Involvement of Akt in mitochondria-dependent apoptosis induced by a cdc25 phosphatase inhibitor naphthoquinone analog

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Abstract Vitamin K-related analogs induce growth inhibition via a cell cycle arrest through cdc25A phosphatase inhibition in various cancer cell lines. We report that 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (DDN), a naphthoquinone analog, induces mitochondria-dependent apoptosis in human promyelocytic leukemia HL-60 cells. DDN induced cytochrome *c* release, Bax translocation, cleavage of Bid and Bad, and activation of caspase-3, -8, -9 upon the induction of apoptosis. Cleavage of Bid, the caspase-8 substrate, was inhibited by the broad caspase inhibitor z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk), whereas cytochrome *c* release was not affected, suggesting that activation of caspase-8 and subsequent Bid cleavage occur downstream of cytochrome *c* release. DDN inhibited the activation of Akt detected by decreasing level of phosphorylation. Overexpression of constitutively active Akt protected cells from DDN-induced apoptosis, while dominant negative Akt moderately enhanced cell death. Furthermore, Akt prevented release of cytochrome *c* and cleavage of Bad in DDN-treated HL-60 cells. Taken together, DDN-induced apoptosis is associated with mitochondrial signaling which involves cytochrome *c* release via a mechanism inhibited by Akt.

Key words: Naphthoquinone analog; Apoptosis; Cytochrome *c*; Bad; Akt

1. Introduction

Menadione (vitamin K3) is a synthetic compound among the vitamin K family of molecules. Menadione has increasingly been of interest because it is shown to exhibit a broad range of antitumor activity in human cells and imposed lower level of toxicity than other cancer chemotherapeutic drugs of quinone structure, such as doxorubicin, daunorubicin, and mitomycin C [1–4]. It has been reported that menadione induces growth arrest and apoptosis in various cancer cell lines. In human hepatoma cells, menadione has an inhibitory effect on the activity of cdc25 phosphatase, which is involved in cell cycle regulation through dephosphorylating cyclin-dependent kinases (CDK) [2]. A thioether analog of menadione that inhibits cell growth exerts its effects via sulfhydryl arylation of cellular protein tyrosine phosphatase [3,4]. In addition, menadione induces G1 arrest by generating superoxide in several cell lines [3]. Despite a broad range effect of menadione on growth suppression of cancer cells, its hydrophobicity presents difficulties for use as an anticancer drug. Recently, 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (DDN), a new naphthoquinone analog with polar groups has been described as the potent growth inhibitor and apoptotic inducer [5]. Previous studies have shown that DDN induces G1 arrest through cdc25A phosphatase inhibition, and induces growth inhibition more potently than other analogs and natural vitamin K3, without producing ROS in hepatocarcinoma cells [5]. Recently, we have shown that DDN induces cell death in HeLa cells via an apoptotic pathway by inhibiting transcriptional activity of NF-κB [6]. However, the signal transduction in DDN-induced apoptosis has not been well established.

Apoptosis is an important regulatory mechanism that eliminates unwanted cells during the development and maintenance of cell homeostasis. The regulation of mitochondrial membrane integrity and the release of cytochrome *c* from mitochondria are important processes during apoptosis, and the Bcl-2 family proteins serve as critical regulators of mitochondria-dependent apoptotic pathways [7,8]. For instance, Bax is a proapoptotic member of the Bcl-2 family that resides in the cytosol and translocates to mitochondria upon induction of apoptosis. Bax induces cytochrome *c* release and caspase activation in vivo and in vitro. In contrast, antiapoptotic Bcl-2 and Bcl-xL proteins prevent the release of cytochrome *c* in the presence of apoptotic stimuli and promote cell survival [9–11]. In many cases, caspases are activated during apoptosis through mitochondria-dependent pathways and sequential activation of these caspases cleaves various cellular substrates [12,13]. In addition, a balance between survival and death signal transduction pathways is important in controlling apoptosis. For apoptosis to effectively occur, survival pathways must be inhibited or fail to be activated. In this regard, inhibition of the key antiapoptotic kinase Akt is important for inducing apoptosis by decreasing antiapoptotic signaling in cells [14–17].

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Abbreviations: zVAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethylketone; DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; IETD-pNA, N-acetyl-Ile-Glu-Thr-Asp-p-nitroanilide; LEHD-pNA, *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide; FITC, fluorescein isothiocyanate

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In this report, we examined signaling pathways during naphthoquinone analog-induced apoptosis in HL-60 cells, with particular focus on mitochondria-dependent cell death. DDN induces apoptosis through activating caspases and alterations in subcellular redistribution of Bax and cytochrome *c*. In addition, a constitutively active form of Akt inhibits DDN-induced apoptosis and cytochrome *c* release. Our data show that DDN induces apoptosis by regulating multiple signaling pathways that involve decreasing antiapoptotic signals in addition to increasing proapoptotic signals.

2. Materials and methods

2.1. Materials

DDN was purchased from Sigma-Aldrich. Lipofectamine was obtained from Life Technologies. Fetal bovine serum was from Gibco BRL, enhanced chemiluminescence (ECL) kit from Amersham Pharmacia, caspase-3, -8, -9 substrates from Biomol, and Hoechst 33258 from Molecular Probes, Inc. Antibody to cytochrome *c* was from Pharmingen. Antibodies to Bax, Bid, Bad, Akt, pAkt (Ser473) and horseradish peroxidase (HRP)-conjugated secondary antibody were from Santa Cruz Biotechnology, Inc. z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) was from Enzyme System Products.

2.2. Cell viability and internucleosomal DNA fragmentation

HL-60 cells were washed with serum-free RPMI. DDN, zVAD-fmk or vehicle was diluted into serum-free RPMI at the indicated concentrations. Cell viability was determined by the trypan blue exclusion method at different time points. For DDN-induced apoptosis, HL-60 cells were maintained in serum-free RPMI for 2 h before experiments. To analyze apoptosis, HL-60 cells were treated with DDN (1 μM) for 24 h and resuspended in buffer containing 1 μg/ml annexin-fluorescein isothiocyanate (FITC) as described by the manufacturer's protocol (Zymed, South San Francisco, CA, USA). After a 10-min incubation, the cells were washed, stained with 1 μg/ml propidium iodide, and then mounted on a slide. In five separate fields, both the total number of cells and the number of annexin-positive cells were counted. DNA fragmentation and staining nuclei with Hoechst 33258 were performed as described previously [17]. Apoptosis was also measured using an enzyme-linked immunosorbent assay (ELISA) based on detection of histone-associated DNA fragments in the cytoplasm of apoptotic cells according to the manufacturer's instructions (Roche, Castle Hill, Australia).

2.3. Subcellular fractionation

The basic methodology for the preparation of mitochondria and cytosol fractions was modified from a previous report [18]. Briefly, HL-60 cells (3×10^6) at the end of the treatment were harvested and washed with ice-cold phosphate-buffered saline (PBS). Cells were resuspended in 500 μl of buffer A (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium ethylenediamine tetraacetic acid (EDTA), 1 mM sodium ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin, 1 mM leupeptin, 1 μg/ml chymostatin). To lyse the cells, the cell suspension was passed five times through a 26-gauge needle fitted to a syringe. Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at $1000\times g$ at 4°C for 10 min. The resulting supernatant was subjected to $10\,000\times g$ centrifugation at 4°C for 20 min. The pellet fraction (i.e. mitochondria) was first washed with the above buffer A containing sucrose and then solubilized in 50 μl of TNC buffer (10 mM Tris–acetate, pH 8.0, 0.5% NP-40, 5 mM $CaCl_2$). The supernatant was recentrifuged at $100\,000\times g$ (4°C, 1 h) to generate cytosol.

2.4. Western blot analysis

Cells were solubilized with ice-cold lysis buffer containing 1% Triton X-100, 50 mM NaCl, 25 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 10 μg/ml leupeptin. Insoluble materials were removed by centrifugation at $10\,000\times g$ for 10 min. Extracted proteins (50 μg/well) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on polyacrylamide gels, and were electrophoretically transferred onto Immobilon-P membrane. Blocking was performed in Tris-buffered saline containing 5% skimmed milk powder and 0.1% Tween-20. The membranes were probed with antibodies against caspases, cytochrome *c*, Bid, Bax, Bad, Akt or phospho-Akt. Detection was performed with ECL system. Protein content was determined with Bradford method using bovine serum albumin as a standard.

2.5. Measurement of caspase activity

Cell lysates were incubated with the colorimetric substrates, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA) (caspase-3) or *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (IETD-pNA) (caspase-8) or Ac-*N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-pNA) (caspase-9), to measure caspase activity according to the protocol suggested by the manufacturer. Reactions were assembled in microtiter plate wells by adding 160 μl of buffer B (100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM dithiothreitol (DTT), and 0.5 mM EDTA) containing 100 μM substrate to wells containing 50 μg of cytosolic protein in 40 μl of buffer A. Plates were incubated at 37°C for 1 h. Release of free pNA, which absorbs at 405 nm, was monitored continuously.

2.6. Transient transfection

Cells were transfected with pECE-MyrAkt or pCMV6-Akt-K179M [19] using lipofectamine reagent according to the manufacturer's protocol. Briefly, HL-60 cells were incubated in opti-MEM media along with 1 μg of plasmid DNA and lipofectamine (10 μl) for 4 h at 37°C. After transfection, cultures were maintained in serum-depleted medium for 15–17 h prior to DDN treatment.

3. Results

3.1. Naphthoquinone analog induces apoptosis in HL-60 cells

To test the cell death effect of DDN, HL-60 cells were treated with different concentrations of DDN for 24 h, and cell viability was examined with trypan blue dye exclusion. As shown in Fig. 1A, DDN induced cell death in a dose-dependent manner, with a concentration of 1 μM DDN resulting in death of 75% of the cell population. The broad caspase inhibitor zVAD-fmk reduced DDN-induced cell death by 50%. To further investigate whether DDN induces apoptosis, we also evaluated translocation of phosphatidylserine to outer surface of the cytoplasmic membrane using annexin-FITC. Annexin-positive cells were increased in DDN-treated cells (Fig. 1B). DNA fragmentation characteristic of apoptosis was evident in cells treated with 1 μM DDN for 24 h, suggesting that a population of the cells underwent apoptotic cell death (Fig. 1C).

3.2. Naphthoquinone analog induces alterations in subcellular redistribution of cytochrome c and Bax

The release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol is a prominent downstream event of apoptotic cell death [7,8]. As shown in Fig. 2, cytosolic cytochrome *c* was increased time-dependently in DDN-treated cells. Since Bax has been shown to induce cytochrome *c* release from mitochondria to the cytosol in conjunction with apoptosis in several cell lines and translocation of Bax to mitochondrial outer membrane is a primary event in triggering mitochondrial function [9,11,18], we examined the subcellular distribution of Bax in DDN-treated HL-60 cells. Cytosolic Bax was decreased and mitochondrial Bax was increased time-dependently in accordance with increase in cytosolic cytochrome *c* (Fig. 2). Bax translocation and cytochrome *c* release were not inhibited by z-VAD-fmk, indicating that subcellular redistribution of cytochrome *c* or Bax is not dependent on caspase activation.

3.3. Naphthoquinone analog induces activation of caspases

Recent evidences indicate that many chemotherapeutic agents function proximal to the executioner caspases [20,21]. We examined whether DDN induces activation of caspase-3. Cells treated with 1 μM of DDN resulted in a time-dependent processing of caspase-3 (Fig. 3A). Activation of caspase by DDN was also confirmed by measuring the enzyme activity using the colorimetric substrate, Ac-DEVD-pNa. DEVDase activity was increased in a time-dependent fashion similar to the proteolytic processing of procaspase-3 (Fig. 3B). Cleavages of caspase-8 and -9 were also induced by DDN (Fig. 3A). When comparing the time course for processing of caspase-3 with caspase-8 and -9, it was difficult to determine which was activated first, since caspase-8, -9 and -3 were activated with similar kinetics. Recently, it is reported that caspase-8 cleaves Bid in the death receptor apoptotic pathway, and the cleaved Bid can directly act on mitochondria to trigger cytochrome *c* release [22]. To investigate whether caspase-8 activation induced by DDN preceded the release of cytochrome *c*, we examined the effect of DDN on Bid cleavage and the effect of caspase inhibitor on cytochrome *c* release. As shown in Fig. 3C, Bid was cleaved into a fragment of 15 kDa in DDN-treated cells. Cleavage of Bid was inhibited by the broad caspase inhibitor zVAD-fmk, whereas cytochrome *c* release was not affected by zVAD-fmk. These results indicate that DDN-induced activation of caspase-8 and subsequent processing of Bid occur downstream of cytochrome *c* release.

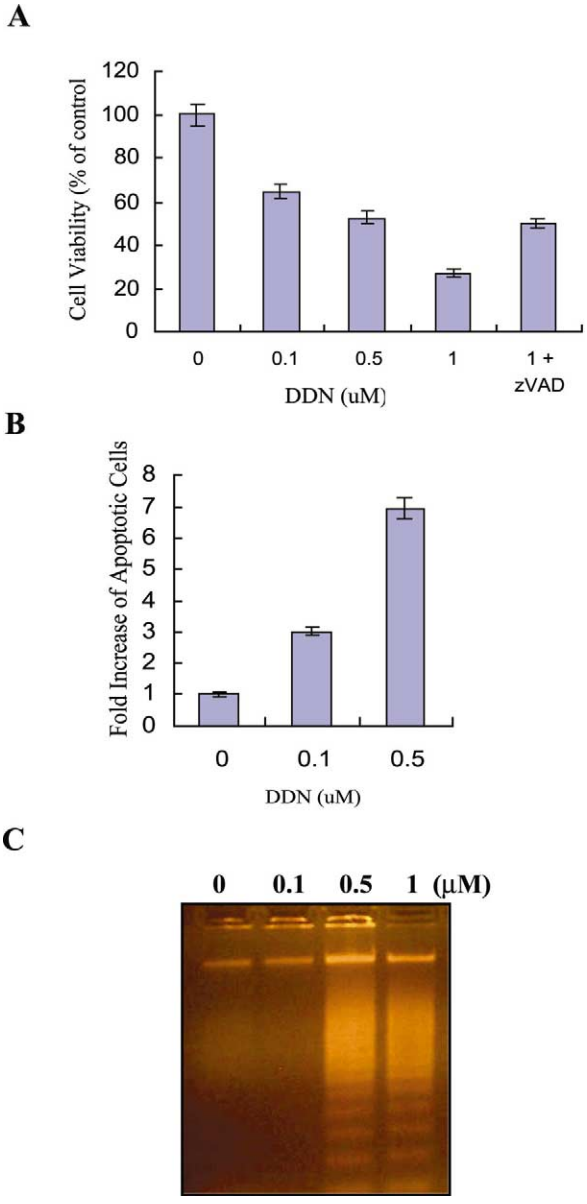

Fig. 1. DDN induces apoptotic cell death in HL-60 cells. A: Cells were treated with DDN at various concentrations for 24 h, and cell viability was determined by trypan blue exclusion method. B: Apoptotic cells determined by annexin staining of HL-60 cells treated with DDN for 24 h. C: DNA fragmentation was investigated in a dose-dependent manner. Total genomic DNA was prepared, separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

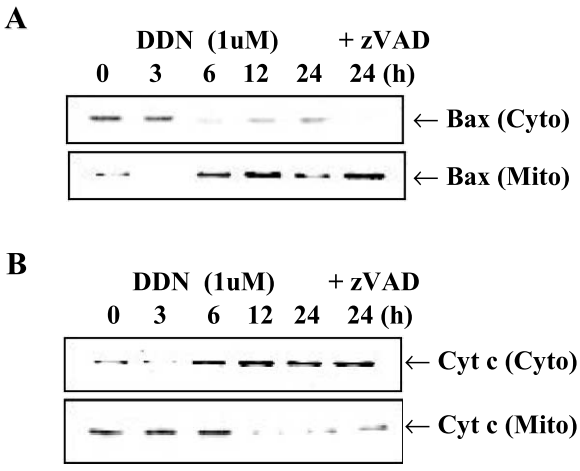

Fig. 2. DDN induces subcellular redistribution of cytochrome *c* and Bax. Cells were treated with 1 μM DDN for the indicated time points, and cytosolic extracts were prepared as described in Section 2. Equal amounts of lysates were loaded, separated by SDS–PAGE and immunoblotted with anti-Bax antibody or anti-cytochrome *c* antibody.

3.4. Akt inhibits cell death and the release of cytochrome c induced by DDN

Akt inhibits apoptosis and the processing of procaspases in their active forms by delaying mitochondrial changes [23]. To further understand the mechanism by which DDN induces cell death, we examined the effect of Akt signaling on DDN-induced apoptosis. HL-60 cells were expressed with a constitutively active form of Akt containing the Src myristoylation signal sequence, and treated with 1 μM of DDN. DDN inhibited the activation of Akt detected by decreasing levels of phosphorylation (Fig. 4A). Expression of MyrAkt significantly protected cells from DDN-induced apoptosis, whereas expression of dominant negative Akt moderately enhanced cell death (Fig. 4B, C). Furthermore, Akt prevented release of cytochrome *c* in DDN-treated HL-60 cells. As shown in Fig. 4D, little cytochrome *c* protein was detected in the cytosolic fraction, when active Akt was overexpressed. Recent studies suggested that caspase-dependent cleavage of Bad may be one of the major mechanisms in the process of apoptosis in response to various apoptotic stimuli [24,25]. Bad is a proapoptotic Bcl-2 family of proteins, and its proapoptotic activity is regulated primarily by phosphorylation in response

to survival factors [25]. Since Bad can be phosphorylated by Akt, and inhibition of Akt prevents phosphorylation of Bad, we examined the possible involvement of Bad in DDN-induced apoptosis. No significant change in the level of phospho-Bad following DDN treatment was observed (data not shown). However, DDN treatment induced cleavage of Bad to generate a ~16-kDa cleavage product. Overexpression of MyrAkt or treatment with zVAD-fmk blocked DDN-induced cleavage of Bad (Fig. 5).

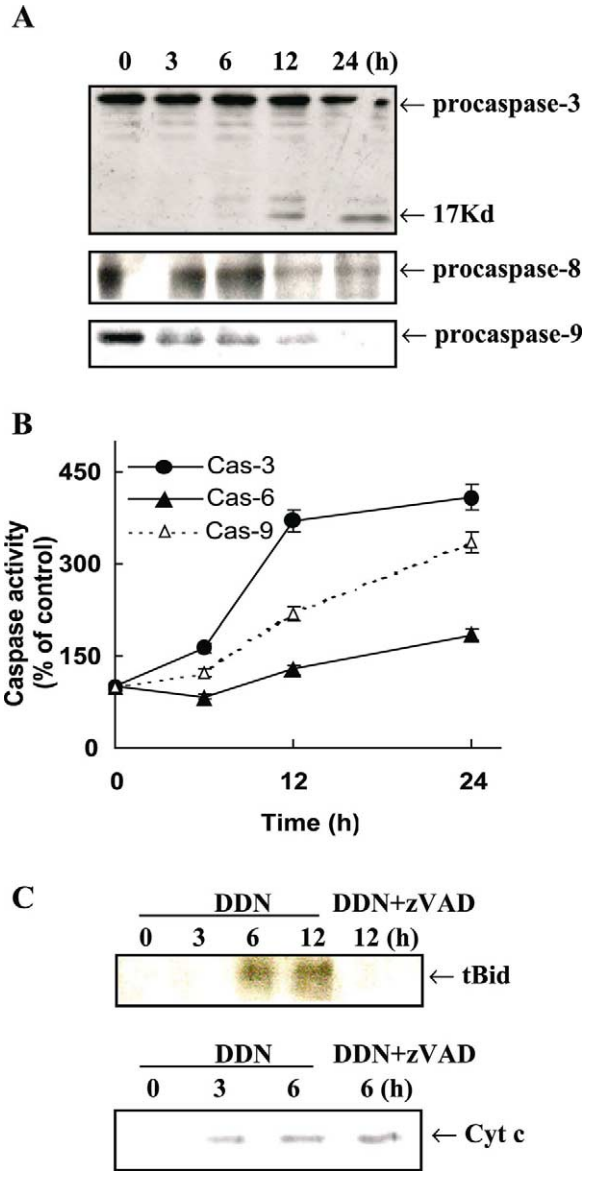

Fig. 3. Induction of caspase activity and Bid cleavage by DDN. A: Processing of caspases by DDN. HL-60 cells were treated with 1 μM DDN for the indicated time points. Cell lysates were prepared, and then analyzed by immunoblotting for the processing of caspase-3, -8, -9. B: At the indicated times, samples of cell extracts were assessed for their ability to hydrolyze the peptides DEVD-pNA, IETD-pNA and Ac-LEHD-pNA. C: Cleavage of Bid, but not cytochrome *c* release is prevented by zVAD-fmk. HL-60 cells were treated with 1 μM DDN in the presence or absence of zVAD-fmk (50 μM), and then analyzed by Western blot. Results shown are representatives of three separate experiments.

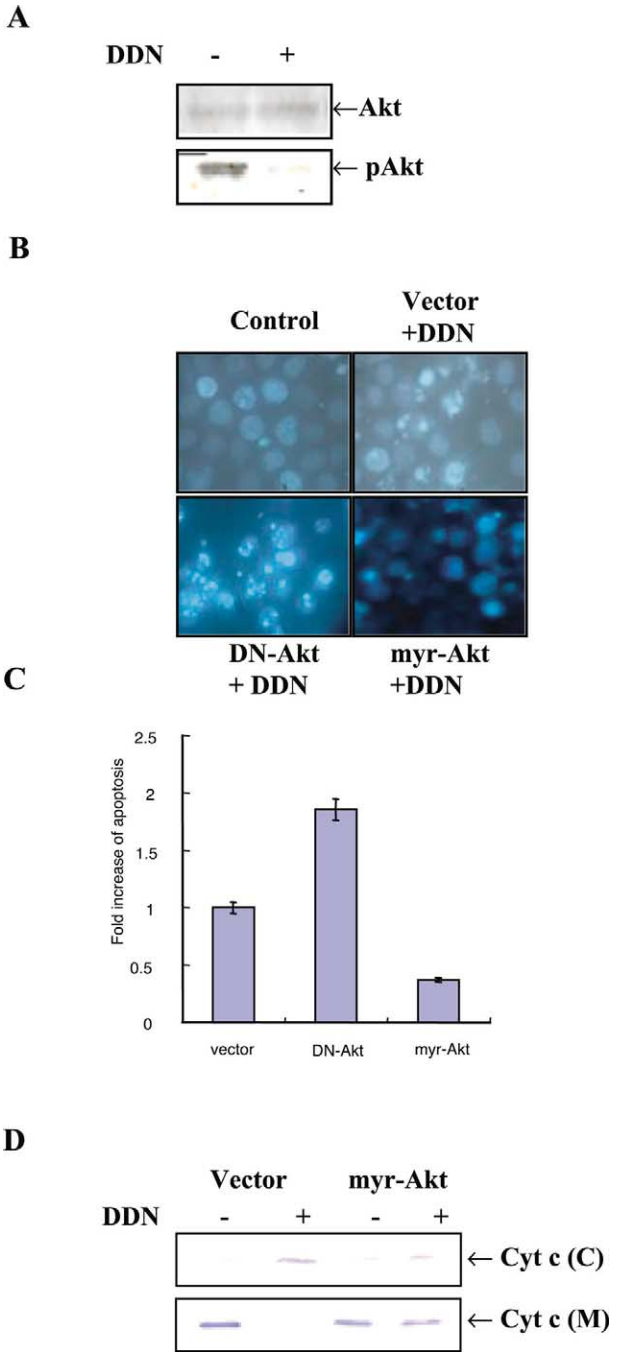

Fig. 4. Akt inhibits cell death and cytochrome *c* release induced by DDN. HL-60 cells were transfected with vector, MyrAkt or dominant negative Akt-K179M and treated with 1 μM DDN for 24 h. A: Akt and phospho-Akt protein levels were analyzed by Western blot of total cell lysates. B: Hoechst staining of cells treated with 1 μM DDN for 24 h. C: Apoptosis was measured using an ELISA based on detection of histone-associated DNA fragments in the cytoplasm of apoptotic cells. HL-60 cells were treated with 1 μM DDN for 24 h. D: Cells were treated with 1 μM DDN for 24 h, and cytosolic and mitochondrial fractions were prepared, separated by 15% SDS–PAGE followed by immunoblot analysis.

4. Discussion

Vitamin K-related analogs have been found to be potent growth inhibitors of tumor cells. The growth inhibitory actions of vitamin K have been ascribed to cdc25 inhibition and

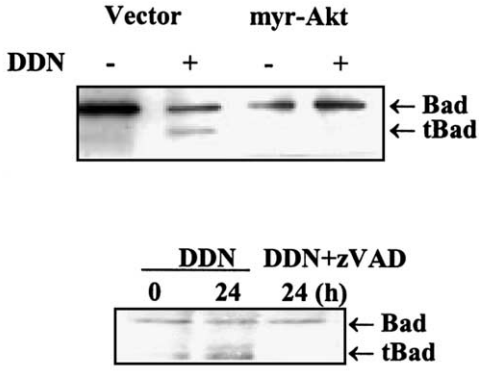

Fig. 5. DDN induces cleavage of Bad. HL-60 cells were treated with 1 μM DDN in the presence or absence of zVAD-fmk (50 μM) for 24 h. Cell lysates were separated by SDS–PAGE and subjected to Western blot analysis.

cell cycle arrest [2–4]. Previously, we have reported that DDN induces growth inhibition in various cancer cell lines and shows antitumor activity in vivo, and presented evidence that DDN induces apoptosis in HL-60 cells via regulating the expression of Bcl-2 family proteins [26]. In an effort to characterize the molecular mechanisms by which DDN induces apoptosis in HL-60 cells, we evaluated whether mitochondrial signaling is involved in the initiation of apoptosis. Mitochondria play a major role in the apoptotic process through the release of cytochrome *c* and other proapoptotic proteins that normally reside in the intermembrane space between the inner and outer mitochondrial membrane [7,8,10]. It is known that the release of cytochrome *c* from mitochondria is an important process during apoptosis induced by chemotherapeutic agents, and several proapoptotic and antiapoptotic proteins are involved the upstream and downstream of mitochondria. Cytochrome *c* is released into cytosol following the induction of apoptosis by many different stimuli including CD95, tumor necrosis factor, and chemotherapeutic agents [7,9,21]. In death ligand-induced apoptosis, activation of caspase-8 cleaves a cytosolic substrate Bid, leading to release of cytochrome *c*. Therefore, release of cytochrome *c* is secondary to caspase activation. However, in irradiation or chemicals such as staurosporine- and etoposide-induced apoptosis, cytochrome *c* release appears to be caspase-independent as it is not inhibited by the broad spectrum cell-permeable caspase inhibitor, zVAD-fmk [27,28]. In this study, cytochrome *c* release induced by DDN was not inhibited by zVAD-fmk, but the Bid cleavage was blocked by the caspase inhibitor. These results suggest that Bid cleavage occurs downstream of mitochondrial cytochrome *c* release, and caspase-8 can be activated downstream of mitochondria in DDN-treated cells, suggesting that the release of cytochrome *c* is an early event in DDN-induced apoptosis. However, the possibility that cleavage of Bid through the caspase pathway independent of mitochondria may not be excluded. We also demonstrated Bax translocation from the cytosol to the mitochondria, which was accompanied by cytochrome *c* release. Previous studies have shown that ectopic expression of Bax induces mitochondria to release cytochrome *c*, caspase activation, and apoptosis [11,13]. It is also reported that Bax translocates from cytoplasmic location to the mitochondria upon apoptosis induction [11]. Therefore, our findings indicate that subcellular redistribution of Bax, which was associated with cytochrome *c* release, is an important process in DDN-induced apoptosis.

Akt is known to be an important survival factor in signal transduction pathways involved in cell growth, and considered to be a possible target for cancer therapy [16,19,21,29]. Akt inhibits apoptosis by inactivating proapoptotic proteins such as Bad, procaspase-9, and forkhead and by activating antiapoptotic proteins such as NF-κB and cyclic adenosine monophosphate (cAMP)-response element binding protein [29]. The present data show that DDN inhibits the activation of Akt detected by decreasing levels of phosphorylation, thereby attenuating the activity of antiapoptotic pathway. Consequently, overexpression of constitutively active Akt protects cells from DDN-induced apoptosis. Akt is found to influence the mitochondrial death signaling because the overexpression of Akt caused inhibition of cytochrome *c* release induced by DDN. It was shown that active Akt inhibits ultraviolet (UV)- and etoposide-induced cytochrome *c* release in Rat1 fibroblast [23], and staurosporine-induced Bax translocation to mitochondria in HeLa cells [30]. These data indicate that inhibition of Akt is an important mechanism for mitochondrial steps of death signaling induced by various apoptotic stimuli. The molecular mechanisms by which Akt inhibits the Bax translocation and cytochrome *c* release are not well understood. Recent studies have shown that Bad is cleaved by a caspase(s) during apoptosis induced by TNF-α, CD95, TRAIL or interleukin (IL)-3 deprivation [24,25]. The truncated Bad has shown higher affinity for Bcl-xL, and it is a potent inducer of cytochrome *c* release [24]. This implies that deprivation of survival factors induces cleavage of Bad, resulting in dissociation of Bad from 14-3-3 proteins and translocation to the mitochondria, where Bad interacts with Bcl-xL and Bcl-2 and antagonizes their antiapoptotic functions [24]. In this study, we observed that DDN induced cleavage of Bad that is inhibited by caspase inhibitor and Akt overexpression. It is not clear whether there is a direct link between cleavage of Bad and cytochrome *c* release in DDN-induced apoptosis, because we found that cleavage of Bad by DDN was caspase-dependent, while cytochrome *c* release was caspase-independent. Therefore, it is suggested that there is an alternative mechanism for Akt intervening cytochrome *c* release in DDN-induced apoptosis.

In conclusion, DDN-induced apoptosis in HL-60 cells is associated with mitochondrial signaling which involves trans-

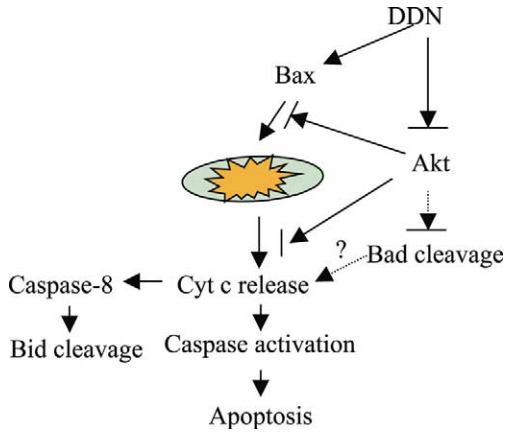

Fig. 6. Schematic illustration for the potential mechanisms involved in the DDN-induced apoptotic pathway.

location of Bax, followed by cytochrome *c* release (Fig. 6). DDN induces apoptosis through the activation of caspase-3, -9, -8 downstream of mitochondria, and through the inhibition of upstream signaling Akt, hence leading to inhibition of survival signals, in addition to increasing death signals.

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