by the MTT assay, and the trypan blue dye exclusion assay was performed to confirm and verify cell viability. Cells were seeded at a density of 1×10^5 cells/3 mL cell culture medium into a 12-well plate. After 24 h of incubation, the cells were treated with vehicle (PBS) or 4 μg/mL concentrations of **1** in medium for 24 h, respectively. MTT solution was added to each well (1.2 mg /mL) and incubated for 4 h. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 570 nm in an ELISA plate reader. For the trypan blue dye exclusion assay, cells were seeded at a density of 1×10^5 cells/well onto a 12-well plate for 24 h; then **1** was added to the medium at various indicated times and concentrations. After incubation, cells exposed to 0.2% trypan blue were counted in a hemocytometer.

Assessment of Apoptosis. The accumulation of sub G1 population in Ca9-22 cells was determined by flow cytometry. Cells were seeded onto 6 cm dishes and treated with or without the indicated **1** for 24 h. Cells were then washed twice with ice-cold PBS and collected by centrifugation at 200*g* for 5 min at 4 °C. Cells were fixed in 70% (v/v) ethanol at 4 °C for 30 min. After fixation, cells were treated with 0.2 mL of DNA extraction buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid buffer, pH 7.8) for 30 min, centrifuged, and resuspended in 1 mL of propidium iodide staining buffer (0.1% Triton X-100, 100 μg/mL RNase A, 500 μg/mL propidium iodide in PBS) at 37 °C for 30 min. Cytometric analyses were performed using a flow cytometer (FACS Calibur, Becton Dickinson) and CellQuest software. Approximately 10 000 cells were counted for each determination.

Apoptotic cells were quantified by annexin V and propidium iodide double staining using a staining kit purchased from PharMingen (San Diego, CA). In brief, 10^6 cells were grown in 35 mm diameter plates and were labeled with annexin V-flourescein isothiocyanate (10 μg/mL) and propidium iodide (20 μg/mL) before harvesting. After labeling, all plates were washed with binding buffer and harvesting by scraping. Cells were resuspended in binding buffer as a concentration of 2×10^5 cells/mL before analysis by flow cytometry.

Protein Extraction and Western Blotting. Cells were harvested and extracted with lysis buffer [50 mM Tris-HCl (pH 7.5), 137 mM sodium chloride, 1 mM EDAT, 1% Nonidet P-40, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μg/mL aprotinin]. The lysates were centrifuged at 20000*g* for 30 min, and the protein concentration in the supernatant was determined with a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and then were electrotransferred to PVDF membrane. The membrane was blocked with a solution containing 5% nonfat dry milk TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h and washed with TBST buffer. The indicated primary antibodies were incubated, washed, and monitored by immunoblotting using specific antibodies. These proteins were detected by enhanced chemiluminescence.

Preparation of Cytosolic Fraction for Assessment of Cytochrome *c*. The mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/mL phenylmethylsulfonylfluoride (PMSF), 8 μg/mL aprotinin, and 2 μg/mL leupeptin, pH 7.4). The cells were broken by passing 10 times through a 22 gauge needle. Nonlysed cells and nuclei were pelleted by centrifugation for 10 min at 750*g*. The supernatant was then centrifuged at 100000*g* for 15 min. This pellet, representing the mitochondrial fraction, was resuspended in buffer A. The supernatant was again centrifuged at 100000*g* for 1 h. The supernatant from this final centrifugation step represents the cytosolic fraction.

Assays of Caspase-3 and -9 Activities. After different treatments, cells (10^6 cells /mL) were collected and washed three times with PBS and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM ethylene glycol tetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 18000*g* for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM of enzyme-specific colorigenic substrates at 37 °C for 1 h. The alternative activities of caspase-3 and caspase-9 were determined as cleavage of the colorimetric substrate by measuring the absorbance at 405 nm.

Flow Cytometric Detection of $\Delta\Psi$m. To assess the change in $\Delta\Psi$m, **1**-treated cells were incubated with 40 nM rhodamine 123 for 15 min at 37 °C. The rhodamine 123 data were recorded using an FL1 photomultiplier. Sample data (10 000 cells) were used to prepare histograms with the CellQuest data analysis program (Becton Dickinson). Dead cells and debris were excluded from the analysis by electronic grating of forward and side scatter measurements.

Statistical Analysis. Differences between treated and control cells were analyzed by Student's *t* test, where $p < 0.05$ was considered significant.

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Supporting Information Available: Western blot analysis of **1**-induced expression levels of Bcl-2 family proteins and effects of **1** on cytochrome *c* release, mitochondrial membrane potential, and caspase activation are available free of charge via the Internet at http://pubs.acs.org.

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