

# Melanoma Differentiation Associated Gene-7/Interleukin-24 Potently Induces Apoptosis in Human Myeloid Leukemia Cells through a Process Regulated by Endoplasmic Reticulum Stress

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## ABSTRACT

Melanoma differentiation associated gene-7 (*mda-7*)/interleukin-24 (IL-24), a member of the IL-10 cytokine gene family, preferentially induces cell death in neoplastic epithelial cells types while sparing their normal counterparts. The effects of *mda-7*/IL-24 in acute myeloid leukemia (AML) cells have not been extensively characterized. Treatment with recombinant GST-MDA-7/IL-24 potently induced apoptosis in diverse myeloid leukemia cell types including U937, HL60, MV4-11, EOL-1, and MLL/ENL cells. MDA-7/IL-24 also markedly induced apoptosis in and suppressed the colony-forming capacity of primary AML blasts but exerted minimal toxicity toward normal CD34<sup>+</sup> hematopoietic progenitor cells. MDA-7/IL-24 lethality was associated with pronounced endoplasmic reticulum (ER) stress induction in leukemia cell lines and primary AML blasts, manifested by the accumulation of growth arrest and DNA damage-inducible protein 34 (GADD34), 78-kDa glucose-regulated protein (GRP78)/BiP, inositol-requiring

enzyme 1 $\alpha$  (IRE1 $\alpha$ ), and eukaryotic initiation factor 2 $\alpha$  phosphorylation. It is noteworthy that short hairpin RNA (shRNA) knockdown of IRE1 $\alpha$ , GADD34, or GRP78/BiP significantly enhanced MDA-7/IL-24-mediated apoptosis, indicating a protective role for these molecules against MDA-7/IL-24 lethality. MDA-7/IL-24 also down-regulated the antiapoptotic protein Mcl-1 and sharply increased expression of the proapoptotic proteins Bim and Noxa. Ectopic Mcl-1 expression or shRNA knockdown of Bim or Noxa significantly attenuated MDA-7/IL-24-mediated leukemia cell death. Finally, knockdown of Bax or Bak significantly reduced MDA-7/IL-24 lethality. Together, these findings indicate that MDA-7/IL-24 potently induces apoptosis in human myeloid leukemia cells through a process regulated by ER stress induction, Mcl-1 down-regulation, and Bim and Noxa up-regulation. They also suggest that MDA-7/IL-24 warrants further investigation in myeloid leukemia.

## Introduction

Melanoma differentiation associated gene-7/interleukin-24 (MDA-7/IL-24), a cytokine classified as a member of the IL-10 family, was initially identified through a subtraction hybridization approach combined with a differentiation therapy

model of human melanoma as a gene up-regulated during terminal differentiation (Jiang et al., 1995). MDA-7/IL-24 potently inhibits cell growth and induces apoptosis in various epithelial cancers in vitro and in vivo, including melanoma, glioma, breast, colorectal, lung, ovarian, and prostate cancers, among others (Fisher, 2005). In contrast to its effects on transformed cells, MDA-7/IL-24 has shown only minimal lethality toward normal cells (Gupta et al., 2006a). *mda-7*/IL-24 is currently undergoing clinical evaluation (phase I) in patients with melanoma and other advanced solid tumors and has shown significant preliminary evidence of activity.

The ability of MDA-7/IL-24 to inhibit cell growth of and to induce apoptosis in tumor cells has been attributed to mul-

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**ABBREVIATIONS:** MDA-7/IL-24, melanoma differentiation associated gene-7/interleukin-24; GST, glutathione transferase; L-CFU, leukemic colony-forming units; GADD, growth arrest and DNA damage-inducible protein; IRE1 $\alpha$ , inositol-requiring enzyme 1 $\alpha$ ; eIF2 $\alpha$ , eukaryotic initiation factor 2 $\alpha$ ; PARP, poly(ADP-ribose) polymerase; UPR, unfolded protein response; MLL-ENL, eleven-nineteen-leukemia; GRP78, 78-kDa glucose-regulated protein; AML, acute myeloid leukemia; ER, endoplasmic reticulum; shRNA, short hairpin RNA; AIF, apoptosis-inducing factor; CFU-GM, myeloid colony-forming units; GFP, green fluorescent protein; PI, propidium iodide; FAB, French-American-British classification.

multiple mechanisms including perturbations in signaling pathways (e.g., p38 mitogen-activated protein kinase and c-Jun NH<sub>2</sub>-terminal kinase), antiangiogenic actions, immune modulation, bystander antitumor activity, autocrine regulation, induction of oxidative damage, and modulation of the expression of pro- and antiapoptotic Bcl-2 family members (Fisher, 2005; Dash et al., 2010a). Recent attention has focused on the capacity of MDA-7/IL-24 to initiate endoplasmic reticulum stress (ER stress) in transformed cells. In response to diverse ER stress inducers, cells trigger a well orchestrated evolutionarily conserved response referred to as the unfolded protein response (UPR). This process involves inhibition of protein synthesis, induction of protein chaperones, and enhanced protein degradation (ER-assisted degradation). Initially, the UPR serves as an adaptive/cytoprotective response permitting cells to clear the mass of infolded or misfolded protein accumulated within the ER. However, if the stress within the ER becomes too severe, the UPR promotes cell death (Scorrano et al., 2003; Bernales et al., 2006; Ron and Walter, 2007). In this context, our group has recently reported that MDA-7/IL-24 localizes to the ER compartment, physically binds to the ER resident chaperone protein GRP78/BiP, and triggers ER stress, manifested by the induction of DNA damage-inducible genes such as GADD153, GADD45 $\alpha$ , and GADD34, calcium release into the cytosol, PKR-like endoplasmic reticulum kinase activation, eIF2 $\alpha$  phosphorylation, and protein translation inhibition (Sarkar et al., 2002; Gupta et al., 2006b; Park et al., 2008). In addition, MDA-7/IL-24 lethality is regulated by members of the Bcl-2 family. For example, the antiapoptotic protein Mcl-1, which is known to protect cells from ER stress-induced cell death (Fritsch et al., 2007; Rahmani et al., 2007a), markedly attenuated *mda-7/IL-24* lethality in prostate cancer cells (Dash et al., 2010b). Together, these findings suggest a role for ER stress in the regulation of MDA-7/IL-24-mediated cytotoxicity toward transformed cells.

Although MDA-7/IL-24 has been extensively studied in epithelial tumors, the therapeutic potential of this molecule has been minimally explored in a limited number of hematologic malignancies, such as chronic lymphocytic leukemia (Sainz-Perez et al., 2006) or chronic myeloid leukemia (Dong et al., 2008). As a consequence, very little is known about the activity or mechanism of action of this molecule in acute myeloid leukemia (AML) cells. The results of this study indicate that MDA-7/IL-24 potently induces apoptosis in diverse human myeloid leukemia cell types, including primary leukemic blasts, but is relatively sparing toward normal CD34<sup>+</sup> hematopoietic cells. Moreover, the present findings provide the first evidence of a functional role for components of the ER stress machinery in the regulation of MDA-7/IL-24 lethality toward AML cells.

## Materials and Methods

**Cells and Constructs.** Human leukemia U937 (myelomonocytic), HL60 (promyelocytic), MV4-11, EOL1 (both FLT3-mutated), and MLL-ENL (leukemia stem cell-like) (Barabé et al., 2007) cells were cultured as reported previously (Rahmani et al., 2003). U937 cells overexpressing Mcl-1 were described previously. U937 cells in which GADD34 and GRP78/BiP were knocked down were obtained by infecting U937 cells with lentiviruses carrying specific short hairpin RNA (shRNA) constructs (Santa Cruz Biotechnology, Santa Cruz, CA). Stable clones from a single cell were selected in the presence of puromycin (1  $\mu$ g/ml) and tested for the expression of the corresponding proteins. U937 cells

stably expressing shRNA directed against Bax or Bak were described previously (Rahmani et al., 2009). Knockdown of Bim or Noxa was obtained by transfecting U937 cells with microRNA-adapted shRNA constructs specifically designed against human Bim or Noxa (Open Biosystems, Huntsville, AL). The sequences used were Bim, 5'-GCAG-GCTGAACCTGCAGATAT-3', and Noxa, 5'-ACACATACTTACATACT-TATA-3'. U937 cells transfected with shRNA constructs against GFP were used as controls for various shRNA-expressing cells.

**Isolation of Patient-Derived Leukemic Blasts.** Leukemic blasts were obtained with informed consent from the bone marrow of patients with relapsed AML, FAB subtype M2. These studies have been sanctioned by the Investigational Review Board of Virginia Commonwealth University/Medical College of Virginia, and all patients provided informed consent. In each case, the percentage of blasts in the peripheral blood was >70%. Bone marrow was collected into heparinized syringes, and mononuclear cells were isolated as described previously (Rahmani et al., 2005).

**Isolation of CD34<sup>+</sup> Cells.** Normal CD34<sup>+</sup> cells were obtained with informed consent from the bone marrow of patients undergoing routine diagnostic procedures for nonmyeloid hematopoietic disorders (e.g., thrombocytopenia, iron-deficiency anemia). CD34<sup>+</sup> cells were isolated from mononuclear cell preparations using an immunomagnetic bead separation technique (Miltenyi Biotec Inc., Auburn, CA) as described previously (Rahmani et al., 2007b). In some experiments, normal CD34<sup>+</sup> cells were isolated from cord blood and processed as above. CD34<sup>+</sup> cells were resuspended in RPMI medium containing 10% fetal calf serum and exposed to agents exactly as described above for human leukemia cells.

**Reagents.** GST-MDA-7/IL-24 was generated as described in detail previously (Su et al., 2003).

**Assessment of Apoptosis.** Apoptotic cells were routinely identified by Annexin V-fluorescein isothiocyanate staining as described previously (Rahmani et al., 2002).

**Leukemic and Normal Hematopoietic Colony Formation Assays.** The colony-forming capacity of leukemic (L-CFU) and normal CD34<sup>+</sup> cells (CFU-GM) was monitored by methylcellulose colony-forming assays as we have described recently in detail (Rahmani et al., 2009).

**Western Blot Analysis.** Western blot analysis was performed using the whole-cell lysates as described previously in detail (Rahmani et al., 2002). The primary antibodies used in this study were caspase-3, Bax, Bcl-2, and Mcl-1 (BD Pharmingen, San Diego, CA); caspase-8 and Noxa (Alexis Corp, San Diego, CA); caspase-4 (Stressgene Bioreagents, Ann Arbor, MI); poly(ADP-ribose) polymerase (PARP) (BIOMOL Research Laboratories, Plymouth Meeting, PA); IRE1 $\alpha$ , phospho-eIF2 $\alpha$ , extracellular signal-regulated kinase 1/2, Bcl-xL, and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA). GADD34, GRP78/BiP, AIF, cytochrome *c* (Santa Cruz Biotechnology, Santa Cruz, CA); and Bim, Bak, and  $\alpha$ -tubulin (Calbiochem, San Diego, CA).

**Subcellular Fractionation.** Leukemic cells ( $4 \times 10^6$ ) were lysed using digitonin buffer, after which cytosolic and membrane fractions were separated by centrifugation as described previously (Rahmani et al., 2005). The purity of cytosolic fraction was routinely confirmed by Western blot using antibodies against cytochrome *c* oxidase subunit IV, which resides strictly in the inner mitochondrial membrane.

**Statistical Analysis.** The significance of differences between experimental conditions was determined using the Student's *t* test for unpaired observations.

## Results

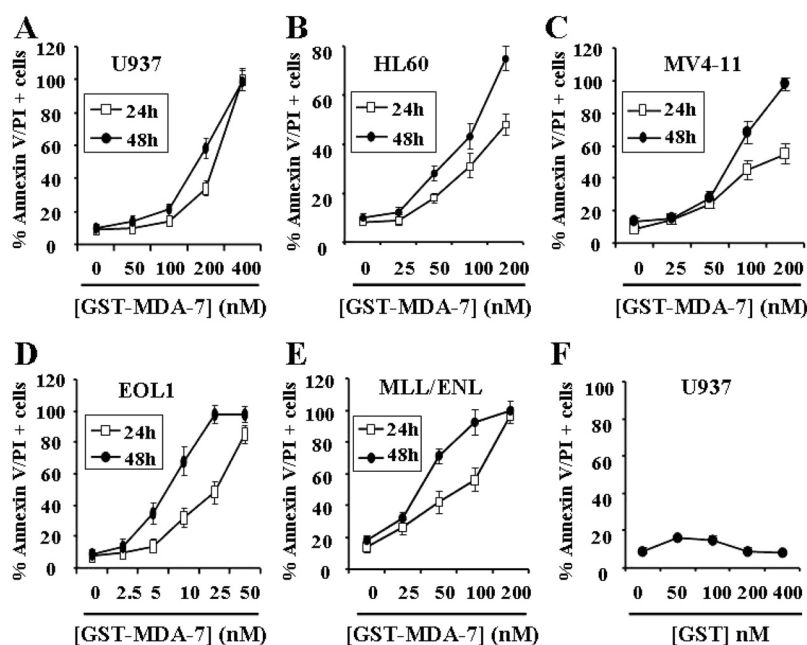
**GST-MDA-7/IL-24 Potently Induces Apoptosis in Association with Profound Mitochondrial Injury and Caspase Activation in Various Human Myeloid Leukemia Cell Lines and in Primary AML Cells.** Previous studies in various epithelial tumors have demonstrated that GST-MDA-7/IL-24 potently induces apoptosis in transformed cells but exerts only minimal toxicity toward normal cells

(Gupta et al., 2006a). To determine whether MDA-7/IL-24 triggered myeloid leukemia cell death, dose-response studies of GST-MDA-7/IL-24 were performed in various myeloid leukemia cell types, including U937 myelomonocytic, HL60 promyelocytic, MV4-11, and EOL1 FLT3-mutated AML and MLL-ENL-expressing cells. As shown in Fig. 1A, 24- to 48-h exposure to MDA-7/IL-24 induced pronounced cell death in U937 cells, reflected by Annexin V/PI positivity, when administered at concentrations  $\geq 200$  nM. It is noteworthy that MDA-7/IL-24 was even more potent in inducing apoptosis in HL60, MV4-11, and MLL/ENL cells, and particularly in EOL1 cells, in which only 5 to 10 nM concentrations were sufficient to induce extensive cell death after 48-h treatment (Fig. 1, B–E). It is noteworthy that GST protein alone had no effects on cell death in U937 cells (Fig. 1F) or the other cell lines (data not shown).

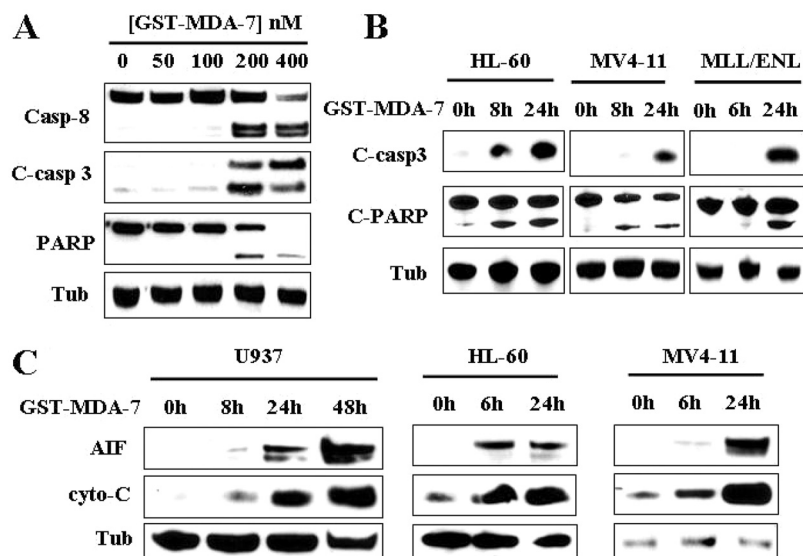
To confirm that MDA-7/IL-24 triggered apoptosis in AML cells, dose-response studies with GST-MDA-7/IL-24 were

performed to monitor caspase activation and mitochondrial injury in U937, HL60, MV4-11, and MLL-ENL cells. It is noteworthy that concentrations of GST-MDA-7/IL-24 that effectively induced cell death in U937 cells (e.g., 200 nM; 48 h) resulted in a pronounced increase in caspase-3/-8 activation and PARP cleavage (Fig. 2A). Cleavage of caspase-3 and PARP were also observed in HL60, MV4-11, and MLL-ENL-expressing cells (Fig. 2B). Significantly, these effects were associated with a pronounced increase in cytosolic release of cytochrome *c* and AIF as shown in U937, HL60, and MV4-11 cells (Fig. 2C). Together, these findings indicate that GST-MDA-7/IL-24 effectively induces mitochondrial injury and apoptosis in diverse myeloid leukemia cell types.

**GST-MDA-7/IL-24 Induces Pronounced Caspase Activation and a Marked Reduction of the Clonogenic Capacity of Primary AML Blasts but Is Relatively Sparing toward Normal CD34<sup>+</sup> Progenitor Cells.** To determine whether MDA-7/IL-24-mediated apoptosis is re-



**Fig. 1.** GST-MDA-7/IL-24 potently induces apoptosis in various human myeloid leukemia cells. U937 (A), HL60 (B), MV4-11 (C), EOL1 (D), and MLL/ENL (E) cells were exposed for 24 h (□) or 48 h (●) to the designated concentration of GST-MDA-7/IL-24, after which the percentage of apoptotic cells was determined by Annexin V/PI analysis as described under *Materials and Methods*. F, U937 cells were exposed to GST protein alone for 48 h, after which the extent of cell death was determined as above. Values represent the means for three separate experiments  $\pm$  S.D.



**Fig. 2.** GST-MDA-7/IL-24 induces profound mitochondrial injury and caspase activation in human myeloid leukemia cells. A, U937 cells were exposed to the designated concentration of GST-MDA-7/IL-24 for 48 h, after which protein lysates were prepared and subjected to Western blot analysis to monitor caspase-3, caspase-8 cleavage/activation, and PARP degradation. B, HL60, MV4-11, and MLL/ENL cells were exposed to GST-MDA-7/IL-24 (100 nM for HL60 and MV4-11, and 50 nM for MLL/ENL) for the designated intervals, after which Western blot assays were performed as in A. C, U937, HL60, and MV4-11 cells were treated with GST-MDA-7/IL-24 (200 nM for U937 cells and 100 nM for HL60 and MV4-11 cells) for the designated intervals, after which mitochondria-free cytosolic fractions were obtained as described under *Materials and Methods* and subjected to Western blot analysis to monitor the release of cytochrome *c* and AIF into the cytosol. For this and all subsequent Western blot assays, lanes were loaded with 20  $\mu$ g of protein; blots were subsequently reprobed with anti-tubulin (Tub) antibodies to document equivalent loading and transfer. The blots shown are representative of three or more independent experiments.

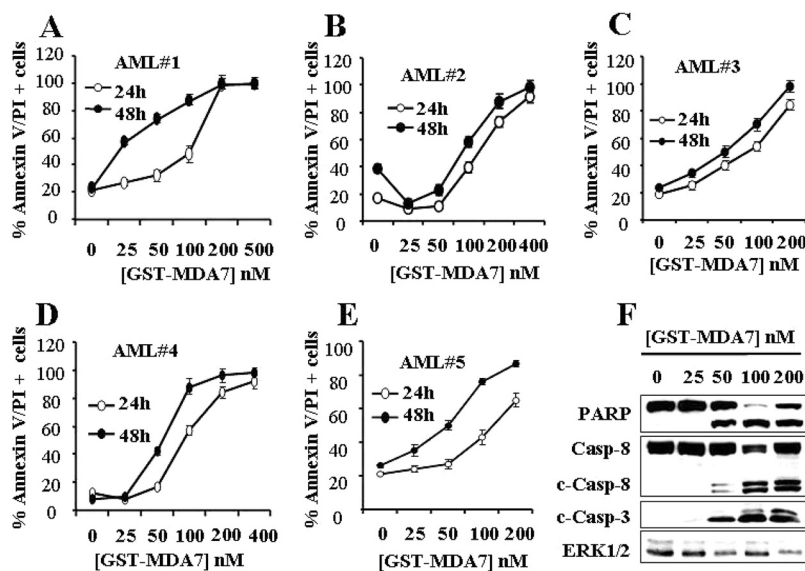
stricted to AML cell lines, parallel dose-response studies with GST-MDA-7/IL-24 (24–48 h) were performed in five primary AML samples (all FAB M2). As shown in Fig. 3, A–E, patterns of cell death induction measured by Annexin V/PI positivity for all samples were virtually identical with those observed in continuously cultured myeloid leukemia cell lines. To establish whether GST-MDA-7/IL-24 triggers apoptosis in primary cells, primary AML blasts isolated from patient 3 were exposed to GST-MDA-7/IL-24 for 48 h, after which caspase activation and PARP cleavage were monitored. As shown in Fig. 3F, GST-MDA-7/IL-24 induced a marked increase in caspase-3 and caspase-8 cleavage as well as PARP degradation at concentrations effective in increasing Annexin V/PI positivity ( $\geq 50$  nM). Similar results were observed in other primary AML cell specimens (data not shown). It is noteworthy that these effects were associated with a very pronounced reduction in L-CFU, which was essentially eliminated at 100 nM GST-MDA-7/IL-24 (Fig. 4A). In contrast, treatment with 100 nM GST-MDA-7/IL-24 for 48 h, which substantially induced cell death in AML blasts (e.g.,  $\geq 80\%$ ), exerted relatively modest toxicity toward normal CD34<sup>+</sup> progenitor cells isolated from three normal subjects (Fig. 4B). Furthermore, the colony-forming capacity of normal CD34<sup>+</sup> cells (CFU-GM) was reduced only minimally by an identical exposure (Fig. 4C). Together, these findings indicate that GST-MDA-7/IL-24 potently induces apoptosis in and markedly inhibits the colony-formation capacity of primary AML cells but exerts only modest toxicity toward normal hematopoietic cells.

**Evidence for ER Stress Induction by GST-MDA-7/IL-24 in Various Human Leukemia Cells Including Primary AML Blasts.** Previous studies from several groups, including our own, suggested that MDA-7/IL-24 induces ER stress in epithelial tumors (Gupta et al., 2006b; Park et al., 2008). To determine whether MDA-7/IL-24 induces ER stress in leukemia cells, U937 cells were exposed to GST-MDA-7/IL-24 (200 nM) for varying intervals, after which time-course analysis of various UPR components expression was performed. As shown in Fig. 5A, MDA-7/IL-24 triggered a dose-dependent induction of the classic UPR markers GRP78/BiP, GADD34, and IRE1 $\alpha$ , comparable with the effects of the classic ER stress-inducer thapsigargin. In addition, time

course analysis revealed that phosphorylation of eIF2 $\alpha$  was first detected within 30 min of exposure to GST-MDA-7/IL-24 (200 nM), and substantially increased by 6 h. After 24 h of treatment, the extent of eIF2 $\alpha$  phosphorylation returned to basal levels. Induction of GADD34 and GRP78/BiP was first noted at 3 and 6 h, respectively, and increased over the ensuing 48 h. In accord with these events, cleavage/activation of caspase-4, caspase-2, caspase-3, and caspase-8 were first noted within 6 h of exposure to GST-MDA-7/IL-24, and became more pronounced at later intervals (i.e., 24 or 48 h) (Fig. 5C). It is noteworthy that the observed induction of IRE1 $\alpha$ , GADD34, and phosphorylation of eIF2 $\alpha$  by GST-MDA-7/IL-24 was recapitulated in diverse leukemia cell lines including HL60-, MV4-11-, and MLL-ENL-expressing cells (Fig. 5D).

Parallel studies were performed in primary blasts isolated from two patients with AML. As shown in Fig. 6, GST-MDA-7/IL-24 significantly induced GADD34 and IRE1 $\alpha$  expression in both specimens. Additional analysis of cells obtained from patient 2, for which sufficient material was available, revealed that GST-MDA-7/IL-24 also enhanced eIF2 $\alpha$  phosphorylation and GRP78/BiP accumulation. Together, these findings indicate that GST-MDA-7/IL-24 induces various classic UPR-related proteins in human leukemia cells, including primary AML blasts, consistent with ER stress induction in these cells.

**IRE1 $\alpha$ , GRP78/BiP, and GADD34 Play Functional Roles in Regulating GST-MDA-7/IL-24-Mediated Cell Death in Leukemia Cells.** To assess the functional significance of up-regulation of IRE1 $\alpha$ , GRP78/BiP, or GADD34 by GST-MDA-7/IL-24, U937 cell lines were generated in which these molecules were stably knocked down with specific shRNAs. As shown in Fig. 7A, cells transfected with shIRE1 $\alpha$ , GRP78/BiP, or shGADD34 exhibited marked reductions in the expression of these proteins. It is noteworthy that each of these cells was significantly more sensitive to GST-MDA-7/IL-24-mediated cell death compared with control cells transfected with shRNA against GFP ( $P \leq 0.05$ ; Fig. 7B). These findings suggest that induction of the UPR-related proteins IRE1 $\alpha$ , GRP78/BiP, and GADD34 by GST-MDA-7/IL-24 plays a significant functional role in regulating



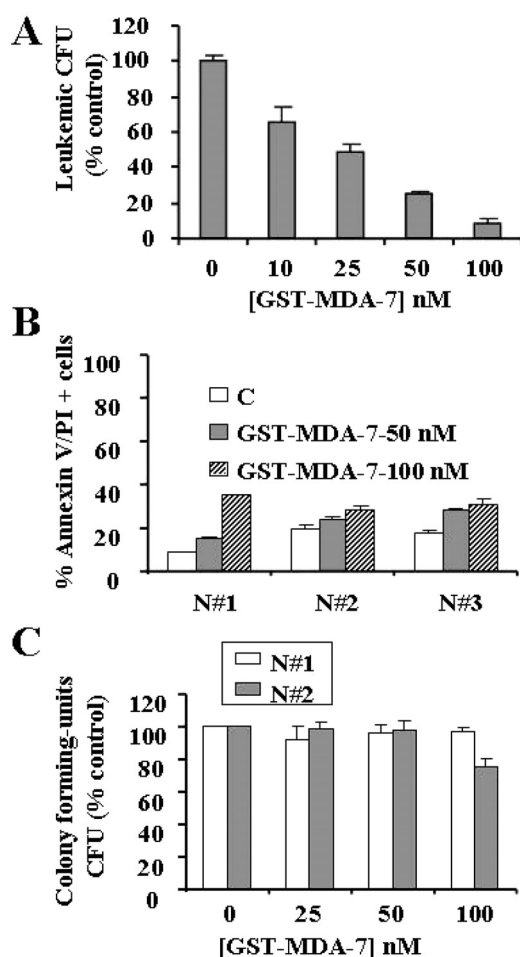
**Fig. 3.** GST-MDA-7/IL-24 potently induces apoptosis in primary AML cells. A to E, leukemic blasts were isolated as described under *Materials and Methods* from the bone marrow of five patients with AML (FAB classification M2) and exposed to the designated concentrations of GST-MDA-7/IL-24 for 24 or 48 h, after which the extent of apoptosis was determined using Annexin V/PI staining assay. On the other hand, protein lysates were prepared from patient 3 and subjected to Western blot analysis to monitor caspase-3 and caspase-8 cleavage/activation and PARP degradation.

cell death and reinforce the notion that GST-MDA-7/IL-24 kills leukemia cells through an ER stress-regulated process.

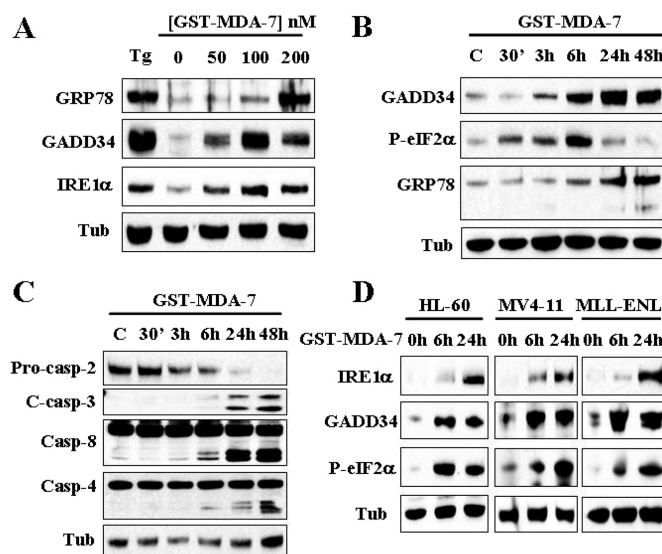
**GST-MDA-7/IL-24-Mediated Lethality Involves Down-Regulation of Mcl-1 and Up-Regulation of Noxa and Bim in Human Leukemia Cells.** In view of the central role that Bcl-2 family members, particularly Mcl-1, Bim, and Noxa, play in survival/death decisions of malignant hematopoietic cells (Moulding et al., 2000; Derenne et al., 2002), expression and function of these proteins were examined in leukemic cells exposed to GST-MDA-7/IL-24. It is noteworthy that exposure of U937 cells to GST-MDA-7/IL-24 resulted in a down-regulation of the antiapoptotic protein Mcl-1 and significant up-regulation of the proapoptotic proteins Bim and Noxa (Fig. 8A). Similar results were observed in HL60 (Fig. 8B) and MV4-11 (Fig. 8C) cells and in primary blasts (Fig. 8D). Treatment with GST-MDA-7/IL-24 also resulted in the time-dependent down-regulation of Bcl-xL and marked cleavage of Bax. In contrast,

no major changes were noted in Bak protein levels, and only slight cleavage of Bcl-2 was observed at later intervals (i.e., 24–48 h).

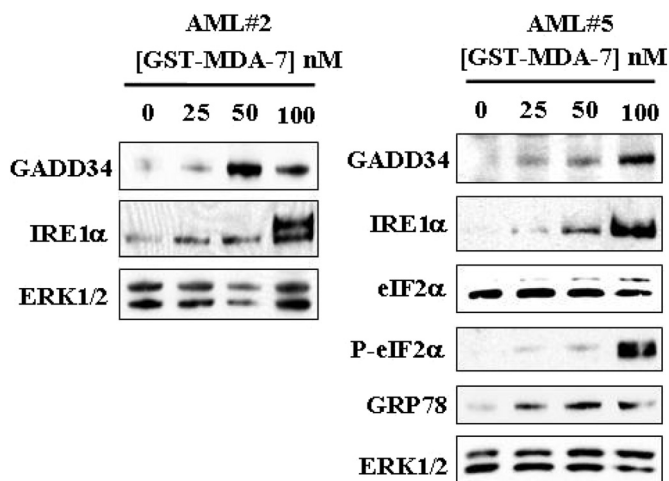
To determine whether up-regulation of Noxa and Bim contributed to MDA-7/IL-24 antileukemic activity, parallel studies were performed in U937 cells in which Bim or Noxa were knocked down by shRNA (Figs. 9, A and B, insets, respectively). It is noteworthy that these cells were significantly more resistant to MDA-7/IL-24-mediated apoptosis ( $P \leq 0.05$  in each case; Fig. 9, A and B). Parallel studies were performed in U937 cells ectopically expressing Mcl-1. As shown in Fig. 9C, these cells were substantially less susceptible to MDA-7/IL-24 lethality compared with their control counterparts ( $P \leq 0.01$ ). Together, these findings indicate that Mcl-1



**Fig. 4.** GST-MDA-7/IL-24 induces a marked reduction in the clonogenic potential of primary AML blasts but is relatively sparing toward normal CD34<sup>+</sup> progenitor cells. A, leukemic blasts isolated from patient 3 were plated in methylcellulose in the presence of the designated concentrations of GST-MDA-7/IL-24 for 14 days, after which L-CFU were enumerated and expressed as a percentage relative to untreated cells. B, normal CD34<sup>+</sup> cells isolated from three normal subjects (N#1–3) were exposed to GST-MDA-7/IL-24 (50 or 100 nM) for 48 h, after which cell death was determined by flow cytometry using the Annexin V staining assay. C, normal CD34<sup>+</sup> cells (N#1–2) were plated in methylcellulose in the presence of the designated concentrations of GST-MDA-7/IL-24 for 8 days, after which CFU-GMs were enumerated and expressed as in A. Each sample was analyzed in triplicate; values represent the means  $\pm$  S.D.



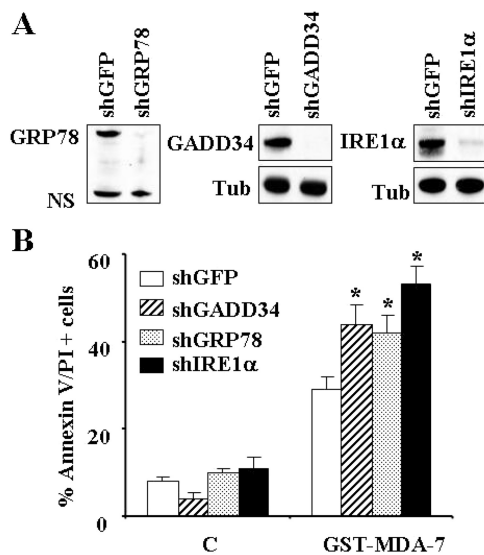
**Fig. 5.** GST-MDA-7/IL-24 triggers UPR response in leukemia cells. U937 cells were exposed to the designated concentrations of GST-MDA-7/IL-24 for 48 h (A) or to 200 nM GST-MDA-7/IL-24 for the designated intervals (B and C), after which protein lysates were prepared and subjected to Western blot analysis using the indicated antibodies. D, HL60, MV4-11, and MLL-ENL were exposed to GST-MDA-7/IL-24 (100 nM for HL60 and MV4-11, and 50 nM for MLL/ENL) for the designated intervals, after which Western blot analysis was performed using indicated antibodies.



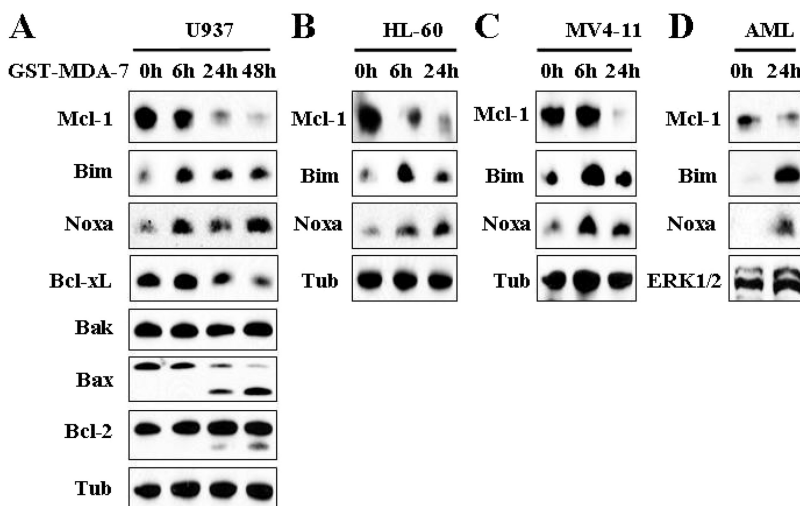
**Fig. 6.** GST-MDA-7/IL-24 induces UPR response in primary AML cells. Leukemic blasts isolated from two patients (2 and 5) with AML were exposed to the designated concentrations of GST-MDA-7/IL-24 for 48 h, after which cells were lysed and subjected to Western blot analysis using the indicated antibodies.

down-regulation and Bim and Noxa up-regulation play significant functional roles in regulating the lethality of GST-MDA-7/IL-24.

In view of the critical role that Bim plays in Bax/Bak activation (Kuwana et al., 2005), the functional role of Bax and Bak in GST-MDA-7/IL-24-mediated apoptosis was investigated. To this end, U937 cells, in which Bax or Bak were stably knocked down with specific shRNA (Rahmani et al., 2009), were used. As shown in Fig. 9D, Bax or Bak knock-down cells were significantly more resistant to GST-MDA-7/IL-24-mediated apoptosis than control counterparts ( $P \leq 0.05$  in each case). Together these findings indicate that Bax and Bak play significant functional roles in leukemia cell death induced by GST-MDA-7/IL-24.



**Fig. 7.** Functional roles of IRE1 $\alpha$ , GRP78/BiP, and GADD34 in regulating GST-MDA-7/IL-24-mediated cell death in leukemia cells. **A**, U937 cells were infected with lentiviruses carrying shRNA constructs against IRE1 $\alpha$ , GRP78/BiP, GADD34, or their control GFP counterparts and selected with puromycin for 2 weeks, after which Western blot analysis was performed to monitor the level of knockdown of these proteins. **B**, these cells were treated with GST-MDA-7/IL-24 (150 nM) for 48 h, after which the extent of apoptosis was monitored using Annexin V/PI analysis assay. Values represent the means for three separate experiments  $\pm$  S.D. \*, significantly higher than values for shGFP-transfected cells ( $p < 0.05$  in each case).



**Fig. 8.** GST-MDA-7/IL-24 down-regulates Mcl-1 and Bcl-xL and up-regulates Noxa and Bim in human leukemia cells. U937 (**A**), HL60 (**B**), MV4-11 (**C**) cells, or leukemic blasts (**D**) were exposed to GST-MDA-7/IL-24 for the designated intervals, after which Western blot analysis was performed. The GST-MDA-7/IL-24 concentrations used were 200 nM for U937 cells and 100 nM for HL60, MV4-11, and leukemic blasts.

## Discussion

Previous studies from several laboratories, including our own, have shown that MDA-7/IL-24 inhibits the growth of and induces apoptosis in cancer cells through multiple mechanisms, including perturbations in signaling pathways (e.g., p38 mitogen-activated protein kinase, c-Jun NH<sub>2</sub>-terminal kinase, and signal transducer and activator of transcription 3) antiangiogenic actions, immune modulation, bystander antitumor activity, induction of reactive oxygen species, and modulation of the expression of pro- and antiapoptotic Bcl-2 family members (Fisher, 2005; Dash et al., 2010a). We and others have reported that MDA-7/IL-24 initiates ER stress in epithelial tumor cells (Gupta et al., 2006b; Park et al., 2008). However, the precise contribution of ER stress to MDA-7/IL-24-induced cell death remains to be defined. In this context, our group has shown that in HeLa cells, MDA-7/IL-24 binds to the ER-resident chaperone GRP78/BiP in association with several DNA damage-related genes (Sarkar et al., 2002; Gupta et al., 2006b), raising the possibility that this phenomenon may contribute to lethality. In contrast to the case of epithelial malignancies, the effects of MDA-7/IL-24 have been minimally explored in acute myeloid leukemia cells, and the functional role that ER stress plays in MDA-7/IL-24-mediated antileukemic actions has not been investigated previously.

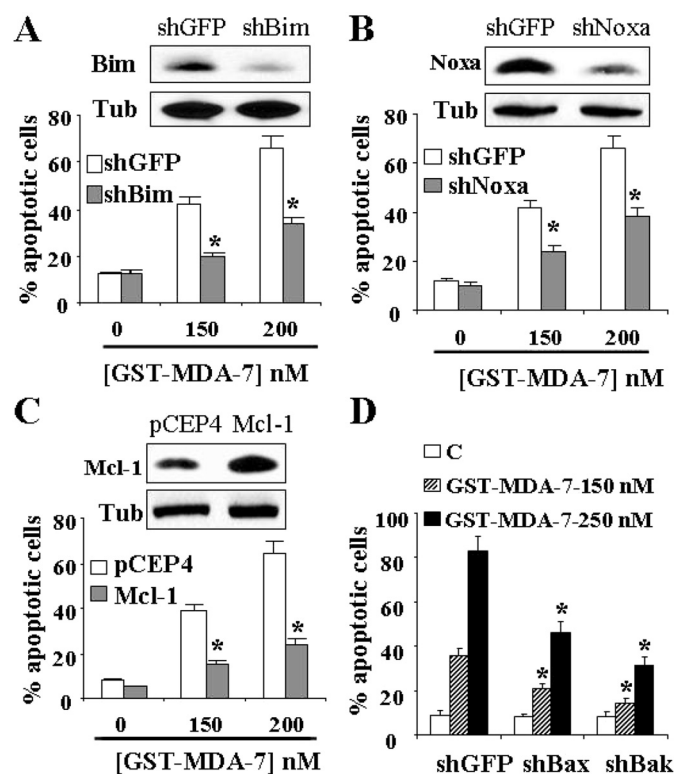
The results of the present study indicate that GST-MDA-7/IL-24 strikingly induces cell death in diverse human leukemia cell types and in primary AML blasts. It is noteworthy that the concentrations of MDA-7/IL-24 used in the present study were comparable with those shown previously to induce cell death in solid tumor cells (Sauane et al., 2004). It is noteworthy that at concentrations that induced cell death in  $\geq 80\%$  of cultured and primary AML cells, MDA-7/IL-24 exerted relatively minimal toxicity toward normal hematopoietic cells (CD34<sup>+</sup>). Such findings are consistent with previous reports demonstrating that MDA-7/IL-24 preferentially targets transformed versus normal cells (Sarkar et al., 2002). The present findings stand in contrast to previous results suggesting that MDA-7/IL-24 promotes the survival of chronic lymphocytic leukemia cells (Sainz-Perez et al., 2006), although this phenomenon was subsequently found to be proliferation-dependent (Sainz-Perez et al., 2008). Such a discrepancy could reflect cell type-specific effects of MDA-7/

IL-24. In this context, Qian et al. (2008) described the induction of apoptosis in B lymphoblastic leukemia cells by a conditionally replicating *mda-7/IL-24* adenovirus. On the other hand, stable transfection of chronic myeloid leukemia cells (K562) or Nawalma lymphoma cells with MDA-7/IL-24 inhibited cell growth without inducing apoptosis (Dong et al., 2008). In this context, recent studies suggest that the ability of enforced expression of adenoviral *mda-7/IL-24* to induce apoptosis in leukemia cells is attenuated by autophagy (Yang et al., 2010). Regardless of the mechanisms responsible for these discrepancies, our findings indicate that diverse human myeloid leukemia cells are highly sensitive to GST-MDA-7/IL-24-mediated apoptosis.

The present results also demonstrate that MDA-7/IL-24 triggers a response characteristically associated with ER stress induction, including up-regulation of GRP78/BiP, IRE1 $\alpha$ , and GADD34 expression, as well as eIF2 $\alpha$  phosphorylation, in various myeloid cells, including primary blasts. These findings suggest that, as observed in epithelial tumor

cells (Gupta et al., 2006b; Park et al., 2008) and in a myelodysplastic syndrome cell line (Mutz-1) stably transfected with MDA-7/IL-24 (Qian et al., 2008), GST-MDA-7/IL-24 potently induces ER stress in myeloid leukemia cells. However, to date, the functional role of ER stress in MDA-7/IL-24 lethality has not been investigated in myeloid leukemia or in hematopoietic malignancies in general. It is noteworthy that functional studies using shRNA directed against GRP78/BiP revealed that induction of this molecule significantly protected myeloid leukemia cells from MDA-7/IL-24 lethality. GRP78/BiP is a major ER-resident chaperone and sensor that is induced by various physiological and nonphysiological ER stresses and that signals downstream to the three major arms of the UPR (PKR-like endoplasmic reticulum kinase, IRE1, and ATF6) (Bertolotti et al., 2000). GRP78/BiP is known to protect cells against various stimuli, particularly those triggering ER stress, in diverse cell types (Shu et al., 2008). It has also been reported to play a protective role against MDA-7/IL-24 lethality in GBM cells (Park et al., 2008). The observation that MDA-7/IL-24 induced GRP78/BiP and that knockdown of this molecule significantly sensitized AML cells to GST-MDA-7/IL-24 lethality is consistent with its established role as a cytoprotective factor in the face of ER stress inducers. In addition, we have reported previously that GST-MDA-7/IL-24 localizes to the ER compartment and physically binds to GRP78/BiP (Gupta et al., 2006b), raising the possibility that MDA-7/IL-24 may disrupt GRP78/BiP function, leading to apoptosis initiation. In this setting, down-regulation of GRP78/BiP by shRNA may lower the threshold for MDA-7/IL-24-mediated interruption of GRP78/BiP cytoprotective functions. Further studies will be necessary to confirm this hypothesis.

The induction of ER stress response is a dynamic process that can serve either protective functions (generally at early intervals) or that can contribute to cell death, often at later intervals after prolonged stress. The temporal relationship governing the relative abundance of pro- versus antiapoptotic components of the UPR can therefore determine whether a cell lives or dies in response to ER stress (Lin et al., 2007). To add to the complexity, components of the UPR can exert either cytoprotective or prodeath effects, depending on the cell context and stimulus. For example, GADD34 (MyD116), a protein initially described as regulator of myeloid differentiation (Lord et al., 1990), has been shown to play a proapoptotic role in some contexts (i.e., SW480 cells exposed to ionizing radiation) (Adler et al., 1999). On the other hand, GADD34 has been shown to protect cells against apoptosis triggered by energy depletion, possibly a consequence of inhibitory effects on mammalian target of rapamycin activity (Watanabe et al., 2007). In addition, pharmacological inhibition of GADD34 by salubrinal has been shown to promote cell death in multiple myeloma cells (Schewe and Aguirre-Ghiso, 2009) and pancreatic  $\beta$ -cells (Cnop et al., 2007). In the present study, the finding that GADD34 shRNA knockdown significantly enhanced apoptosis suggests that GADD34 plays a protective role against MDA-7/IL-24 lethality in myeloid leukemia cells. Although exceptions exist, IRE1 $\alpha$  is also believed to play primarily a cytoprotective role against ER stress-mediated cell death (Lin et al., 2007; Rahmani et al., 2007a). Consistent with these findings, the present results indicate that IRE1 $\alpha$  induction protects leukemia cells from MDA-7/IL-24 lethality.



**Fig. 9.** Knockdown of Bim or Noxa or enforced expression of Mcl-1 renders cells less susceptible to GST-MDA-7/IL-24 lethality. U937 cells in which Bim (A, inset) or Noxa (B, inset) was stably knocked down with shRNA, and their control counterpart shGFP-transfected cells were lysed, and protein lysates were subjected to Western blot analysis to monitor down-regulation of Bim and Noxa. Cells were then exposed to GST-MDA-7/IL-24 (150 or 200 nM) for 48 h, after which the extent of cell death was determined using the Annexin V/PI staining assay (A and B). C, U937 cells ectopically expressing Mcl-1 or the empty vector (pCEP4) were exposed to GST-MDA-7/IL-24 for 48 h, after which the extent of cell death was monitored as above. Protein lysates were also prepared from cells before treatment and Mcl-1 protein levels were monitored by Western blot analysis (C, inset). \*, significantly less than values obtained for pCEP4 cells ( $P < 0.01$ ). D, U937 cells in which Bak or Bax was stably knocked down with shRNA or their control counterpart cells transfected with GFP-shRNA (shGFP) were exposed to GST-MDA-7/IL-24 for 48 h, after which the extent of cell death was monitored using the Annexin V/PI staining assay. For all studies, values represent the means for three separate experiments  $\pm$  S.D. For A, B, and D, \*, significantly lower than values obtained for shGFP-transfected cells ( $P < 0.05$ ).

A question remaining to be answered is why GST-MDA-7/IL-24 preferentially targets leukemic blasts compared with normal CD34<sup>+</sup> progenitor cells. There is evidence, albeit indirect, to suggest that this effect is unlikely to be explained by differential expression of MDA-7/IL-24 receptors (e.g., IL-20R1/IL20R2 or IL-22R1/IL-20R2) on the surface of leukemia blasts versus normal CD34<sup>+</sup> progenitor cells. First, GST-MDA-7/IL-24 does not seem to require classic MDA-7/IL-24 receptors to kill cells as much as cells lacking functional MDA-7/IL-24 receptors, such as A549 cells, remain fully sensitive to GST-MDA-7/IL-24 (Dent et al., 2005). Second, endogenous expression of MDA-7/IL-24 via adenoviral delivery also exhibits selectivity toward transformed versus normal cells (Sarkar et al., 2002). An alternative explanation for MDA-7/IL-24 selectivity is that the high levels of ER stress characteristically displayed by transformed cells may lower the apoptotic threshold for an ER stress inducer such as MDA-7/IL-24.

The down-regulation of Mcl-1 by GST-MDA-7/IL-24 observed in various AML cell types, including primary blasts, as well as evidence of a requirement for this process in GST-MDA-7/IL-24 lethality, are consistent with our recent findings in prostate cancer cells (Dash et al., 2010b). In addition, previous studies from our group and others have shown that Mcl-1 plays a critical role in protecting cells against ER stress induced by diverse agents, including sorafenib, thapsigargin, and tunicamycin (Fritsch et al., 2007; Rahmani et al., 2007a). Furthermore, induction of ER stress can contribute to Mcl-1 down-regulation by inhibiting its translation (Rahmani et al., 2005, 2007a). In this context, the present findings involving Mcl-1 further implicate ER stress in MDA-7/IL-24 lethality in myeloid leukemia. This notion is reinforced by the observation that MDA-7/IL-24 induced up-regulation of Noxa and Bim, and that these proapoptotic proteins played important functional roles in MDA-7/IL-24 lethality. Both Noxa and Bim have been shown to be involved in ER stress-mediated cell death (Puthalakath et al., 2007; Wang et al., 2009). Finally, the observation that both Bax and Bak contributed functionally to MDA-7/IL-24 lethality implicate activation of the mitochondrial apoptotic pathway in myeloid leukemia cell death. However, the specific molecular link between ER stress and mitochondrial dysfunction remains to be determined.

In summary, the present findings demonstrate that MDA-7/IL-24 strikingly induces apoptosis in both cultured and primary human myeloid leukemia cells and provide, for the first time, evidence of a functional contribution of ER stress induction in the regulation of MDA-7/IL-24-mediated leukemia cell death. The observation that equivalent MDA-7/IL-24 exposures exerted relatively little toxicity toward normal CD34<sup>+</sup> hematopoietic cells suggests a possible role for this molecule in future antileukemic strategies. Finally, the present findings raise the possibility that agents capable of interfering with cytoprotective UPR-related proteins such as GRP78/BiP, IRE1 $\alpha$ , or GADD34 might effectively enhance the antileukemic activity of MDA-7/IL-24. Studies designed to test this hypothesis are currently underway.

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