DC-81-Indole Conjugate Agent Induces Mitochondria Mediated Apoptosis in Human Melanoma A375 Cells

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DC-81, an antitumor antibiotic produced by the *Streptomyces* species, belongs to pyrrolo[2,1-*c*] [1,4]-benzodiazepine (PBD), which are potent inhibitors of nucleic acid synthesis. We previously reported an efficient synthesis of PBD hybrids linked with indole carboxylates. This is the first demonstration on the mechanism of the anticancer effect of PBD hybrid (IN6CPBD) agent on human melanoma A375 cells. IN6CPBD-treated cells exhibited higher cytotoxicity than DC-81 and displayed several features of apoptosis, including an increase in the sub-G1 population, a significantly increased annexin V binding, a degradation of caspase-3, and poly (ADP-ribose) polymerase (PARP) cleavage. Because degradative changes associated with apoptosis are often preceded by the disruption of mitochondrial function, the assessment of mitochondrial function in IN6CPBD-treated cells is worthy of investigation. Our data revealed that treatment of A375 cells with IN6CPBD resulted in the loss of mitochondrial membrane potential ($\Delta\Psi_{mt}$), a decrease in intracellular pH (pHi), a reduction of ATP synthesis, increased reactive oxygen species (ROS) generation, and cytochrome *c* release. Collectively, our studies indicate that IN6CPBD induces apoptosis in A375 cells through a mitochondrial dysfunction pathway, leading to caspase-3 substrate PARP cleavage and subsequent apoptotic cell death.

Introduction

Many compounds have been discovered that bind to and interact with the B-form of DNA and can inhibit nucleic acid synthesis and block DNA transcription. Pyrrolo[2, 1-*c*] [1,4]-benzodiazepines (PBDs) are a group of potent, naturally occurring antitumor antibiotics produced by the *Streptomyces* species (*1*). The cytotoxic and antitumor effects of these compounds are believed to arise from the modification of DNA, which leads to the inhibition of nucleic acid synthesis and the production of excision-dependent single- and double-strand breaks in cellular DNA (*2*, *3*). These antibiotics have been proposed to covalently bond to N2 of guanine to form a neutral minor groove adduct (*4*−*7*).

Although the naturally occurring PBDs have potent anticancer activity, they have been precluded from clinical application because of side effects (*8*). Therefore, hybrid compounds with active moieties of known antitumor and antiviral agents are being designed and synthesized to provide highly sequence-selective DNA-interactive properties and antitumor activity (*9*). An indole moiety incorporated into natural and synthetic anticancer agents, such as CC-1065 (*10*), bizelesin (*11*), UTA-6026 (*12*), and K-252a (*13*), shows potent cytotoxicity. These results encouraged us to design and synthesize a hybrid (IN6CPBD, Figure 1) from DC-81 coupled with an indole carboxylate moiety (*14*). We expected that IN6CPBD would recognize more DNA base pairs and bind sequence-selectively to the macromolecule.

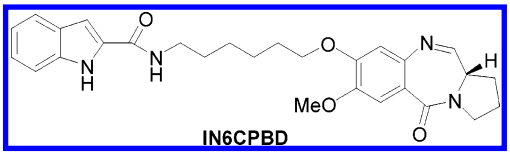

Figure 1. Structure of IN6CPBD agent.

Apoptosis is an important phenomenon in cytotoxicity induced by anticancer drugs. The execution of apoptosis, or programmed cell death (*15*), is associated with characteristic morphological and biochemical changes mediated by a series of gene regulation and cell-signaling pathways. Recently, perturbation of mitochondrial function has been shown to be a key event in the apoptotic cascade (*16*). Anticancer drugs may damage the mitochondria by increasing the permeability of the outer mitochondrial membrane, which is associated with the collapse of the mitochondrial membrane potential ($\Delta\Psi_{mt}$), because a decline in $\Delta\Psi_{mt}$ can disturb intracellular ATP synthesis, generation of reactive oxygen species (ROS), altered mitochondrial redox ratio, intracellular pH, translocation of

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cytochrome *c* to the cytosol, and degradation of caspase-3/PARP. In this regard, we have initiated experiments aimed at characterizing the mitochondrial function of IN6CPBD agent on human melanoma A375 cells, a rapidly proliferating and malignant cell line resistant to radio and chemotherapy (*17*).

The aim of this study was to investigate whether IN6CPBD agent possessed more cytotoxicity than DC-81 and verify whether IN6CPBD agent induced antiproliferation, leading to an aberrant mitochondrial function and subsequent apoptotic cell death.

Experimental Procedures

Cell Culture. Human melanoma cell line A375, purchased from American Type Culture Collection (Manassas, VA), was maintained in Dulbecco's minimal essential medium (DMEM) supplemented with10% FCS, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate (Gibco, BRL). A375 cells were passaged at confluence after treatment with 5 mM EDTA (Gibco, BRL) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

MTS Cell Proliferation Assay. A commercially available kit (CellTiter96 Aqueous proliferation assay kit, Promega, Madison, WI) was used to detect the proliferation according to the manufacturer's instructions. A375 cells were seeded in a 96-well plate at a cell density of 2500 cells/well. After overnight incubation, the IN6CPBD agent at indicated concentrations was added to the culture media and incubated for 24 h. The MTS reagent contains tetrazoliumsalt,[3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2*H*-tetrazolium], premixed with the electron coupling reagent (phenazine ethosulfate), was added into each well at 20 μL. The plate was then incubated for 1−2 h at 37 °C. The optical density value was detected by a microplate reader (MRX-II, Dynex technology, Chantilly, VA), whose detecting and reference wavelengths were set at 490 and 690 nm, respectively.

sub-G1 Region Analysis. A375 cells were treated with various concentrations (0−4 μM) of IN6CPBD for 24 h. Cells were harvested by trypsinization and centrifugation. Cell pellets were resuspended in 50% cold ethanol and fixed at −20 °C. After fixation, cells were washed once with cold PBS and incubated in 0.5 mL of PBS containing 100 μg/mL RNase A for 20 min at 37 °C. Cells were harvested by centrifugation at 400*g* for 5 min, and 250 μL of PBS containing 50 μg/mL propidium iodide (PI) was added to the pellet. Thirty minutes later, the DNA contents of 10,000 events were measured by a FACSscan flow cytometer (Elite ESP, Beckman Coulter, Brea, CA). Histograms were analyzed using Windows Multiple Document Interface software (WinMDI). Cells with DNA content less than that in untreated cells in G0/G1 were considered apoptotic.

Annexin V and PI Binding Assay. To assess the simultaneous observation of the early phase of apoptotic and necrotic features, A375 cells were treated with different agents at 4 μM or treated with various concentrations (0−4 μM) of IN6CPBD for 24 h. Cells were harvested by trypsinization and centrifugation and measured by cytometry by adding annexin V-FITC to 10^6 cells per sample according to the manufacturer's specifications (Bender MedSystems, Vienna, Austria). Simultaneously, the cells were stained with PI. Flow cytometry data were analyzed by WinMDI software.

Analysis of Mitochondrial Membrane Potential ($\Delta\Psi_{mt}$). A375 cells were cultured in 6-well plates and allowed to reach exponential growth for 24 h before treatment. The cells were harvested 24 h after treatment with compound (DC-81, IN6CPBD) at a concentration of 4 μM or treated with graded concentrations of IN6CPBD. The medium was removed and the adherent cells trypsinized. The cells were pelleted by centrifugation at 400*g* for 5 min and stained in a 100 nM/mL $DiOC_6$ dye for 30 min at room temperature and washed with PBS twice and resuspended in PBS. The samples were analyzed for fluorescence (FL-1 detector, filter 530/30 nm bandpass) by a FACSscan flow cytometer (Elite ESP, Beckman Coulter, Brea, CA).

Intracellular pH Measurement. For the measurement of intracellular pH (pHi), A375 cells were treated with various concentrations (0−4 μM) of IN6CPBD for 24 h. After cells were harvested and centrifuged, carboxy-SNARF-1-AM (Molecular Probes, Eugene) was added to give a final concentration of 10 μM, and the cells were incubated for 30 min at 37 °C. Carboxy-SNARF-1-AM enters cells passively as a nonpolar ester and is then hydrolyzed by the intracellular esterases into a polar compound so that it cannot leave membrane-intact cells. At the end of the incubation, the cells were then analyzed by flow cytometry.

ATP Content Bioluminescence Assay. The amount of intracellular ATP was determined by bioluminescent assay on the basis of the measurement of the light output of the luciferin−luciferase reaction. The luciferin−luciferase was purchased as a kit from ThermoLabsystems Luminoskan Ascent (Vantaa, Finland). A375 cells were treated with graded concentrations of IN6CPBD for 24 h, then cells were harvested and lysed after treatment with ice-cold RIPA buffer, and cell extracts were obtained. After centrifugation to remove cell debris, we collected supernatants for ATP measurement. The amount of ATP was determined by the ATP monitoring kit.

Determination of Intracellular ROS Level. To evaluate intracellular reactive oxygen species (ROS) levels, two fluorescent dyes of 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) and hydroethidine (HE, Molecular Probes) were used to clarify this issue. The nonpolar DCFH-DA is converted to the polar derivative DCFH by esterases when it is taken up by the cell. DCFH is nonfluorescent but is rapidly oxidized to the highly fluorescent DCF by intracellular H_2O_2 or nitric oxide. HE oxidation is particularly sensitive to superoxide anions, hydroxyl radicals, and peroxynitrite. In addition, superoxide dismutase (SOD, Sigma) or catalase (Sigma), an effective $O_2^{-\bullet}$ or H_2O_2 scavenger, was also used in this study. Cells were pretreated with SOD (100 U/mL) or catalase (800 U/mL) before 0 and 4 μM IN6CPBD treatment. Four hours later, DCFH-DA (10 μM) or HE (10 μM) was immediately added to cultured cells for 30 min at 37 °C. The fluorescence of the samples was measured with a flow cytometer. The 2′,7′-dichlorofluorescein (DCF) and HE data were recorded using FL-1 and FL-2 photomultipliers, respectively.

Determination of Intracellular Cytochrome *c* Level. After treatment with IN6CPBD (0−4 μM) for 24 h, cells were washed three times with cold PBS, re-suspended in cell lysis buffer to a concentration of 1.5×10^6 cells/mL, and then incubated for 1 h at room temperature with gentle mixing. After centrifugation to remove cell debris, the supernatant was diluted at least 5-fold and assayed immediately or aliquoted and store at ≤ −70 °C for cytochrome *c* measurement. The total amount of intracellular cytochrome *c* was determined according to the protocol provided with the cytochrome *c* immunoassay kit (R&D Systems, Minneapolis, MN).

Protein Extraction and Western Blot Analysis. Total cell extracts from cultured A375 cells were obtained by lysing the cells in ice-cold RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 100 μg/mL PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 100 μg/mL NaF. After centrifugation at 14,000*g* for 30 min, protein in the supernatants was quantified by the Bradford method (Bio-Rad). Forty micrograms of protein per lane was applied in 10% SDS−poly acrylamide gel. After electrophoresis, protein was transferred from the gel to the polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were blocked at room temperature for 1 h in PBS + 0.1% Tween 20 (PBS-T) containing 5% skim milk. After briefly rinsing with PBS-T, the membrane was incubated with primary antibody at room temperature for 2 h or at 4 °C overnight. Rabbit polyclonal antibodies against CPP32 (H-277) and PARP (H-250) was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against actin was purchased from Chemicon Int. Inc. (Temecula, CA). The membrane was incubated with the corresponding horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h. Membranes were washed with PBS-T four times for 15 min, and the

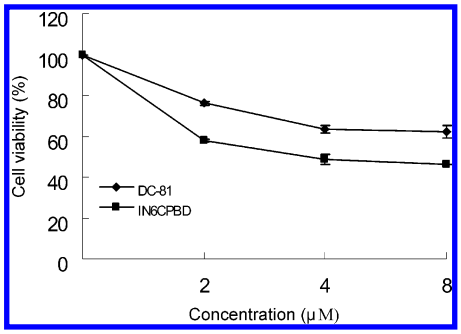

Figure 2. Dose−response curves for compounds tested against A375 cells. Cells were seeded in a 96-well plate at 2500 cells per well and cultivated overnight until cell attachment. Compounds at the indicated concentration were added into the culture media in triplicate and incubated for 24 h before MTS was added. The conversion of MTS to formazan was measured at 490 nm. The absorbance is directly proportional to the number of living cells.

protein blots were visualized with Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA). The relative amounts of specific proteins were quantified by densitometry scanning of X-ray films and analyzed by Eagle Eye Image System (Stratagene, La Jolla, CA).

Statistical Analysis. All results were expressed as means values ± standard deviation (SD) and analyzed by using the statistical analysis system (SPSS, SPSS Inc., Chicago, IL). Differences among groups were analyzed by Student's *t*-test. *P* values <0.05 were considered as significant for all statistical tests.

Results

Effect of IN6CPBD on Cell Viability. DC-81 and IN6CPBD were assessed for their in vitro cytotoxicity on human melanoma A375 cells. The activity of mitochondrial dehydrogenase enzymes, detectable by catalyzing MTS reagent, correlated with cell viability (*18*). The cytotoxic effects of the two compounds DC-81 and IN6CPBD agent on A375 cells was examined using an MTS cell proliferation assay. The cell viability of A375 cells treated with agents at different dosages after 24 h is shown in Figure 2. The inhibitory effect is dependent on drug concentration. At concentrations of 2 μM, IN6CPBD agent exhibited a higher inhibitory activity compared to that of DC-81 on A375 cells.

Effect of IN6CPBD on Cellular sub-G1. Because hypodiploid DNA content (sub-G1 material) is characteristic of apoptosis and reflects fragmented DNA, the sub-G1 population in A375 cells after IN6CPBD treatment was measured. As shown in Figure 3, the sub-G1 DNA peaks after 0, 1, 2, and 4 μM IN6CPBD treatment were 2.9 ± 0.5%, 6.5 ± 1.3%, 7.7 ± 1.2%, and 21.2 ± 1.5%, respectively. IN6CPBD treatment of A375 cells resulted in a markedly increased accumulation of sub-G1 phase cells that were dose dependent.

IN6CPBD Induces Externalization of PS. Fluorescein isothiocyanate (FITC)-conjugated annexin V has been utilized to detect the externalization of phosphatidylserine that occurs at an early stage of apoptosis. Propidium iodide (PI) is used as a marker of necrosis due to cell membrane destruction (*19*). To elucidate whether IN6CPBD induces more apoptotic cells than DC-81, treatment of A375 cells with 4 μM for 24 h induced apoptosis effects in 2.7 ± 0.1% (DC-81) and 13.3 ± 0.8% (IN6CPBD) of annexin V-FITC cells (Figure 4A). Although IN6CPBD are highly potent antitumor agents, the precise mechanism of action remains unclear. To further characterize whether IN6CPBD-induced cell death involved apoptosis or necrosis, we performed a biparametric cytofluorimetric analysis using annexin V and PI double staining. As shown in Figure 4B, 3.5% of the cells were apoptotic (annexin V^+/PI^-), whereas only 0.1% were necrotic (annexin V^+/PI^+) under the untreated control group. Following 1−4 μM IN6CPBD treatment, we found a marked increase of apoptotic cells (from 9.3 to 30.1%); in contrast, there was no obvious change of necrotic cells (from

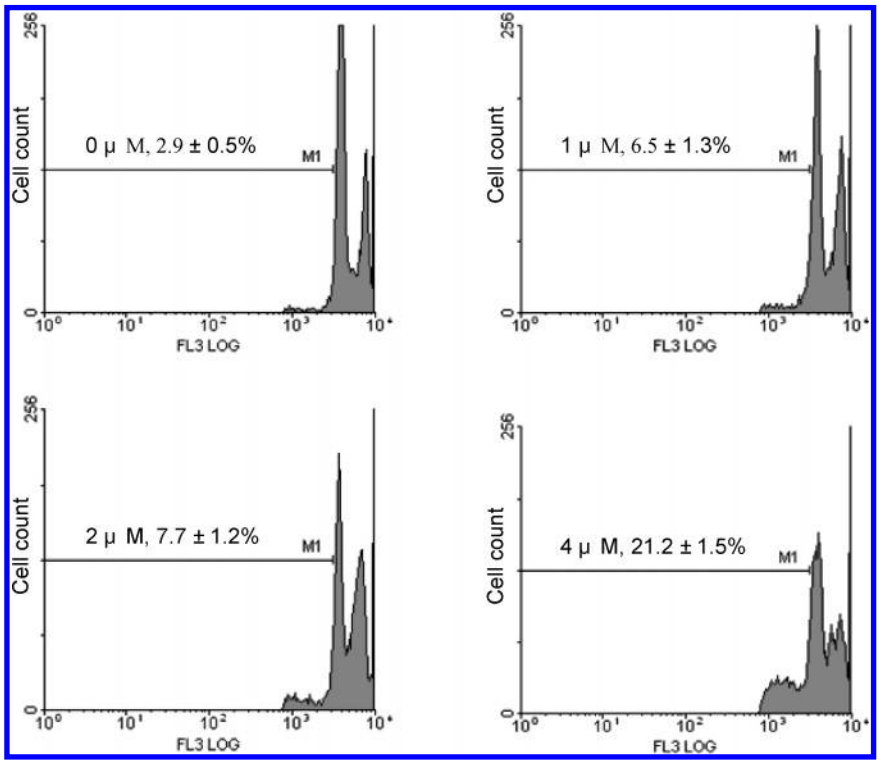

Figure 3. Effect of IN6CPBD on cellular sub-G1 content. A375 cells were treated with graded concentrations of IN6CPBD for 24 h and stained with PI. Approximately 10,000 cells from each group were analyzed with the FACScan flow cytometer. Data represent the percentage of cell counts and display sub-G1.

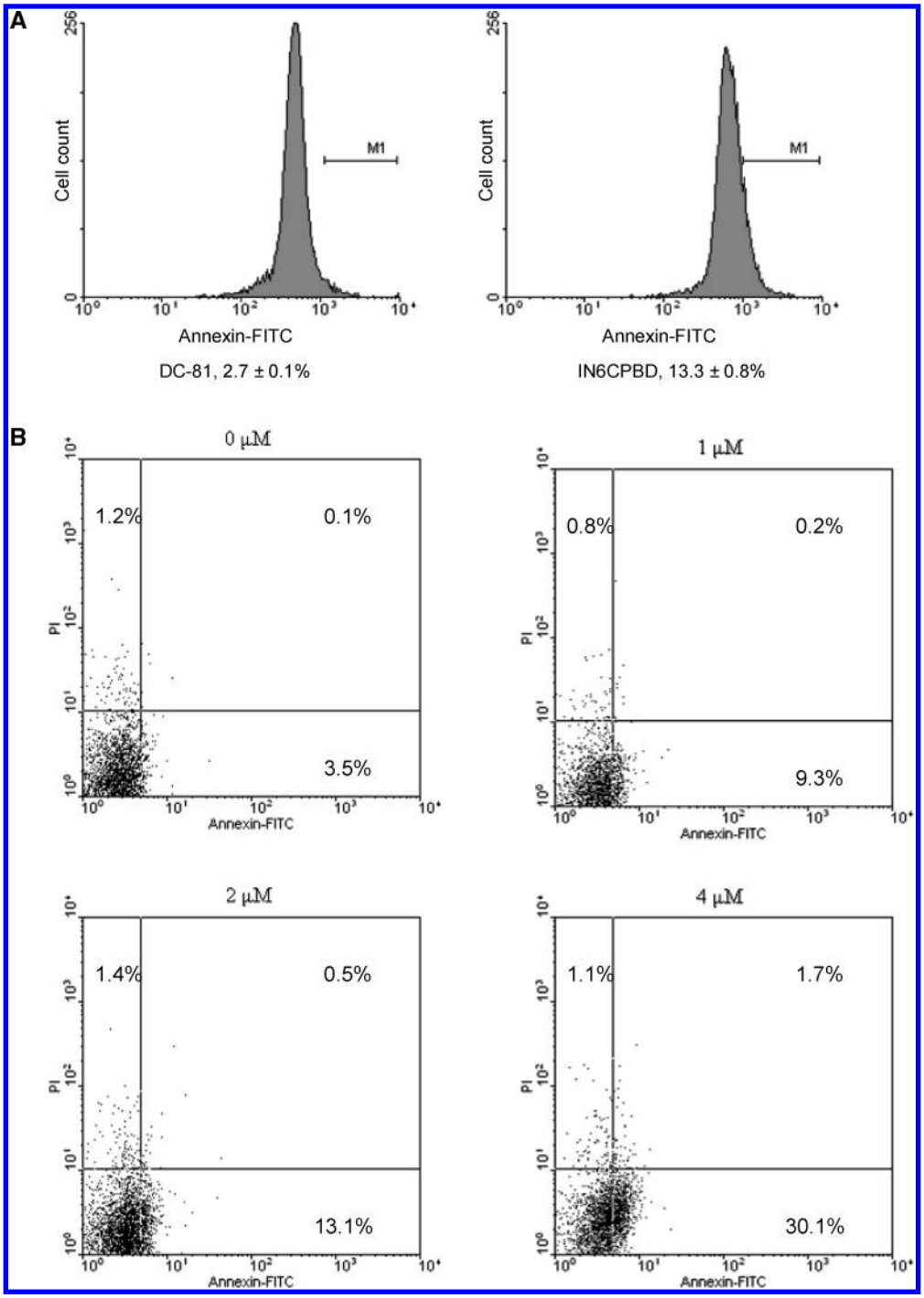

Figure 4. IN6CPBD induces externalization of PS. (A) To elucidate whether IN6CPBD induces more apoptotic cells than DC-81, cells were cultured with agents at a concentration of 4 μM for 24 h and then stained with annexin V-FITC, and approximately 10,000 cells from each group were analyzed by FACS. (B) Dot plots for A375 cells treated with graded concentrations of IN6CPBD for 24 h and then stained with PI and an annexin V−FITC conjugate specifically detecting the exposure of PS residues at the cell surface. Approximately 10,000 cells from each group were analyzed by flow cytometry. Data shown are of a representative experiment repeated three times with similar results.

0.2 to 1.7%). A375 cells were observed to undergo IN6CPBD-induced apoptosis in a dose-dependent manner.

IN6CPBD Induced Mitochondrial Membrane Potential ($\Delta\Psi_{mt}$) Disruption. Previous studies have suggested that a decline of $\Delta\Psi_{mt}$ may be an early event in the process of cell death. Therefore, we investigated whether $\Delta\Psi_{mt}$ disruption was involved in agent-induced apoptosis. A375 cells were harvested 24 h after treatment with compound (DC-81, IN6CPBD) at a concentration of 4 μM or treated with graded concentrations of IN6CPBD, then analyzed by flow cytometry after $DiOC_6$ dye labeling. The dye binds to the inner and outer membrane of mitochondria and undergoes a red shift in fluorescence during membrane depolarization. IN6CPBD exhibited a marked reduction in cellular uptake of the fluorochrome compared to that of DC-81(Figure 5A). As demonstrated in Figure 5B, cells treated with doses higher than 1 μM exhibited significant decline of $\Delta\Psi_{mt}$ in A375 cells. Compared with that of the untreated control, $\Delta\Psi_{mt}$ was significantly reduced in A375 cells exposed to 2 ($p < 0.05$) and 4 μM ($p < 0.01$) IN6CPBD, respectively. The decrease in fluorescence intensity reflects the collapse of $\Delta\Psi_{mt}$, which generally defines an early but already irreversible stage of apoptosis (*20*). These results reveal that exposure of

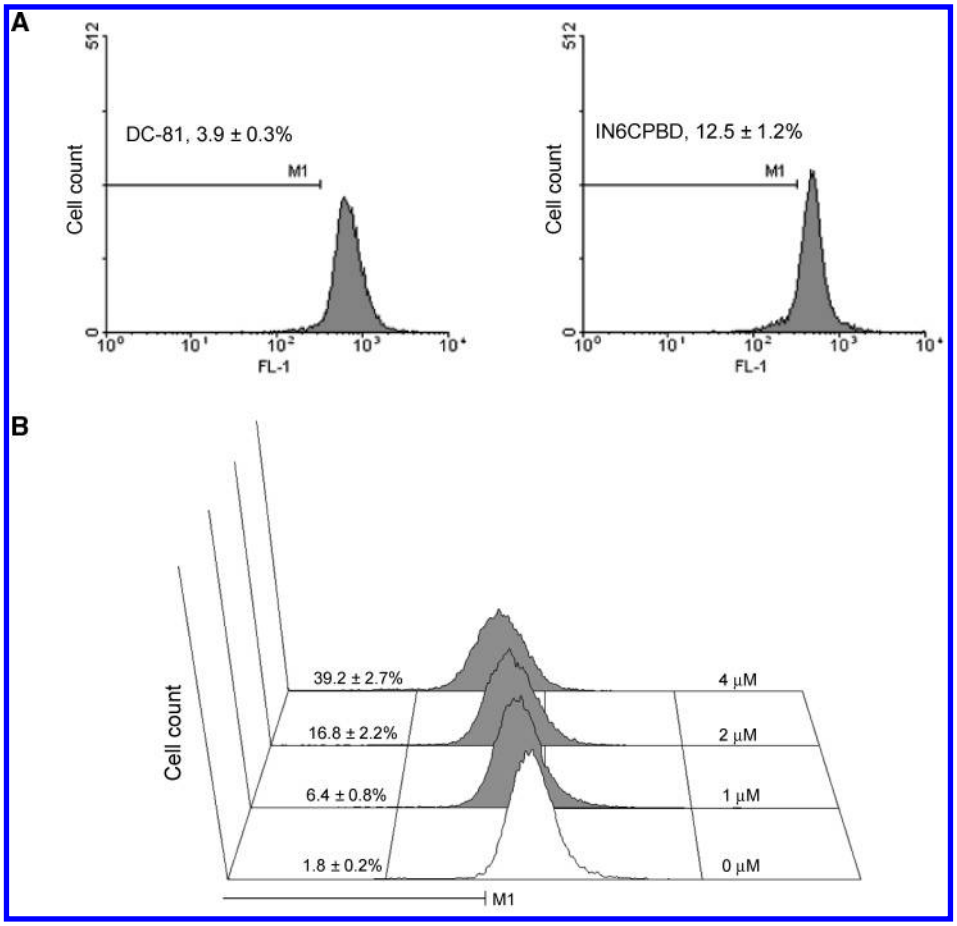

Figure 5. Effect of IN6CPBD on the mitochondrial membrane potential ($\Delta\Psi_{mt}$). (A) To clarify whether IN6CPBD has more $\Delta\Psi_{mt}$ disruption than DC-81, cells were cultured with agents at a concentration of 4 μM for 24 h and then stained with $DiOC_6$, and approximately 10,000 cells from each group were analyzed immediately by flow cytometry. (B) $\Delta\Psi_{mt}$ of A375 cells after exposure to IN6CPBD (0−4 μM) for 24 h. Approximately 10,000 cells from each group were analyzed by flow cytometry. The number in M1 indicates the percentage of cells with reduced $\Delta\Psi_{mt}$.

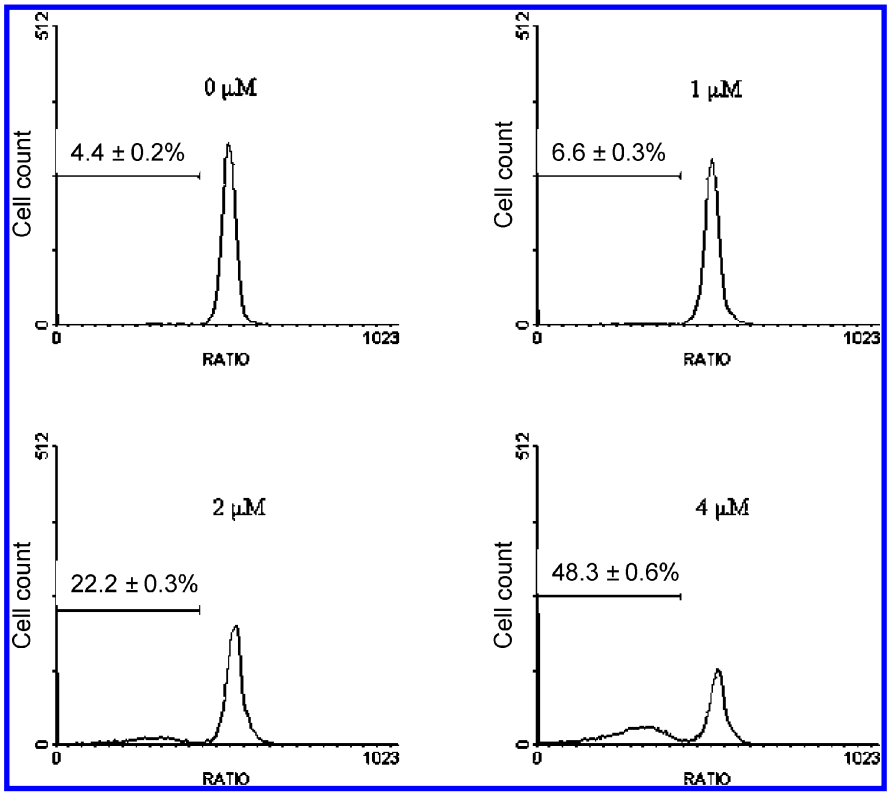

Figure 6. Intracellular pHi in A375 cells after IN6CPBD treatment. Histogram of fluorescence ratio (635/575 nm) vs cell count in A375 cells with various concentrations of IN6CPBD treatment and stained with carboxy-SNARF-1-AM. Approximately 10,000 cells from each group were analyzed immediately by flow cytometry.

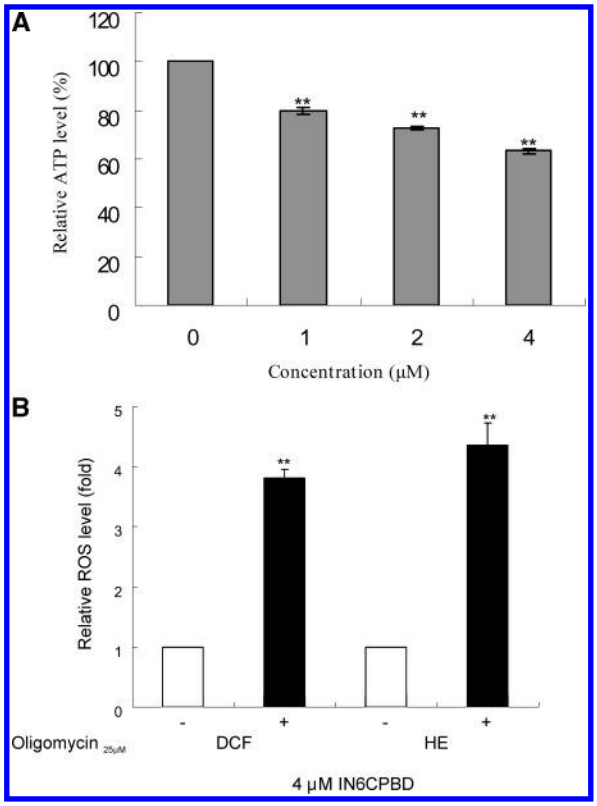

Figure 7. Effect of IN6CPBD on the intracellular ATP levels of the A375 cells. (A) A375 cells were treated with 0, 1, 2, and 4 μM IN6CPBD for 24 h. Relative ATP levels were calculated as the percentage of the 0 μM level. (B) Relative ROS level (DCF and HE) of A375 cells were obtained after 4 μM IN6CPBD treatment with or without oligomycin (25 μM), a mitochondria-specific F_0F_1ATP synthase inhibitor. Approximately 10,000 cells from each group were analyzed by flow cytometry. ** $p < 0.01$ as compared to the control.

melanoma A375 cells to IN6CPBD inducing the drop of $\Delta\Psi_{mt}$ may be a possible cause for the apoptotic process.

IN6CPBD Reduced Intracellular pHi in A375 Cells. It has been reported that the pHi is reduced during cellular quiescence and decreased cell activity (*21*). To investigate whether reduced pHi is due to IN6CPBD, A375 cells were treated with pH sensitive fluoroprobe carboxy-SNARF-1-AM. Following exposure of cells to IN6CPBD (0−4 μM), a distinct subpopulation of cells were 4.4 ± 0.2%, 6.6 ± 0.3%, 22.2 ± 0.3%, and 48.3 ± 0.6%. A representative histogram is shown in Figure 6.

IN6CPBD Reduced Intracellular ATP Content in A375 Cells. ATP is the central parameter of cellular energetics, metabolic regulation and cellular signaling; therefore, determination of intracellular ATP is worthwhile in the characterization of cellular physiology. Intracellular ATP was measured by continuously monitoring ATP production by firefly luciferase luminescence. Compared with that of the untreated control, the intracellular ATP content of A375 cells decreased about 20%, 30%, and 40% after 1, 2, and 4 μM IN6CPBD treatment, respectively (Figure 7A). Moreover, to elucidate whether increase in ROS level might be due to the decrease in cellular ATP synthesis, cells were pretreated with a mitochondria-specific F_0F_1ATP synthase inhibitor, oligomycin (25 μM) before 4 μM IN6CPBD treatment. Our results showed that pretreatment with oligomycin increased the formation of ROS (DCF, HE) (Figure 7B).

IN6CPBD Induced ROS Generation. To determine whether ROS is involved in IN6CPBD-induced apoptosis, we measured the production of intracellular H_2O_2 and $O_2^{-\bullet}$ directly using DCFH-DA and the HE probe. Our results showed that IN6CPBD significantly increases intracellular H_2O_2 and $O_2^{-\bullet}$ levels. In addition, catalase and SOD significantly abrogated the increased ROS production of A375 cells treated with 4 μM IN6CPBD (Figure 8A and B).

IN6CPBD Induced Cytochrome *c* Release. An important consequence of mitochondrial dysfunction is the translocation of cytochrome *c* from the mitochondrial intermembrane space to the cytosol (*22*, *23*). As shown in Figure 9, cells treated with doses higher than 1 μM IN6CPBD exhibited significant increase of the intracellular cytochrome *c* levels in A375 cells.

IN6CPBD Induced Caspase-3/PARP Degradation. Poly (ADP-ribose) polymerase (PARP) has been identified as a substrate for caspase-3. Data from Western blot experiments showed that activation of two apoptotic signals and degradation of PARP and caspase-3 were observed when A375 cells were exposed to 4 μM IN6CPBD (Figure 10).

Discussion

DNA is a target for many antitumor drugs currently used in clinics. However, there are only a few DNA-interactive agents that bind to DNA with high sequence selectivity. Selectivity is generally thought to favor the targeting of the rapidly growing tumor cells. The development of low molecular weight molecules with the highly sequence-selective DNA-interactive properties is a present study of interest. We have previously reported an efficient synthesis of DC-81 (*24*), as a starting point for the design of novel dimeric and conjugate agents that would be expected to be more biologically potent. We next combined DC-81 and an indole 2-carbonyl moiety to synthesize IN6CPBD designed to have much higher sequence selectivity in DNA interactivity.

In this study, we used an MTS cell proliferation assay to evaluate the cytotoxicity of tested compounds in human melanoma A375 cells. Our results indicated that IN6CPBD is more effective as an antiproliferative agent than DC-81. One can speculate that this is because IN6CPBD recognizes more DNA binding sites and increases the stability of the drug/DNA complex. In a previous article (*14*), we have demonstrated that hybrid agents bind to DNA more efficiently than DC-81 in A2058 cells on the basis of the inhibition of restriction endonuclease *BamH*I. In the present article, we extend our observation to the antiproliferative effect of IN6CPBD, which may be associated with cellular apoptosis. Our results showed that the IN6CPBD agent induces a markedly increased accumulation of sub-G1 phase cells and triggers apoptosis as revealed by the externalization of annexin V-targeted PS residues at the periphery of the cells. It has been hypothesized that the sensitivity of tumor cells to IN6CPBD might be associated with the DNA repair process that involves p53 (*25*). Thus, to elucide whether cellular apoptosis might be due to the inhibition of nucleic acid synthesis, the expression of the p53 tumor suppressor gene is determined. Data from Western blot analysis showed a significant decrease in the expression of p53 protein level of cells treated at concentrations ≥ 2 μM IN6CPBD as compared to the control group (data not shown). Our results suggests that cells treated by IN6CPBD resulted in a decrease in p53 expression, DNA repair deficiency, DNA synthesis block, and cell cycle arrest, ultimately leading to A375 cell apoptosis.

Many reports have indicated that mitochondria may play a critical role in the commitment of cells to apoptosis (*16*), this prompted us to investigate whether mitochondria were the target organelles in agent-induced apoptosis. Mitochondria play a central role in cellular homeostasis, and their homeostatic center is the mitochondrial membrane potential ($\Delta\Psi_{mt}$). Thus, the

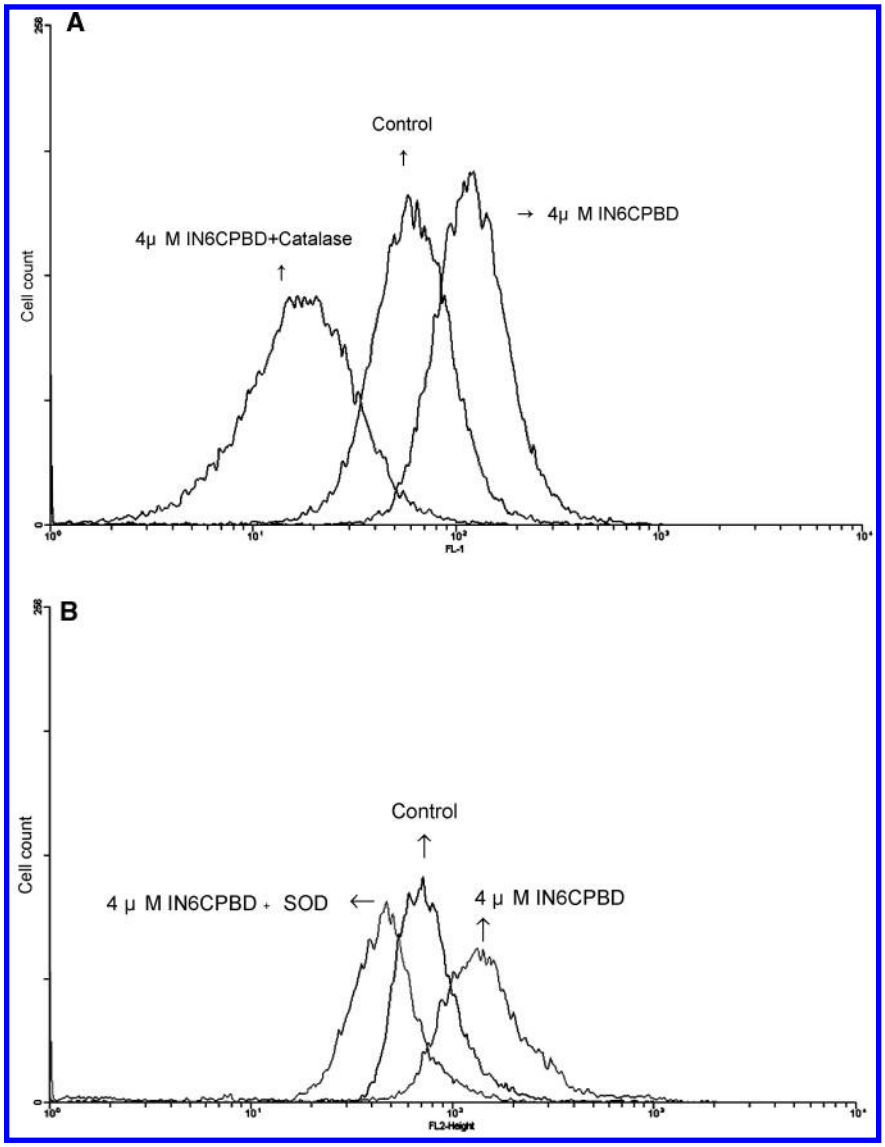

Figure 8. Effect of IN6CPBD on reactive oxygen species (ROS) generation in A375 cells. Cells were untreated or incubated with 4 μM IN6CPBD for 4 h, and the ROS production (dichlorofluorescein, DCF (A); hydroethidine, HE (B) fluorescence) was measured. As a control, ROS was measured in the presence of catalase and SOD, H_2O_2 and $O_2^{-\bullet}$ scavengers, respectively. Approximately 10,000 cells from each group were analyzed by flow cytometry. Similar results were obtained in three independent experiments.

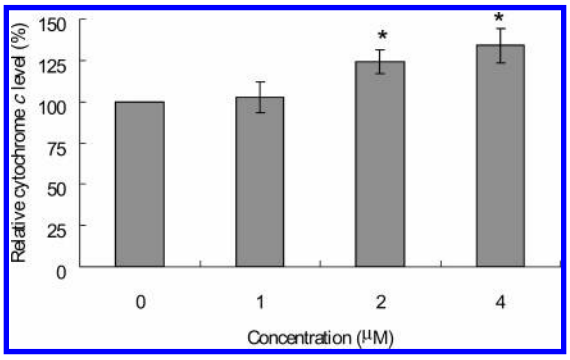

Figure 9. Effect of IN6CPBD on the intracellular cytochrome *c* levels of the A375 cells. The change of intracellular cytochrome *c* levels was determined using the experimental protocol. Relative cytochrome *c* levels were calculated as the percentage of the 0 μM level. * $p < 0.05$ as compared to the control.

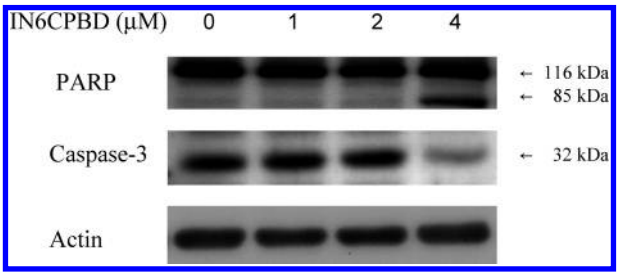

Figure 10. Effect of IN6CPBD on degradation of apoptotic molecules (PARP and caspase-3). After exposure to different concentrations of IN6CPBD for 24 h, cell lysates were collected, and the expression levels of caspase-3 and PARP were determined by Western blot analysis with specific antibodies. Similar results were observed in three separate experiments.

assessment of $\Delta\Psi_{mt}$ in cells is worthy of investigation. In this study, IN6CPBD exhibited more $\Delta\Psi_m$ disruption than DC-81. In addition, A375 cells treated with dosages higher than 1 μM IN6CPBD exhibited a marked decrease in $\Delta\Psi_{mt}$. pHi plays an important role in a variety of cellular processes, including enzymatic activity, receptor-mediated signal transduction, ion transport, and homeostasis. The pH sensitive fluoroprobe carboxy-SNARF-1-AM assay was employed in this study. Our data revealed that concentration higher than 1 μM IN6CPBD on A375 cells caused a significant reduction in the fluorescence ratio because of the development of apoptotic cell death.

Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in eukaryotes. A study is in progress to investigate whether decreased intracellular ATP is due to IN6CPBD treatment. Our study showed a decrease of intracellular ATP content in response to IN6CPBD treatment. Although mitochondria-dependent depletion of ATP would by itself damage cells because of the failure to provide energy for membrane pumps, a greater hazard is the generation of ROS (Figure 7B). Accumulation of ROS has been shown to be an effective mechanism to activate programmed cell death (*26*). We found that ROS was involved in apoptotic effect induced by IN6CPBD treatment. In addition, application of catalase and SOD, which H_2O_2 and $O_2^{-\bullet}$ scavenge, abrogated the enhanced ROS of A375 cells treated with IN6CPBD.

The release of cytochrome *c* to the cytosol represents another consequence of the mitochondrial permeability transition. Cytosolic cytochrome *c* has been implicated in the activation of caspases 3 and 9 (*23*, *27*). These cysteine−aspartate specific proteases play a key role in the downstream events associated with the apoptotic cascade. We found an increase in cytosolic cytochrome *c* in A375 cells treated with at least 2 μM IN6CPBD. Furthermore, we also observed that IN6CPBD exposure resulted in a degradation of caspase-3 and cleavage of the DNA repair enzyme, PARP.

The data gathered in the present study demonstrated that cells exposed to IN6CPBD initiate mitochondrial changes including a decline in $\Delta\Psi_{mt}$, a decrease in pHi, a reduction of ATP synthesis, increased ROS generation, and cytochrome *c* release. In addition, IN6CPBD also induced a marked increase of sub-G1 accumulation, phosphatidylserine translocation, and caspase-3/PARP degradation to identify apoptotic cells. In conclusion, our study confirms for the first time that IN6CPBD induces apoptosis in A375 cells and that IN6CPBD-induced apoptosis may involve mitochondrial dysfunction. These findings suggest that IN6CPBD may represent a promising compound that may be of interest in cancer chemoprevention.

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