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Induction of the Intrinsic Apoptotic Pathway by 3-Deazaadenosine Is Mediated by BAX Activation in HL-60 Cells

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3-Deazaadenosine (DZA), a potent inhibitor of *S*-adenosylhomocysteine hydrolase, was previously proposed to induce intrinsic apoptosis in human leukemic cells. In the present study, we analyzed the mechanism underlying the DZA-induced intrinsic apoptotic pathway. DZA activated typical caspase-dependent apoptosis in HL-60 cells, as demonstrated by an accumulation of hypo-diploidic cells, the processing of multiple procaspases and an inhibitory effect of z-VAD-Fmk on this cell death. During DZA-induced apoptosis, cytochrome c (cyt c) was released into the cytosol. This was neither prevented by z-VAD-Fmk and nor was it associated with the dissipation of mitochondrial membrane potential ($\Delta\Psi_m$). Prior to the release of cyt c, BAX was translocated from the cytosol to mitochondria and underwent oligomerization. Finally, the overexpression of BCL-XL protected HL-60 cells from apoptosis by blocking both the cyt c release and BAX oligomerization. Collectively, these findings suggest that DZA may activate intrinsic apoptosis by stimulating BAX activation and thereby the release of cyt c.

Key Words: Apoptosis, BAX protein, BCL-XL protein, Cytochrome c, 3-Deazaadenosine

INTRODUCTION

It was known that 3-deazaadenosine (DZA), an analog of adenosine, is an inhibitor of *S*-adenosylhomocysteine (Ado-Hcy) hydrolase (EC3.3.1.1) [1]. The inhibition of Ado-Hcy hydrolase by DZA leads to the accumulation of both Ado-Hcy and S-adenosylmethionine (Ado-Met), a co-factor for methyl transfer reactions, reflecting the perturbation of trans-methylation reactions. Because DZA is also able to serve as a substrate for Ado-Hcy hydrolase, the interaction between DZA and Ado-Hcy hydrolase results in the accumulation of 3-deazaadenosylhomocysteine (DZA-Hcy) in cultured cells [2] and the liver [3]. It is also well known that DZA exerts a wide variety of biological functions such as anti-human immunodeficiency virus (HIV) activity [4], anti-inflammatory effects [5], alterations in the expression of genes like collagen IV and ICAM-1 [6,7], the inhibition of TNF-α and IL-1β expression [8], and an inhibitory effect on nuclear NF-κB transcriptional activity [9]. In addition, DZA has been reported to induce apoptosis in human and murine leukemic cell lines such as HL-60, U937 and L1210 cells [10-12].

In a previous study, we reported that the apoptotic effect of DZA on human leukemic cells involves a process where DZA enters leukemic cells through adenosine transporters and subsequently induces caspase-dependent apoptosis [11]. DZA-induced apoptosis was found to be dependent on neither *de novo* protein synthesis nor the inhibition of Ado-Hcy hydrolase. Furthermore, it was shown that DZA stimulates the release of cytochrome c (cyt c) from mitochondria in human leukemic U937 cells to induce the intrinsic apoptotic pathway [13]. However, the mechanism underlying DZA-induced intrinsic apoptosis is currently unclear. In this study, we addressed this issue by conducting an analysis of the effect of DZA on mitochondrial permeability transition, BAX activation as well as the effects of caspase inhibitors and BCL-XL on DZA-induced apoptosis in human leukemic HL-60 cells.

METHODS

Chemicals

DZA and z-VAD-Fmk were purchased from Sigma-Aldrich (St Louis, MO) and Tocris Bioscience (Ellisville, MO), respectively. Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich and were of molecular biology grade.

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ABBREVIATIONS: Ado-Hcy, *S*-adenosylhomocysteine; Ado-Met, *S*-adenosylmethionine; BH, BCL-2 homology; cyt c, cytochrome c; DZA, 3-deazaadenosine; MMP, mitochondrial membrane potential; MOMP, mitochondrial outer membrane permeabilization.

Cell culture

HL-60 cells from the American Type Culture Collection (ATCC, Manassas, VA) were maintained in RPMI 1640 media supplemented with 20 mM HEPES (pH 7.4), 25 mM sodium bicarbonate, 50 μg/ml gentamicin and 10% heat-inactivated FBS (Hyclone Laboratories Inc., Logan, UT).

Apoptosis assay

HL-60 cells treated as described in the figure legends, were fixed in ice-cold 70% ethanol and stained with propidium iodide (PI, 0.2 mg/ml) followed by flow cytometric analysis (FACSCalibur, BD bioscience, San Jose, CA) as previously reported [14]. Cell cycle distribution was analyzed using Cell Quest and Modfit software (BD bioscience).

Immunoblot analysis

Immunoblot analysis was performed as previously reported [15]. Briefly, HL-60 cells treated as indicated, were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% Na-deoxycholate, 1% NP-40, 1 mM EDTA) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Equal amount of proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA). After hybridization with primary and secondary antibodies, the proteins were detected by enhanced chemiluminescence reagents (GE Healthcare, UK). Mouse antibodies against caspase-3 and cyt c were purchased from BD Bioscience. Rabbit antibodies against caspase-9 and caspase-8 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and LABVISION Co. (Fremont, CA), respectively. Mouse anti-porin was obtained from Calbiochem (San Diego, CA).

Assays of cyt c release and BAX oligomerization

To detect the release of cyt c from mitochondria by DZA, immunoblot analyses of the cytosolic and mitochondrial fractions were performed as described in a previous report [16]. A MitoProbe^{TM} JC-1 Assay Kit (Invitrogen, Carlsbad, CA) was used for MMP assays, following the manufacturer's instructions. CCCP (carbonyl cyanide m-chlorophenylhydrazone), a mitochondrial uncoupling reagent which induces the dissipation of MMP, was used as a positive control to verify that JC-1 reacts properly to MMP change. BAX oligomerization was analyzed as previously reported [17,18] with minor modifications. Briefly, purified mitochondria from HL-60 cells were incubated in resuspension buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1% CHAPS) containing 2 mM bismaleidohexane (BMH, Thermo Scientific, Rockford, IL) for 30 min at room temperature. The mitochondria were pelleted and dissolved in 1XSDS/PAGE sample buffer for immunoblot analysis.

Overexpression of BCL-XL

To express BCL-XL in HL-60 cells, human BCL-XL expressing plasmid, pcDNA3.1-/BCL-XL was electro-transfected into HL-60 cells with microporator (Digital-Bio,

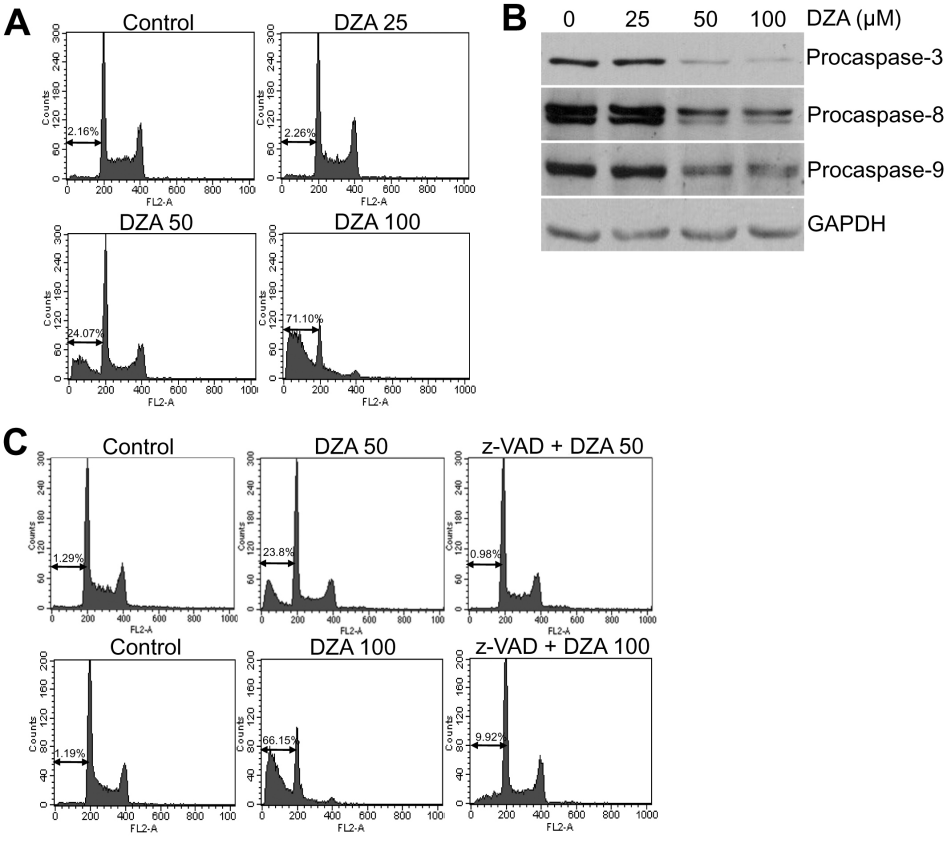

Fig. 1. Induction of caspase-dependent apoptosis in HL-60 cells by DZA. (A) HL-60 cells treated with the indicated micromolar concentrations of DZA for 6 h were fixed, stained with PI and then analyzed by flow cytometry as described in METHODS. (B) HL-60 cells treated the same as described in (A) were subjected to immunoblot analysis against the indicated procaspases. GAPDH was used for equal loading of proteins. (C) After incubating HL-60 cells in the absence or presence of 30 μM z-VAD-Fmk for 1 h, they were treated with the indicated micromolar concentrations of DZA for 6 h further. The cells then were subjected to PI staining and flow cytometric analysis. The figures are representative of three independent experiments.

Suwon, Korea) using the following condition 1270 V, 30 milliseconds and 1 pulse.

RESULTS

DZA activates caspase-dependent apoptosis in HL-60 cells

When HL-60 cells were treated with DZA, cells in the sub-G0/G1 phase, indicating cells with fragmented genomic DNA, accumulated in a dose-dependent manner (Fig. 1A). Consistent with the report [10], DNA fragmentation was observed above 50 μM DZA. Electron microscopic examination indicated that DZA-treated HL-60 cells showed the hallmarks of apoptosis including nuclear fragmentation and apoptotic body formation (data not shown). Parallel to DNA fragmentation, the activation of multiple caspases including initiator caspases such as caspase-8, -9 and effector caspase-3 occurred (Fig. 1B) and z-VAD-Fmk, a pan-caspase inhibitor, almost completely prevented the fragmentation of DNA (Fig. 1C). These findings demonstrate that DZA induces typical caspase-dependent apoptosis in HL-60 cells.

DZA induces cyt c release through PT-independent MOMP

Since it has been reported that cyt c is released by DZA in U937 cells [13] and initiator caspase-9 was activated as shown in Fig. 1B, we checked the release of cyt c. As shown in Fig. 2A, cyt c was released from mitochondria to the cytosol by DZA treatment in a dose-dependent manner. This cyt c release was not prevented by z-VAD-Fmk which completely inhibited apoptosis (Fig. 2B). In addition, neither the dissipation of mitochondrial membrane potential (MMP, $\Delta\Psi_m$) nor mitochondrial permeability transition

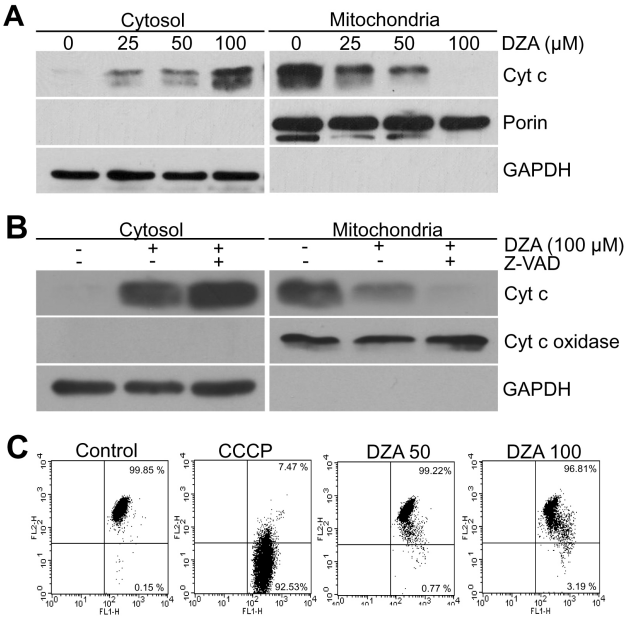

Fig. 2. DZA-induced cyt c release in a PT- and caspase-independent manner. (A) Cytosolic and mitochondrial fractions of HL-60 cells treated with the indicated concentrations of DZA for 6 h were subjected to immunoblot analysis against cyt c. Porin and GAPDH were used for the equal loading of mitochondrial and cytosolic proteins, respectively. (B) HL-60 cells incubated in the absence or presence of 30 μM z-VAD-Fmk for 1 h were treated with vehicle or 100 μM DZA. Six hours later, cytosolic and mitochondrial fractions were prepared and subjected to immunoblot analysis against cyt c. Cyt c oxidase and GAPDH were used for equal loading of mitochondrial and cytosolic proteins, respectively. (C) HL-60 cells treated with vehicle, the indicated micromolar concentrations of DZA for 6 h or 50 μM CCCP for 5 min were incubated with 2 μM JC-1. After 15 min, cells were harvested, washed and analyzed on FACSCalibur with emission filters of FL1 and FL2 for green and red fluorescences, respectively. CCCP was used to confirm that JC-1 adequately reacted to MMP dissipation. The results are representative of three independent experiments.

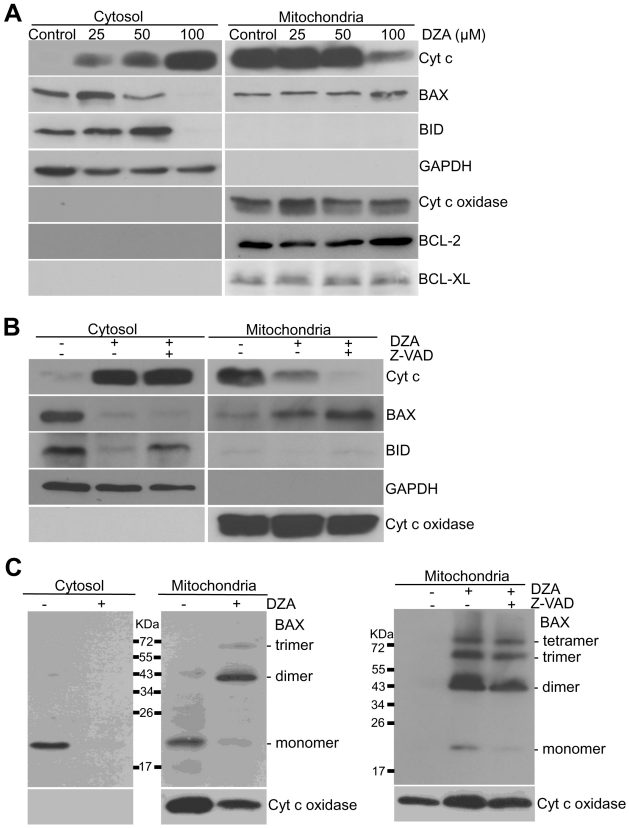

Fig. 3. DZA-induced BAX activation in HL-60 cells. (A) Subcellular fractions of HL-60 cells treated with indicated concentrations of DZA were prepared and subjected to immunoblot analysis against the indicated proteins. GAPDH and cyt c oxidase were used for equal loading of cytosolic and mitochondrial proteins, respectively. (B) Cytosolic and mitochondrial fractions of HL-60 cells incubated with vehicle or 30 μM z-VAD-Fmk for 1 h and then 100 μM DZA for 6 h further were prepared and subjected to immunoblot analysis. Cyt c oxidase and GAPDH were used for equal loading of mitochondrial and cytosolic fractions, respectively. (C) Left, HL-60 cells were treated with vehicle or 100 μM DZA. Right, HL-60 cells incubated in the absence or presence of 30 μM z-VAD-Fmk for 1 h were treated with DZA. After 6 h, cytosolic and mitochondrial fractions were incubated in the presence of 2 mM BMH as described in METHODS. Each fraction was subjected to immunoblot analysis against BAX and cyt c oxidase. The results are representative of three independent experiments.

pore (MPTP) formation was found to be associated with cyt c (Fig. 2C, data not shown). These findings suggest that DZA-induced cyt c release is upstream of caspase activation and may occur through an alteration in the mitochondrial outer membrane (MOM) without the involvement of the mitochondrial inner membrane (MIM), i.e. permeability transition (PT)-independent MOM permeabilization (MOMP).

BAX is activated during DZA-induced apoptosis

Caspase- and PT-independent MOMP is known to be induced mainly by BAX and BAK, BH123 BCL-2 family proteins [19,20]. To determine whether BAX is activated in this apoptosis, we checked the translocation of BAX. In accordance with the release of cyt c, BAX appeared to be translocated to mitochondria as the result of DZA treatment (Fig. 3A). In the case of pretreatment with z-VAD-Fmk, the mitochondrial translocation of BAX was not changed but, rather was accentuated (Fig. 3B), indicating that BAX translocation is upstream or independent of caspase activation. We, next, checked whether BAX in mitochondria was oligomerized to observe its real activation. As shown in Fig. 3C, the BAX that was translocated to mitochondria was consistently oligomerized. This oligomerization was not prevented by pretreatment with z-VAD-Fmk, indicating that mitochondrial BAX is activated without any involvement by caspase.

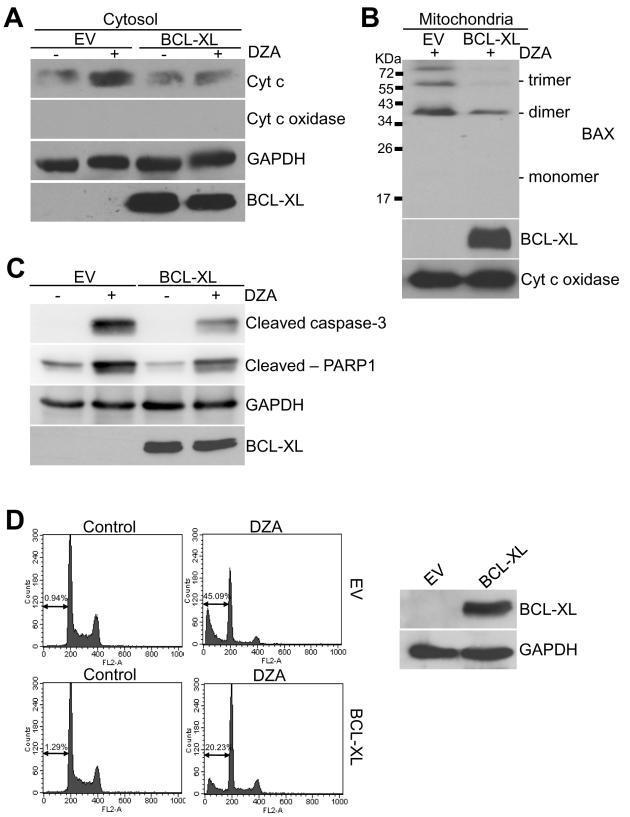

Fig. 4. Inhibition of DZA-induced apoptosis by BCL-XL. (A) HL-60 cells transiently transfected with empty pcDNA3.1(−) vector (EV) or plasmid expressing BCL-XL (pcDNA3.1-/BCL-XL) for 48 h were treated with vehicle or 100 μM DZA. After 6 h, cytosolic fractions were subjected to immunoblot analysis against cyt c. GAPDH were used for equal loading of cytosolic proteins. (B) HL-60 cells transfected the same as in experiment (A) were treated with vehicle or 100 μM DZA for 6 h. Mitochondrial fractions were then treated with 2 mM BMH as described in METHODS and subjected to immunoblot analysis against BAX. Cyt c oxidase was used for equal loading of mitochondrial proteins. (C) HL-60 cells transfected as indicated were treated with vehicle or 100 μM DZA for 6 h and subjected to immunoblot analysis. GAPDH were used for equal loading of cell lysates. (D) HL-60 cells transiently transfected as indicated were treated with vehicle or 100 μM DZA for 6 h. Cells were then fixed and subjected to PI staining and flow cytometric analysis. The figures are representative of three independent experiments.

DZA-induced cyt c release and apoptosis is prevented by BCL-XL

Finally, we examined the effect of BCL-XL, an inhibitor of BAX activation, on DZA-induced cyt c release and apoptosis. When HL-60 cells were transfected with BCL-XL expressing plasmid, pcDNA3.1-/BCL-XL and then treated with DZA, cyt c release and BAX oligomerization were significantly reduced compared to vector-control cells (Fig. 4A, 4B). Accordingly, the activation of caspase-3 as demonstrated by the presence of active caspase-3 and cleaved poly ADP ribose polymerase (PARP)-1 (Fig. 4C) and thus, cell death (Fig. 4D) were significantly less in HL-60 cells overexpressing BCL-XL than in vector-control cells. This observation suggests that DZA induces the release of cyt c and apoptosis by BAX activation.

DISCUSSION

3-Deazaadenosine, an inhibitor of Ado-Hcy hydrolase, has been reported to induce apoptosis in human leukemic cells. Although it has been suggested that the intrinsic apoptotic pathway is implicated in this apoptosis, the mechanism underlying its activation in DZA-induced apoptosis remains still to be clarified. Here, we attempted to address this issue in HL-60 cells.

The findings presented herein indicate that DZA sequentially induced BAX activation, cyt c release, caspase activation and apoptosis. The DZA-induced release of cyt c release to the cytosol was not associated with the collapse of MMP ($\Delta\Psi_m$) or MPTP formation (Fig. 2C, data not shown), suggesting that MIM may not be altered but cyt c release may occur via changes in MOM. Since BAX and BAK are known to play a critical role in inducing PT-independent cyt c release [19,20] and BCL-XL inhibits DZA-induced BAX activation, the release of cyt c and the resulting apoptosis (Fig. 4), it can be concluded that DZA may stimulate the activation of BAX to induce cyt c release in a MMP-independent manner, thus initiating the intrinsic apoptotic pathway. This conclusion, therefore, leads to the notion that the mechanism of BAX activation may be a pivotal step in DZA-induced apoptosis in HL-60 cells.

BAX and BAK can interact with antiapoptotic BCL-2 family proteins such as BCL-2, BCL-XL, MCL-1 and BCL-W as well as pro-apoptotic BH3-only proteins such as BIM, BID and PUMA [21]. In unstressed cells, BAX and BAK are maintained in an inactive state by binding to antiapoptotic BCL-2 family proteins such as BCL-2 and BCL-XL or due to a loss of interaction with BH3-only proteins such as BID, BIM and PUMA to keep cells alive. As a result, BAX can be activated by a decrease in the levels of

antiapoptotic BCL-2 family proteins or by interactions with BH3-only proteins. Although how and why DZA induces BAX activation is not clarified here, it appears that BID is not involved, because BID is processed after the translocation of BAX (Fig. 3A, 3B). BIM, another activator of BAX as well as BCL-2 and BCL-XL were not altered by DZA at either the transcriptional or post-translation levels (Fig. 3A, data not shown). It can therefore be assumed that DZA may activate BAX via BH3-only proteins other than BID and BIM or through a decrease in antiapoptotic BCL-2 family proteins, except BCL-2 and BCL-XL. However, given the reports that mild heat or pH alteration can directly activate BAX and BAK [17,22], the direct role of DZA in BAX activation should be also feasible.

Although DZA is a structural analog of adenosine and functions as both an inhibitor and a substrate for Ado-Hcy hydrolase, the role of Ado-Hcy hydrolase in DZA-induced apoptosis has been regarded with skepticism. Findings reported in our previous study indicated that Ado-Hcy hydrolase is not involved in HL-60 apoptosis by DZA because other Ado-Hcy inhibitors did not activate apoptosis and did not affect DZA-induced apoptosis [11]. Ado-Met and Hcy were reported to induce apoptosis in human colon cancer and HL-60 cells, respectively [23,24] but, their effective concentrations were above 0.5 mM, which may be such a high level that can be hardly reached by inhibiting Ado-Hcy hydrolase in cells. Thus, it is possible that the apoptosis-inducing activity of DZA may not be related to the inhibition of Ado-Hcy hydrolase but its other characteristics. Although it was reported that adenosine has a cytoprotective effect in glial cells [25], adenosine derivatives such as 2-chlorodeoxyadenosine (cladribine) and 2-chloro-2'-arabinofluoro-2'-deoxyadenosine (clofarabine) as well as adenosine itself have apoptosis-inducing effects on various cancer cell lines. Cladribine and clofarabine are converted in a stepwise manner into their triphosphate active forms which act as anticancer agents through binding to important proteins in various intracellular organelles such as nuclei and mitochondria [26]. For example, the triphosphate form of clofarabine can bind to DNA polymerases to inhibit DNA synthesis and APAF-1 to induce caspase-9 activation [27,28]. Likewise, Prus et al. [29] reported that DZA is phosphorylated to DZA-5'-triphosphate which is not involved in functions induced by Ado-Hcy hydrolase inhibition, suggesting that the functions of DZA irrelevant of Ado-Hcy hydrolase would be exerted by DZA-5'-triphosphate. As evidenced by an *in vitro* cyt c release assay, while DZA did not induce cyt c release from mitochondria in the absence or presence of intact cytosol, cytosol from HL-60 cells that had been treated with DZA clearly induced cyt c release (data not shown), implying that DZA must be metabolized to induce cyt c release. It can therefore be speculated that the structural features of DZA similar to adenosine may be important in inducing intrinsic apoptotic pathway and metabolites of DZA such as DZA-5'-triphosphate might induce cyt c release.

In HL-60 cells that transiently or stably overexpress BCL-XL, apoptosis by DZA was not completely prevented and no more than 60% inhibition was found (Fig. 4, data not shown). Since BCL-XL prevents the activation of the intrinsic apoptotic pathway, this incomplete inhibition could indicate that DZA-induced apoptosis is not totally dependent on the intrinsic apoptotic pathway and the presence of other apoptotic mechanisms which are not prevented by BCL-XL. Although its role and location in the DZA-induced apoptotic pathway was not determined here, caspase-8 which initiates extrinsic apoptosis, was also activated (Fig. 1B), implying the involvement of the extrinsic apoptotic pathway. Like the triphosphate forms of clofarabine and cladribine [28], DZA-5'-triphosphate might bind to APAF-1 to activate caspase-9 and apoptosis could then occur even when cyt c release is blocked. It is also possible that DZA could damage mitochondria via pathways other than the BAX- and BCL-XL-related mechanisms and cells with damaged mitochondria would eventually succumb due to other reasons such as metabolic failure. Thus, DZA-induced cell death, which was not inhibited by BCL-XL might be caused by various pathways that are different from intrinsic apoptosis, and genetically solid methods to knock out each gene involved in apoptosis should be employed to dissect the molecular mechanism of DZA-induced apoptosis, and determine the role of intrinsic apoptotic pathway in this apoptosis in the future. Taken together, it can be inferred that metabolites of DZA such as DZA-5'-triphosphate may initiate the signal transduction pathway leading to BAX activation and possibly other apoptotic pathways that remain currently unidentified, depending on the nature of the interactions between DZA metabolites and cellular proteins.

The mechanism by which BAX and BAK are activated has been the focus of intensive research and the target of anticancer therapeutics as well. BH3-mimetic agents, including ABT-263 (Abbot Inc, Abbott Park, IL), that finally lead to BAX and BAK activation and apoptosis have been developed and are under clinical trials [30]. Structural analogs of adenosine such as clofarabine and cladribine have also been tested in applications to anticancer treatment [26, 31]. Based on these facts, DZA might have the potential to be developed as an anti-leukemic therapeutic as well as for apoptosis research. To achieve this, the molecular mechanism involved in DZA-induced BAX activation and other apoptotic mechanisms need to be elucidated in detail in the future.

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