Mitochondrial membrane sensitivity to depolarization in acute myeloblastic leukemia is associated with spontaneous in vitro apoptosis, wild-type TP53, and vicinal thiol/disulfide status

Monica Pallis, Martin Grundy, Julie Turzanski, Reinhard Kofler, and Nigel Russell

Nonresponse to remission-induction chemotherapy, which remains a major problem in acute myeloblastic leukemia (AML), has been linked to cellular resistance to apoptosis. Because the apoptosis induced by chemotherapeutic drugs is mediated by loss of mitochondrial transmembrane potential (MTP), it was postulated that sensitivity to mitochondrial membrane depolarization might be heterogeneous in AML. Using the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (mCCCP), the mitochondrial membrane sensitivity to depolarization (mCCCP concentrations that inhibit 50% of the transmembrane potential [IC₅₀]) in AML blasts was measured and demonstrated marked interclonal heterogeneity, with the existence of comparatively sensitive (median mCCCP IC₅₀, 4 μM) and resistant (median mCCCP IC₅₀, 10 μM) clones. Furthermore, the mCCCP IC₅₀ was inversely associated with spontaneous in vitro apoptosis (P = .001). It was high in cases with mutant TP53 and correlated with the total cellular level of the multidrug resistance-associated protein (P = .019) but not of bcl-2, bax, or bcl-x. It was also found that the dithiol oxidant diamide, in contrast to the monovalent thiol oxidant diethyl maleate, increased the sensitivity of mitochondrial membranes to mCCCP. To confirm that TP53 directly affects MTP in leukemic cells and to establish the role of vicinal thiol oxidation in the TP53-dependent pathway, CEM 4GS leukemia cells with forced, temperature-dependent expression of TP53 were studied. Monobromobimane, which inhibits mitochondrial membrane depolarization by preventing dithiol cross-linking, inhibited depolarization and apoptosis in 4GS cells. It was concluded that in leukemia, TP53 and vicinal thiol/disulfide status are determinants of mitochondrial membrane sensitivity to depolarization, which is in turn associated with spontaneous apoptosis.

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Introduction

Acute myeloblastic leukemia (AML) is a heterogeneous malignant disease in which resistance to induction chemotherapy occurs in 20% to 50% of patients. Chemoresistance may be mediated by overexpression of drug-efflux molecules such as p-glycoprotein (pgp) or multidrug resistance–associated protein (MRP), as demonstrated in several clinical studies. Model systems have predicted that chemoresistance may also be mediated by apoptosis inhibitory pathways, such as those involving bcl-2, bax, or bcl-x. We previously showed that a bcl-2 antisense oligonucleotide sensitized a proportion of AML blasts to the drug cytosine arabinoside. Others found that bcl-2 antisense oligonucleotides induced apoptosis in clinical samples that initially expressed low but not high levels of the protein. However, attempts to find associations between overexpression of any individual member of the bcl family proteins and clinical response in AML have been inconclusive or contradictory, reflecting the complexity of the interactions involved.

Redox mechanisms also have a role in modulating apoptosis. Glutathione, the predominant intracellular reducing thiol, is expressed at high levels in AML, and expression of the glutathione-metabolizing enzyme glutathione transferase π has been associated with poor clinical outcome. The role of other thiol-containing molecules in apoptosis of AML blasts has been little studied. For example, the antioxidant thioredoxin is known to affect cellular sensitivity to apoptosis in adult T-cell leukemia, but its role in AML has not been explored.

Mutations of the oncogene TP53 are associated with poor outcome in AML. TP53 is normally involved in the regulation of apoptosis in damaged cells. This may involve both bcl-2 family and redox pathways: up-regulation of bax by TP53 has been documented, and TP53 is known to regulate expression of several genes involved in redox mechanisms including MRP, glutathione peroxidase, and thioredoxin reductase.

Mitochondrial transmembrane potential (MTP) is considered to have a key role in the control of apoptotic responses, including responses mediated by chemotherapy. Loss of MTP can trigger opening of the permeability transition (PT) pore. PT pore opening has been implicated as a critical stage in apoptosis in isolated mitochondria and several cellular models. PT pore opening allows release of factors that initiate the final, degradative phase of apoptosis. Although PT pore opening does not necessarily accompany membrane depolarization, a reduction in MTP lowers the threshold of pore transition. Both redox pathways and bcl proteins help to control MTP Oxidants and changes in bcl proteins that decrease MTP also induce apoptosis, and conversely, antioxidants and changes in bcl proteins that prevent depolarization.
also inhibit apoptosis.\textsuperscript{4,14} TP53 may play a pivotal role in mitochondrial depolarization. TP53 null murine thymocytes failed to depolarize their mitochondrial membranes in response to both oxidative insult and the drug etoposide.\textsuperscript{35}

Given the large number of bcl family genes and redox pathway genes that possibly modulate MTP in leukemia, it is difficult to unravel which pathways have major importance in resistance to apoptosis. To address this issue, we developed a functional assay of mitochondrial membrane sensitivity to depolarization in cases of primary AML. We then studied the effect of overexpression of bax, bcl-2, and bcl-x\textsubscript{L} on sensitivity to mitochondrial membrane depolarization in AML, along with the role of cellular redox status with particular reference to TP53. Because the data we obtained suggested roles for both TP53 and retinoid thiol oxidation in mitochondrial membrane depolarization in AML blasts, we also used a TP53 conformational mutant leukemic cell line\textsuperscript{21} to investigate the role of retinoid thiol oxidation in the depolarization and apoptosis pathways induced by TP53.

### Materials and methods

#### Materials

The fluorescent dye 3,3\textsuperscript{'}-dihexyloxacarbocyanine iodide (DiOC\textsubscript{6}) was obtained from Molecular Probes (Eugene, OR). Stock solution (1 mM) in ethanol was stored at room temperature. Working solution (400 nM) in phosphate-buffered saline (PBS) was stored at 4°C and used at a concentration of 10 nM in fresh CM (pH 7.3–7.5). The cells were then incubated in sterile Falcon tubes for 90 minutes at 37°C in 5% carbon dioxide (CO\textsubscript{2}) with 40 nM DiOC\textsubscript{6}, with and without mitochondrial toxins. Each condition was set up in triplicate. DiOC\textsubscript{6} fluorescence was measured by using the FL1 channel of a FACScan (Becton Dickinson, Cowley, United Kingdom). In preliminary experiments, we determined that 30 \textmu M mCICCP induced maximum depolarization in primary leukemia samples (data not shown). To calculate the patient mCICCP concentration that inhibits 50% of the transmembrane potential (Δψ\textsubscript{M}), we first calculated a 50% loss of mean fluorescence intensity (MFI) by using the following formula: ([DiOC\textsubscript{6} MFI without mCICCP − DiOC\textsubscript{6} MFI] / DiOC\textsubscript{6} MFI) α 30 \textmu M mCICCP. We then constructed a dose-response curve by using Microsoft Excel software and read the concentration of mCICCP at which a 50% loss of fluorescence occurred.

#### Measurement of bcl-2, bax, bcl-x, and MRP

For measurement of bcl-2, bax, and bcl-x, thawed and rested leukemic cells were fixed and permeabilized in 35% ethanol for 10 minutes, rinsed twice in PBS containing 1% bovine serum albumin and 0.1% sodium azide, and resuspended in the same buffer. Aliquots were then stained with unconjugated antibodies to bcl-x (S-18) or bax (both from Santa Cruz Biotechnolog, Santa Cruz, CA) or with appropriate controls as described previously\textsuperscript{42} by using a fluorescein isothiocyanate, conjugated (FITC)–labeled secondary antibody. A single batch of each polyclonal antibody was used, and a reproducible concentration was established as described previously.\textsuperscript{43} Bcl-2 was measured by using 10 \textmu L FITC anti-bcl-2 or control antibody (both from Dako, High Wycombe, United Kingdom). The unconjugated antibody MRPr1 (Monsano, Uden, The Netherlands) was used according to the manufacturer’s instructions, with the modification that a blocking solution of 20% rabbit serum in PBS was added 30 minutes before the second-layer incubation in FITC rabbit antirat secondary antibodies.

#### Flow cytometry

For flow cytometric analysis, a Becton Dickinson FACScalibur was used with a logarithmic amplifier and Cellquest software. The following variables were measured. For bcl-2, bcl-x, and bax, where the test and control distributions tended to be nonoverlapping, Quantum beads were used to standardize interassay fluorescence and allow quantitation of mean bound fluorochromes by using the FL1 channel of a FACScan (Becton Dickinson, Cowley, United Kingdom). The pgp-positive KG1a and MDR-negative U937 and HL60 myeloid leukemia cell lines were from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). All cell lines used were grown in a culture medium (CM) of RPMI 1640 with 10% fetal-calf serum and 2 mM L-glutamine. 7-AAD was measured by using the FL3 channel of a FACScan (Becton Dickinson, Cowley, United Kingdom). The unconjugated antibody MRPr1 (Monsano, Uden, The Netherlands) was used according to the manufacturer’s instructions, with the modification that a blocking solution of 20% rabbit serum in PBS was added 30 minutes before the second-layer incubation in FITC rabbit antirat secondary antibodies.

#### Flow cytometric measurement of apoptosis

Primary leukemia clones were incubated in 5% CO\textsubscript{2} at a concentration of 5 × 10\textsuperscript{6}/mL in RPMI suspension culture with 10% FCS and 2 mM L-glutamine. 7-AAD was mixed with cultured cells to yield a final concentration of approximately 10 μg/mL. Cultures were incubated for 20 minutes in the dark at room temperature before processing for flow cytometry. 7-AAD was measured in FL3. Cells undergoing apoptotic cell death are represented by 7-AAD–high cells with low forward scatter.\textsuperscript{44} For PI-mediated determination of sub-G\textsubscript{0} cells, cultured cells were first fixed in 70% ethanol at −20°C for 15 minutes. Cells were then rinsed twice in PBS, resuspended in PI at a concentration of approximately 10 μg/mL, and analyzed by flow cytometry.

TP53

Mutations of the TP53 gene in AML samples were investigated by direct sequencing of full-length TP53 complementary DNA as reported previously.\textsuperscript{46}

#### Statistical procedures

SPSS software (SPSS, Chicago, IL) was employed. The Spearman rank correlation was used to assess the significance of associations between
mClCCP IC\textsubscript{50} and expression of cellular proteins. The Wilcoxon signed-rank test was used to investigate the significance of the effect of antioxidants on MTP.

**Results**

**Methodology**

The potentiometric fluorescent dye DiOC\textsubscript{6} can be used to measure mitochondrial membrane depolarization. We investigated use of this dye to measure mitochondrial membrane sensitivity to the protonophore mClCCP. This experimental system was previously used by others\textsuperscript{37,47} to demonstrate that monovalent thiol reagents or ectopic expression of bcl-2 can block the depolarizing effect of mClCCP. For clinical studies, we sought to develop this system into an assay that would be reproducible, sufficiently sensitive to detect differences between clones, and sensitive to mitochondrial but not plasma membrane depolarization. We first determined the saturation plateau for DiOC\textsubscript{6} in leukemic cells after incubation at 37°C; this occurred at 60 to 120 minutes in primary AML cells and cell lines (Figure 1A). Others showed that after prolonged (>5 hours) incubation with DiOC\textsubscript{6}, the plasma membrane as well as the mitochondrial membrane can affect DiOC\textsubscript{6} fluorescence,\textsuperscript{35} so we performed experiments using potassium chloride to depolarize the plasma membranes of cells from patients and thus ensured that this would not be a confounding factor in our system (Figure 1B).

We evaluated use of the pro-oxidant tBHP and the uncoupler mClCCP as agents to depolarize the mitochondria of primary leukemia cells. The tBHP was found to hyperpolarize the mitochondrial membrane before depolarizing (Figure 2). This may be associated with continued formation of a hydrogen ion gradient despite the failure of adenosine diphosphate/adenosine triphosphate exchange as described previously.\textsuperscript{49} For practical purposes, the curve obtained with tBHP was not considered suitable for interpatient comparisons because no saturation plateau was reached, whereas a plateau was reached by using mClCCP.

Because primary leukemia samples may be pgp positive or negative, we were concerned that pgp-mediated efflux of the cationic fluorescent dye DiOC\textsubscript{6} would alter the intracellular availability of the dye and hence the kinetics of its binding to the mitochondrial membrane. We therefore measured DiOC\textsubscript{6} uptake in pgp-positive primary leukemia cells with and without the pgp modulator PSC 833. As shown in Figure 3, at 90 minutes, DiOC\textsubscript{6} fluorescence was greater in the presence of PSC833 than in its absence, a finding compatible with reduced dye efflux. However, when expressed in terms of percentage of control fluorescence, the dose of mClCCP required to induce loss of fluorescence was independent of whether PSC833 was present or absent, indicating that DiOC\textsubscript{6} binding kinetics are unaffected when the overall intracellular dye concentration is reduced by efflux.

**MTP sensitivity to mClCCP in primary AML blasts and cell lines**

The depolarizing response to mClCCP was measured in 27 samples from patients with AML. Examples of dose-response curves are shown in Figure 4. The mClCCP dose required to reduce the fluorescence to 50% of untreated control values (the mClCCP IC\textsubscript{50}) was bimodally distributed in patient clones with modal values at 4 μM and at 10 μM (range, 1-15 μM; overall median, 6.5 μM;
Several cell lines were also studied (Figure 4), and K562 cells were found to have the highest IC₅₀ measured. Because mitochondrial membrane depolarization does not always result in PT opening, we cultured AML blasts overnight with mClCCP to determine the effects of the ionophore on apoptosis. We found that doses in the same range as the IC₅₀ values for mitochondrial membrane depolarization induced apoptosis in primary leukemia clones (Figure 5). Analysis of apoptotic cell death based on 7-AAD was previously validated against other methods by us and others.44,45

AML samples are highly heterogeneous in their need for growth factors to support both clonogenic growth and survival in suspension culture. In some cases of AML, growth factors may be needed to maintain pathways that protect the mitochondrial membrane from depolarization. We therefore hypothesized that samples that undergo the most spontaneous apoptosis in vitro have mitochondrial membranes that depolarize the most readily. We correlated spontaneous in vitro apoptosis (ie, apoptosis in suspension culture in 10% serum without added growth factors) with the mClCCP IC₅₀ in 12 samples and established that a strong correlation existed (r = 0.8; P = .001; Figure 5C).

**Associations between mClCCP IC₅₀ and the expression of bcl-2, bax, and bcl-xL in AML blasts**

Control of the MTP by transfected bcl-2, bax, or bcl-xL was previously demonstrated in model systems, but the importance of protein overexpression in controlling depolarization in primary leukemia cells has not been explored. Bcl-2, bax, and bcl-xL were measured in our patient samples. (Since we failed to find the bcl-x isoform in a previous study, we have assumed that the isoform measured is bcl-xL.) Expression of none of these molecules correlated with the depolarizing response to mClCCP (Table 1). The bcl-x to bax ratio and the bcl-2 to bax ratio were also calculated and determined not to correlate with mClCCP sensitivity (data not shown).

**The role of TP53 in mitochondrial membrane depolarization**

A role for TP53 in depolarizing the mitochondrial membrane has been described. TP53 mutation occurs in approximately 15% of AML cases.20 Cells from 3 patients with AML with a known TP53 mutation were available for inclusion in this study. All 3 had an mClCCP IC₅₀ above the median level (6.5 μM) for the group as a whole (the specific values were 7, 10, and 10 μM, respectively; Table 1). This finding suggested that lack of p53 expression may be associated with resistance to mitochondrial membrane depolarization, but because of the infrequency of the TP53 mutation in patients with AML, we were unable to obtain enough clinical material for a statistical analysis of significance. Therefore, to confirm that TP53 directly affects MTP in leukemic cells, we studied CEM cells with a forced temperature-sensitive expression of wild-type TP53.21 After 18 of hours culture at the

**Figure 4.** Sensitivity of AML blasts to mitochondrial membrane depolarization induced by mClCCP. Each dose was assayed in triplicate. (Ai and Bi) Flow cytometric histograms for DiOC₆ fluorescence at 10 μM mClCCP (unshaded histogram) and untreated control (shaded histogram). Ai illustrates a sensitive sample, and the corresponding dose-response curve is shown in Aii. Bi illustrates a resistant sample, and the corresponding dose-response curve is shown in plot Bii. The horizontal line markers on the dose-response curves indicate the 50th percentile fluorescence, and the perpendicular lines indicate the mClCCP IC₅₀. (C) Summary scatterplot showing the mClCCP IC₅₀ for 27 samples from patients with AML and the CEM C7H2, KG1a, and K562 leukemic cell lines. The cell line data include error bars (± SEM) derived from at least 3 separate experiments.
permissive temperature of 32°C, apoptosis was apparent on PI analysis of decreased DNA in 30% (SE, 4.4%) of cells from the TP53 subline 4G5, and mitochondrial membranes were depolarized at a higher percentage—47% (SE, 4%) of cells (Figure 6)—thereby showing that wild-type p53 expression does induce mitochondrial membrane depolarization. Using the vital dye 7-AAD, which is taken up by apoptotic cells, we also demonstrated that mitochondrial membrane depolarization preceded loss of plasma membrane integrity.

The mClCCP IC50 is associated with MRP expression in primary AML blasts

There is some evidence that TP53 controls genes involved in detoxification of cells affected by oxidative stress.23-25,54 Although most of the mammalian TP53 inducible genes have been only partly identified, control of the MRP promoter has been demonstrated.25 Moreover, we previously found that MRP overexpression is associated with the presence of TP53 mutations in AML cells.55 This led us to examine the correlation between MRP expression and mClCCP IC50 in cases of AML, and we found that there was a significant association (r = 0.58; P = .019; Figure 7).

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IC50 indicates the dose of mClCCP which induces 50% loss of mitochondrial membrane potential; BFM, bound fluorochrome molecules. Correlation coefficients (Spearman r for mClCCP IC50 with bcl family expression) were −0.172 for bcl2, 0.178 for bax, and −0.282 for bcl-x; none of these correlations were significant at the P < .05 level.

Effects of antioxidant inhibitors on mitochondrial membrane depolarization

Because MRP is associated with redox status,56,57 we wondered whether other components of redox status affected the MTP in AML. Permeability transition can be induced by depletion of antioxidants within the mitochondrial space.58-60 However, because
antioxidants are not evenly distributed between the mitochondrion and the cytoplasm, we decided not to measure total antioxidant levels in AML cells but to take the functional approach of measuring the effects of antioxidant inhibition on mitochondrial membrane depolarization. DEM is a monovalent thiol reagent that is commonly used to deplete cellular glutathione and can induce subsequent apoptosis, whereas diamide is a divalent thiol reagent that induces apoptosis through the formation of disulfide bonds in molecules with vicinal thiol groups. We investigated whether sensitivity to mitochondrial membrane depolarization could be augmented by antioxidant depletion through thiol or dithiol oxidation. Thus, we measured DiOC6 fluorescence in the presence of a low dose of mClCCP (3 μM) in the presence and absence of DEM or diamide. For both compounds, 100 μM doses were chosen because preliminary 7-AAD analysis of AML blasts cultured overnight with the compounds indicated that some apoptosis was induced at these doses, whereas higher doses could lead to the death and disintegration of entire clones (data not shown). Others demonstrated previously that death induced by a high dose of antioxidant inhibitors may be necrotic. Diamide, which alone induced a mean of 17.3% depolarization in the 10 samples tested, increased the depolarization induced by low-dose mClCCP from 22% to 53.6% (P = .047; Figure 8), indicating that cross-linking of thiols can sensitize AML blasts to depolarization. In contrast, the monovalent thiol reagent DEM increased the membrane potential in 6 of 8 cases and had no significant effect on depolarization in response to mClCCP (Figure 8).

To further define the role of vicinal thiol oxidation in depolarization of mitochondrial membranes and to determine whether this effect is TP53 dependent, we examined the effect of preventing dithiol oxidation on MTP and apoptosis in temperature-sensitive p53 mutant CEM cells. MBB is a monovalent thiol-reactive agent that prevents vicinal thiol cross-linking, mitochondrial membrane depolarization, and apoptosis. We found that apoptosis was inhibited in the CEM 4G5 cells incubated overnight at the TP53 permissive temperature when these cells were cultured with MBB (Figure 9). In 3 similar experiments, we observed 35.7% ± 1.5% depolarized cells and 21% ± 1% sub-G0 cells after overnight culture without MBB and 14.7% ± 4.0% depolarized cells and 1.7% ± 2.1% sub-G0 cells after culture with MBB (values corrected for depolarized and sub-G0 C7H2 cells). We used PI on permeabilized cells as our primary method for detecting apoptosis in this experiment. However, because this method may fail to discriminate loss of DNA content from cells dying in S or G2M phases, we also examined DiOC6 data plots for evidence of reduced forward scatter as an indicator of cell shrinkage in apoptotic cells. Using this method, we confirmed that apoptosis in 4G5 cells decreased when the cells were cultured with MBB (data not shown). The results of this experiment support a role for vicinal thiol-mediated pathways in TP53-induced apoptosis.

Discussion

Using a sensitive flow cytometric assay, we demonstrated differential sensitivity to mitochondrial membrane depolarization by mClCCP in AML blasts. This assay formed a basis for studying pathways contributing to depolarization resistance that could thus affect apoptosis in AML. Furthermore, we determined that depolarization can be enhanced by diamide, an agent that cross-links vicinal thiols. Surprisingly, sensitivity to mitochondrial membrane depolarization was not significantly associated with total protein levels of bcl-2, bax, or bcl-x but was associated with mutant TP53 and with high expression of MRP. We also determined that sensitivity to mitochondrial membrane depolarization correlates with spontaneous apoptosis in vitro. A high mClCCP IC50 may thus be indicative of autocrine growth factor–mediated—or of growth factor–independent—rescue from apoptosis in AML, which may be clinically relevant in light of the previously established association between autonomous growth of blast cells and reduced patient survival.

An obligate role for mitochondrial membrane depolarization in TP53-mediated apoptosis has been shown in thymocyte and DLD-1 (colorectal cancer) models. Using the CEM C7H2 subclone 4G5 with temperature-sensitive TP53 expression, we...
shown that wild-type TP53 directly induces mitochondrial membrane depolarization in leukemic cells. The regulation of bcl family proteins, particularly bax, by TP53 is well documented, but it was also found that TP53 may also control apoptosis through activation of redox pathway genes. Up-regulation of bax, which is strongly associated with TP53 expression, may not be sufficient to induce apoptosis, as was clearly shown in a temperature-sensitive mutant TP53 erythroleukemia model in which rescue from apoptosis could be achieved by using the receptor tyrosine kinase KIT, although TP53-dependent bax production was high in both rescued and unrescued cells. Antioxidants have been shown to inhibit TP53-induced membrane depolarization and to suppress apoptosis. The antiapoptotic effect of the thiol cross-linking inhibitor MBB on TP53-specific pathways was not directly demonstrated previously, although it is entirely consistent with the previous finding that bcl-2 oligonucleotides can increase in vitro chemosensitivity in some primary AML blasts. Furthermore, bcl-2, bcl-x, or bax are responsible for clinical sensitivity to depolarization, but our data do not support the idea sometimes extrapolated from these studies that variations in total protein expression of bcl-2, bcl-x, or bax are responsible for clinical variability in chemoresistance. In our experiments, absolute levels of bcl-2, bax, or bcl-x did not appear to be important. However, posttranslational differences between bcl proteins that affected the ability of these proteins to associate and move between intracellular compartments cannot be ruled out as factors contributing to differential sensitivity to depolarization and apoptosis.

The central role for mitochondrial depolarization in triggering apoptosis was established in studies showing that apoptosis was abolished when depolarization was blocked. Although mitochondrial membrane depolarization is not part of all apoptotic pathways, it is known to be part of the death pathway of cells exposed to chemotherapeutic drugs such as those commonly used to treat leukemia. Several proteins at the inner mitochondrial membrane or contact sites between inner and outer membranes can affect depolarization, and an aim of this study was to work toward identifying the crucial molecules in AML. The relative contributions of most individual molecules to therapy resistance in clinical samples cannot be assessed easily because there are so many contending molecules (at least 16 bcl proteins alone), and their differential deployment is likely to depend on complex activation and inhibitory pathways. Indeed, in simplified models, factors that affect the sensitivity of the mitochondrial pore were shown to interact: bcl-2 inhibited oxidative stress–induced apoptosis, and conversely, cellular thiol depletion reversed bcl-2–induced resistance to oxidative stress. A quantitative functional assay of sensitivity to a depolarization agonist is therefore useful because it enabled us to measure resistance modulation in primary tumor cells and to determine associations with levels of candidate modulatory molecules.

We found that all 3 cases of AML with mutant TP53 were resistant to mCCICP. Moreover, using the temperature-sensitive CEM sublines, we demonstrated that depolarization is at least partly under TP53 control. Specific genomic targets of TP53 have not been fully characterized. However, one known target is MRP, which is involved in cellular detoxification and is elevated in patients with AML who have TP53 mutations. We do not know why MRP expression was high in cases with a high mitochondrial mCCICP IC50. It may be that MRP is one of several detoxification-related molecules under common regulatory control and should thus be viewed simply as a marker of mitochondrial membrane sensitivity to depolarization. MRP, which associates with glutathione to function as an efflux pump, is coexpressed with gamma-glutamylcysteine synthetase, a rate-limiting enzyme in the formation of glutathione, so it is plausible that MRP expression reflects the intracellular concentration of glutathione.

Expression of redox-related and bcl family proteins is highly heterogeneous in AML. In several cellular models, bcl proteins transfected into hematopoietic cells affected sensitivity to mitochondrial depolarization, but our data do not support the idea that variations in total protein expression of bcl-2, bcl-x, or bax are responsible for clinical variability in chemoresistance. In our experiments, absolute levels of bcl-2, bax, or bcl-x did not appear to be important. However, posttranslational differences between bcl proteins that affected the ability of these proteins to associate and move between intracellular compartments cannot be ruled out as factors contributing to differential sensitivity to depolarization and apoptosis.
References


22. Zorzali M, Szabo I. The mitochondrial permeabil-

23. Petronilli V, Cola C, Massari S, Colonna R, Ber-
trand P. Physiological effectors modify voltage sensing by the cyclosporin A-sensitive permeabil-


25. Salvioli S, Ardidzoni A, Franchessi C, Cossarizza A, et al. Bcl-xL, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess cell death in intact cells: implications for studies on mito-


27. VanderHeiden MG, Schumacker PT, Thompson CB. Bcl-xL antagonizes the mitochondrial dysfunction preceding nuclear apoptosis induced by chemo-

28. Hunter AE, Rogers SY, Roberts IA, Barrett AJ, Russell N. Autonomous growth of blast cells is associated with reduced survival in acute myelo-

29. Miyatachi K, Kelleher CA, Yang YC, et al. Possible roles of cellular thiols other than gluta-


58. Chernyak BV, Bernardi P. The mitochondrial permeability transition pore is modulated by oxidative agents through both pyridine nucleotides and glutathione at two separate sites. Eur J Biochem. 1996;238:623-630.


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