Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance

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Summary

Drug resistance remains a major obstacle to successful cancer treatment. A database of drug-associated gene expression profiles was screened for molecules whose profile overlapped with a gene expression signature of glucocorticoid (GC) sensitivity/resistance in acute lymphoblastic leukemia (ALL) cells. The screen indicated that the mTOR inhibitor rapamycin profile matched the signature of GC sensitivity. We tested the hypothesis that rapamycin would induce GC sensitivity in lymphoid malignancy cells and found that it sensitized to GC-induced apoptosis via modulation of antiapoptotic MCL1. These data indicate that MCL1 is an important regulator of GC-induced apoptosis and that the combination of rapamycin and glucocorticoids has potential utility in lymphoid malignancies. Furthermore, this approach represents a strategy for identification of promising combination therapies for cancer.

Introduction

Multiple studies have demonstrated the potential for gene expression profiling to identify cancers that will recur after treatment with multiagent chemotherapy (Buckhaults, 2006; Heuser et al., 2005; Mintz et al., 2005; van't Veer et al., 2002). The overarching goal for such an approach to cancer therapy is that patients with the highest risk of relapse might be identified and treated with a different, more effective therapy. Unfortunately, in most cases, a more effective alternative does not exist. Therefore, we have focused on how signatures associated with poor prognosis might be used to direct therapeutic approaches in addition to identifying patients with poor prognosis. As relapses are likely to be at least partially a result of intrinsic cellular resistance to the chemotherapeutic agents being used, gene expression signatures associated with relapse might be used to identify approaches to reverse drug resistance. But, given that most patients are treated with multiple agents that likely have different mechanisms of resistance, and in vivo response to therapy is influenced by multiple factors that may not be intrinsic to the cancer cell, it is not immediately obvious how to use such gene expression data to guide therapeutic strategies. While we have previously used gene expression analysis to identify the receptor tyrosine FLT3 as a potential therapeutic target in specific subsets of childhood acute lymphoblastic leukemia due to its differential expression, this approach requires significant prior knowledge about the associated biology in order to make such associations (Armstrong et al., 2003). Therefore, new approaches are needed if gene expression analysis is to guide the identification of therapeutic approaches.

One striking example in which resistance to a cancer therapeutic predicts outcome is resistance to glucocorticoid treatment in childhood acute lymphoblastic leukemia (ALL). Resistance to glucocorticoid-induced apoptosis of primary lymphoblastic

SIGNIFICANCE

Multiple studies have demonstrated that gene expression profiling can be used to identify patients with differing prognoses. However, it has been more difficult to use this information to develop therapeutic approaches. Here we show that disease-associated signatures can be computationally connected to drug-associated profiles in order to identify molecules that reverse a drug resistance signature. Demonstration that rapamycin reverses glucocorticoid resistance via MCL1 modulation prompts investigation of this combination in lymphoblastic leukemia and suggests that such a gene expression-based chemical genomic approach might be used more broadly to identify molecules (drugs) that reverse gene expression signatures associated with poor prognosis and drug resistance.

leukemia cells in vitro predicts a poor prognosis in childhood ALL (Hongo et al., 1997; Kaspers et al., 1997, 1998; Pieters et al., 1991). In vivo response to 7 days of monotherapy with prednisone is also a strong and independent prognostic factor in childhood ALL (Dordelmann et al., 1999; Kaspers et al., 1998). The importance of glucocorticoid resistance in ALL is further highlighted by the fact that it remains a strong predictor of a poor prognosis even when assessed in a subset of ALL cases harboring the t(9;22), cases traditionally considered to have one of the worst outcomes in ALL. Since glucocorticoids (GC) are critical to many biologic processes and are a cornerstone of ALL therapy, there have been many studies of GC and the GC receptor (GR) mechanism of action and resistance that have explored multiple avenues (Tissing et al., 2003). However, the precise explanation for sensitivity versus resistance to GC in ALL remains largely unknown.

Alterations of critical control points in the intrinsic apoptotic pathway are an attractive potential mechanism to explain GC sensitivity and resistance. Defective apoptosis is a hallmark of cancer (Hanahan and Weinberg, 2000), and the ability to activate the apoptotic program is an important determinant of efficacy for anticancer drugs (Fesik, 2005; Reed, 1995). Members of the BCL2 protein family play critical roles in the intrinsic apoptotic program controlling whether an apoptotic signal will result in cell death (Danial and Korsmeyer, 2004). In response to various apoptotic stimuli, proapoptotic BH3-only members induce activation of BAX and BAK, and cytochrome *c* release from mitochondria, thus triggering activation of caspases through the apoptosome complex (Danial and Korsmeyer, 2004). Conversely, antiapoptotic members such BCL2, BCL-X_L, or MCL1 antagonize BH3-only proteins, thereby inhibiting BAX and BAK activation and apoptosis (Danial and Korsmeyer, 2004). The proapoptotic molecules BIM and PUMA are necessary for appropriate GC-induced apoptosis, and BIM expression is rapidly induced after GC treatment of ALL cells (Abrams et al., 2004; Erlacher et al., 2005; Wang et al., 2003). Overexpression of antiapoptotic BCL2 protects CCRF-CEM lymphoblastic leukemia cells from GC-induced apoptosis (Hartmann et al., 1999). Moreover, $Bax^{-/-};Bak^{-/-}$ double knockout (DKO) lymphocytes that have a complete block in the intrinsic apoptotic pathway are resistant to GC (Lindsten et al., 2000; Takeuchi et al., 2005). Finally, high-level expression of the antiapoptotic MCL1 is correlated with in vitro GC resistance (Holleman et al., 2004).

Given that in vitro GC resistance can be accurately assessed in ALL samples, and this resistance is predictive of a poor outcome, we asked whether we could combine a GC sensitivity/resistance gene expression signature with a database of gene expression data derived from a diversity of bioactive small molecules to identify compounds that could modulate this biology. Using this approach, we identified rapamycin as a potential GC resistance reversal agent, and MCL1 as an important modulator of GC-induced apoptosis.

Results

The Connectivity Map identifies rapamycin as a potential GC resistance reversal agent

In order to identify a gene expression signature associated with GC resistance, we performed gene expression analysis on pretreatment ALL samples that were determined to be either sensitive (IC50 < 150 μg/ml prednisolone) or resistant (IC50 > 150 μg/ml prednisolone) to GC-induced apoptosis in vitro (Den Boer et al., 2003; Pieters et al., 1991). RNA was isolated from 13 sensitive and 16 resistant diagnostic ALL samples and hybridized to Affymetrix U133A microarrays. One hundred and fifty-seven probe sets were significantly correlated with the sensitive/resistant distinction after correction for multiple hypothesis testing ($p < 0.0005$) (Figure 1A) (Figure S1 in the Supplemental Data available with this article online). Comparison of our signature with a previously defined signature for GC sensitivity/resistance demonstrated overlap of the resistance signature including increased expression of antiapoptotic MCL1 in resistant cells (Figure S1) (Holleman et al., 2004).

Having defined a signature of GC resistance, we sought to develop a strategy to reverse the signature, and by inference, reverse the resistance phenotype. We hypothesized that a compendium of gene expression profiles representing pharmacologic treatment of cells might be used to identify compounds that modulate the GC resistance signature. To test this hypothesis, we made use of a database of 453 genome-wide expression profiles derived from the treatment of a variety of human cell lines with 164 bioactive small molecules, the majority of which are established pharmaceuticals or compounds with known activities, referred to as the Connectivity Map (Table S1) (Lamb et al., 2006). We used a gene set enrichment metric based on the Kolmogorov-Smirnov statistic (Lamb et al., 2003) to rank order these 453 individual treatment instances by their similarity to the resistance/sensitive signature. Remarkably, the ten instances of the mammalian target of rapamycin (mTOR) inhibitor sirolimus (more commonly known as rapamycin) were ranked near the top of the Connectivity Map list, an observation far beyond that expected by chance ($p < 10^{-5}$) (Figure 1C). This analysis indicated that rapamycin is positively connected with the sensitive versus resistance signature and suggested the hypothesis that this small molecule could induce GC sensitivity in glucocorticoid-resistant ALL cells. Rapamycin inhibits the activity of mTOR, which is activated by PI3K/Akt pathway (Hay and Sonenberg, 2004). Also consistent with the connection of the GC resistance signature to this pathway was the observation that the GC resistance signature was highly enriched in genes associated with the AKT pathway. Specifically, gene set enrichment analysis (GSEA) (Subramanian et al., 2005) using 163 gene sets derived from the BioCarta pathway database (Majumder et al., 2004), indicated that nine gene sets were significantly enriched in the resistant samples as determined by permutation testing ($p < 0.005$) (Figure 1B). The AKT pathway was the most highly enriched gene set in the resistant samples, further suggesting a potential role for the PI3K/AKT/mTOR pathway in GC resistance. The enrichment of AKT pathway-associated genes in GC-resistant samples and the Connectivity Map-based identification of rapamycin as a potential GC resistance reversal agent focused our attention on the PI3K/AKT/mTOR pathway and prompted further assessment of rapamycin.

Rapamycin reverses the glucocorticoid resistance signature in lymphoid cells

In order to further verify the overlap between a rapamycin profile and our GC resistance signature, we assessed overlap between the genes highly expressed in resistant ALL samples and genes that decreased after rapamycin treatment in independent

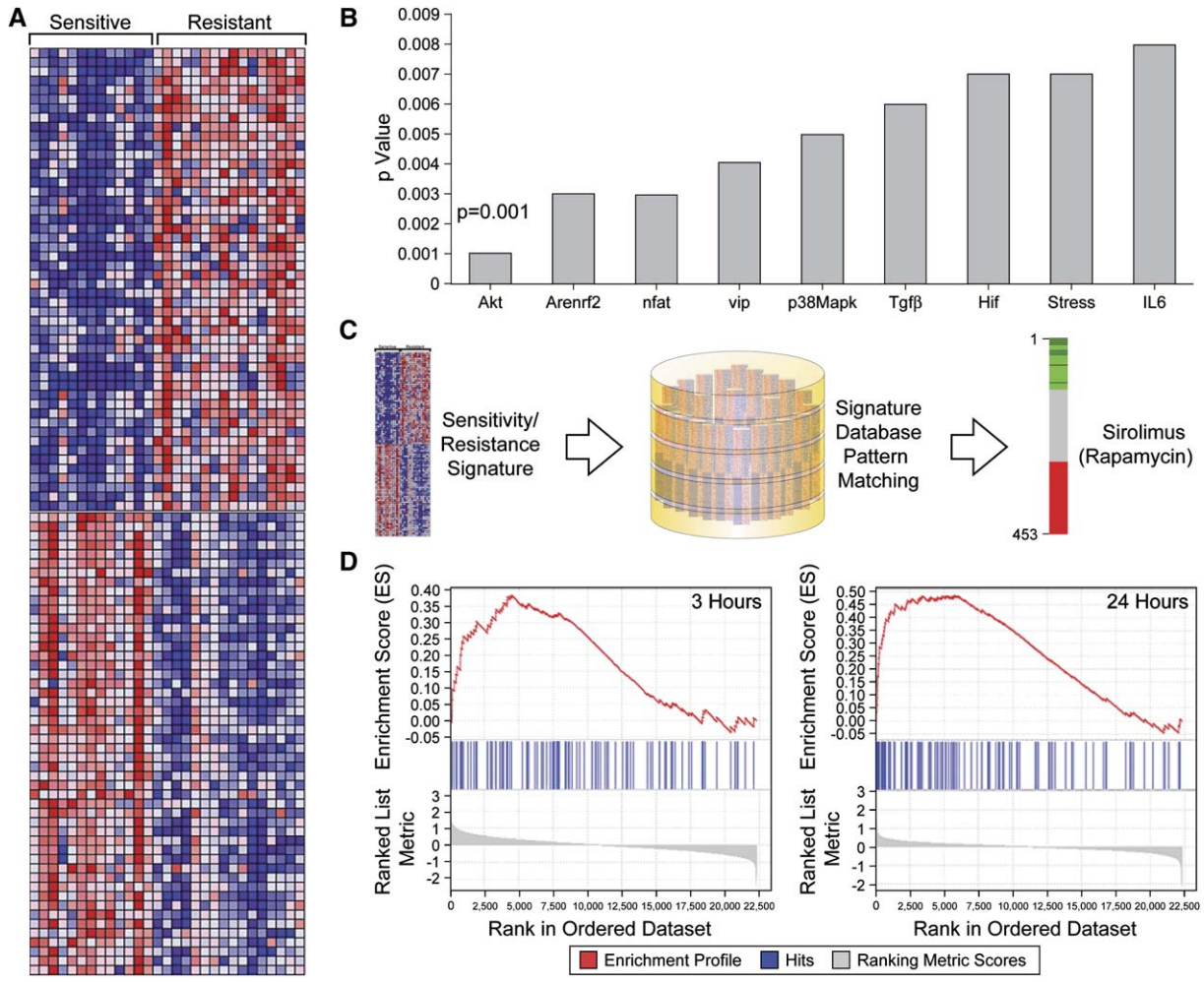

Figure 1. Gene expression-based screening identifies rapamycin as a potential sensitizing agent to GC-induced apoptosis

A: The top 50 probe sets that are correlated with either GC sensitivity or resistance are shown. A detailed list of genes can be found at http://www.broad.mit.edu/cgi-bin/cancer/datasets.cgi/. Permutation analysis identified 157 probes correlated with this distinction ($p < 0.0005$).

B: GSEA analysis was performed using 163 pathway-associated gene sets curated by Biocarta to assess for enrichment of specific pathways in either resistant or sensitive samples. All pathways that demonstrated enrichment with $p < 0.005$ are demonstrated and ordered based on the p value. Note that all gene sets with enrichment were more highly expressed in the resistant ALL samples.

C: Sirolimus (rapamycin) is positively connected with the GC resistance/sensitivity signature. The ''barview'' is constructed from 453 horizontal lines, each representing an individual treatment instance in the Connectivity Map data set, ordered by their corresponding connectivity score with the query signature. Each of the ten rapamycin instances in the data set is colored in black. Colors applied to the remaining instances reflect the sign of their scores (green, positive; gray, null; red, negative).

D: CEM-c1 cells were treated with either DMSO or 10 nM rapamycin for 3 and 24 hr after which the gene expression profile was determined. The 157 probe sets used in 1C were assessed for overlap with those genes more highly expressed in the cells treated with DMSO (resistant) as compared to those treated with rapamycin (potentially sensitive). The blue lines demonstrate where the GC probe sets fall within the 22,000 probe sets ordered from left (1) to right (22,000) based on the DMSO/rapamycin distinction with gene #1 most highly expressed in DMSO-treated cells. The red line represents the running ES score that becomes more positive as probe sets are identified toward the top of the list before they would be expected if randomly distributed. Note that at 24 hr more probe sets (blue lines) are identified toward the top (left) of the ordered list of genes.

experiments. First, we treated the GC-resistant T cell lymphoblastic leukemia cell line CEM-c1 with rapamycin and determined the gene expression profile 3 and 24 hr after rapamycin treatment. Using the genes highly expressed in resistant ALL samples as a gene set, we performed GSEA against the CEM-c1 cell lines that were treated with rapamycin or DMSO for 3 or 24 hr. This analysis demonstrated a progressive overlap between those genes whose expression decreased after rapamycin treatment and genes highly expressed in GC-resistant samples (Figure 1D). We also used GSEA to demonstrate partial reversal of the GC resistance signature in a previously published profile obtained after rapamycin treatment of human B-lymphoma cells (data not shown) (Majumder et al., 2004; Peng et al., 2002). Given the consistent gene expression connection between GC sensitivity/resistance and rapamycin treatment, we performed further studies to assess the efficacy and mechanism of rapamycin reversal of GC resistance.

Rapamycin sensitizes lymphoid malignancy cells to GC

As mTOR is downstream of AKT in some cell types (Gingras et al., 2001; Hay and Sonenberg, 2004), we tested if activated AKT induced GC resistance, and if this resistance could be reversed by rapamycin. We transduced the GC-sensitive mouse T-hybridoma 2B4 cell line (Memon et al., 1995) with a retrovirus

encoding GFP and a constitutively active form of Akt (Myr-Akt). The 2B4 cells expressing Myr-Akt were more resistant to GC-induced apoptosis than either the uninfected parental 2B4 cells (data not shown) or the control virus-transduced 2B4 cells (Figure 2A), indicating that activation of the Akt pathway can confer GC resistance. Next, we determined to what extent the GC resistance induced by Myr-Akt was due to mTOR activation. We treated 2B4 cells expressing Myr-Akt with rapamycin and then tested their GC sensitivity. Treatment with 10 nM rapamycin reversed a portion of the GC resistance induced by Myr-AKT returning the GC sensitivity back to that of wild-type 2B4 cells (Figure 2A). When control 2B4 cells were incubated with 10 nM rapamycin, we noted a further sensitization to GC-induced death (Figure 2A). Furthermore, all of the GC-induced death could be reversed by the GC receptor antagonist RU486 (data not shown), thus demonstrating the specificity of the GC effect. These data indicate that AKT activation can induce GC resistance and that a portion of the resistance works via an mTOR-dependent pathway.

The fact that rapamycin could sensitize 2B4 cells to GC led us to investigate this effect on a panel of human cell lines derived from patients with lymphoid malignancies. First, we demonstrated inhibition of ribosomal S6 phosphorylation by 10 nM rapamycin, thus confirming effective inhibition of mTOR at this concentration (Figure S2A). Multiple cell lines, including a T-ALL cell line (CEM-c1), a B-ALL cell line (697), and multiple Burkitt's lymphoma cell lines (Raji, NAMALWA, Ramos), demonstrated enhanced apoptosis when rapamycin and glucocorticoids were used in combination (Figure 2B). Of note, some human lymphoid leukemia cell lines (such as REH) were not sensitized, likely due to a different mechanism of GC resistance in these lines (Figure 2B). We confirmed that rapamycin alone, at concentrations up to 100 nM, did not induce apoptosis in CEM-c1 (Figure 2C), 697, Ramos, or NAMALWA cell lines (Figure S3). However, after pretreatment with 10 nM rapamycin, the CEM-c1 (Figure 2D), 697 (Figure 2E), Ramos (Figure 2F), and NAMALWA (Figure S2C) cells demonstrated a shift in the IC50 for dexamethasone. To test whether rapamycin treatment simply reduced the apoptotic threshold, we tested other drugs on rapamycin-pretreated CEM-c1 cells. Neither staurosporine-, vincristine-, doxorubicin-, nor etoposide-induced apoptosis was influenced by rapamycin pretreatment (Figure S4). Therefore, rapamycin sensitizes lymphoid malignancy cells to GC-induced apoptosis via a mechanism that does not enhance sensitivity to all drugs.

Rapamycin downregulates antiapoptotic MCL1 in lymphoid malignancy cells

BCL2 family members are critical regulators of the intrinsic apoptotic pathway and play critical roles in GC-induced apoptosis. Therefore, we determined which members of the BCL2 family might regulate GC-induced apoptotic events in these cells. BIM and PUMA are BH3-only members of the BCL2 protein family induced by GC in lymphoid cells and are required for GC-induced apoptosis (Erlacher et al., 2005; Villunger et al., 2003). BIM expression is also induced by glucocorticoids in ALL cells (Abrams et al., 2004; Schmidt et al., 2006). We measured BIM and PUMA levels and confirmed BIM induction in CEM-c1 cells (Figures 3A and 3B) and 697 cells (data not shown). However, we found that rapamycin did not affect the level of *BIM* transcripts or protein (Figures 3A and 3B). Even though PUMA is induced by dexamethasone in some lymphoid cells (Erlacher et al., 2005), it was not induced in CEM/c1, 697, or 2B4 cells (Figure 3A and data not shown). Furthermore, rapamycin did not affect the level of *PUMA* transcripts when cells were treated

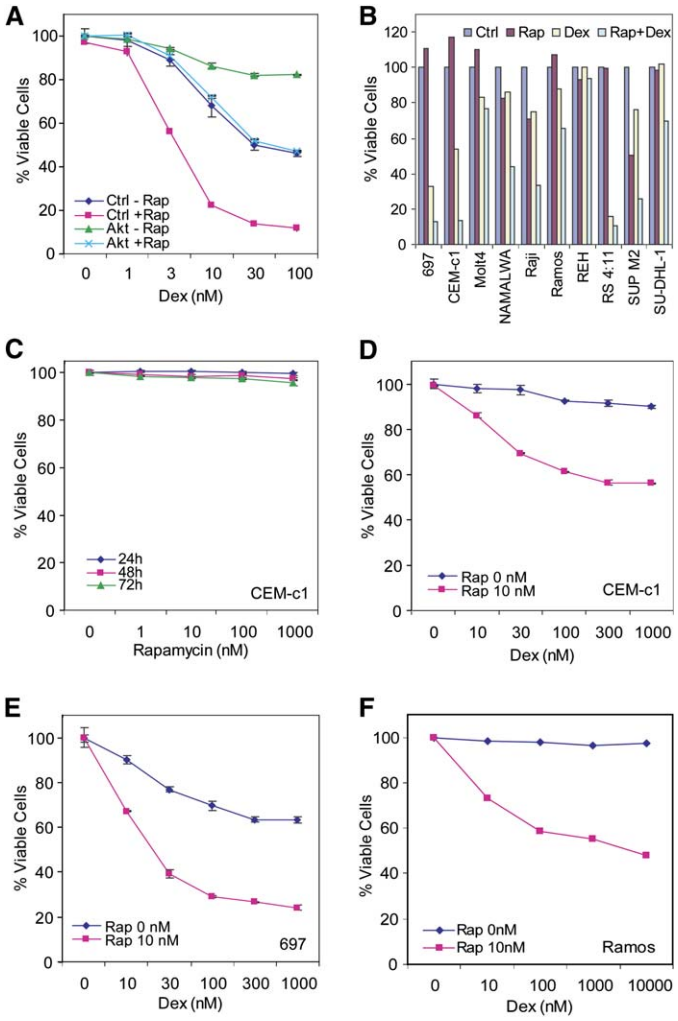

Figure 2. Rapamycin sensitizes lymphoid malignancy cells to GC-induced apoptosis

A: Mouse T cell hybridoma 2B4 cells were engineered to express a constitutively active form of Akt by retroviral transduction. The GFP-positive Akt-transduced or control-transduced cells were treated with an increasing concentration of dexamethasone for 14 hr with or without 10 nM rapamycin, followed by FACS analysis. GFP-positive cells were assessed for viability by Annexin V staining.

B: A panel of lymphoid malignancy-derived cell lines were treated with vehicle control, 10 nM rapamycin, 1 μM dexamethasone, or the combination of dexamethasone and rapamycin, and the percentage of viable cells was determined at 48 hr.

C: Viable cells were assessed in T-ALL CEM-c1 cells after treatment with increasing concentrations of rapamycin at 24, 48, and 72 hr.

D: The percentage of viable cells was determined in CEM-c1 cells 24 hr after treatment with an increasing concentration of dexamethasone ± pretreatment with 10 nM rapamycin.

E: The percentage of viable cells was determined in 697 cells 24 hr after treatment with an increasing concentration of dexamethasone ± 10 nM rapamycin.

F: The percentage of viable cells was determined in Ramos cells 48 hr after treatment with an increasing concentration of dexamethasone ± 10 nM rapamycin.

Error bars represent the mean ± standard deviation.

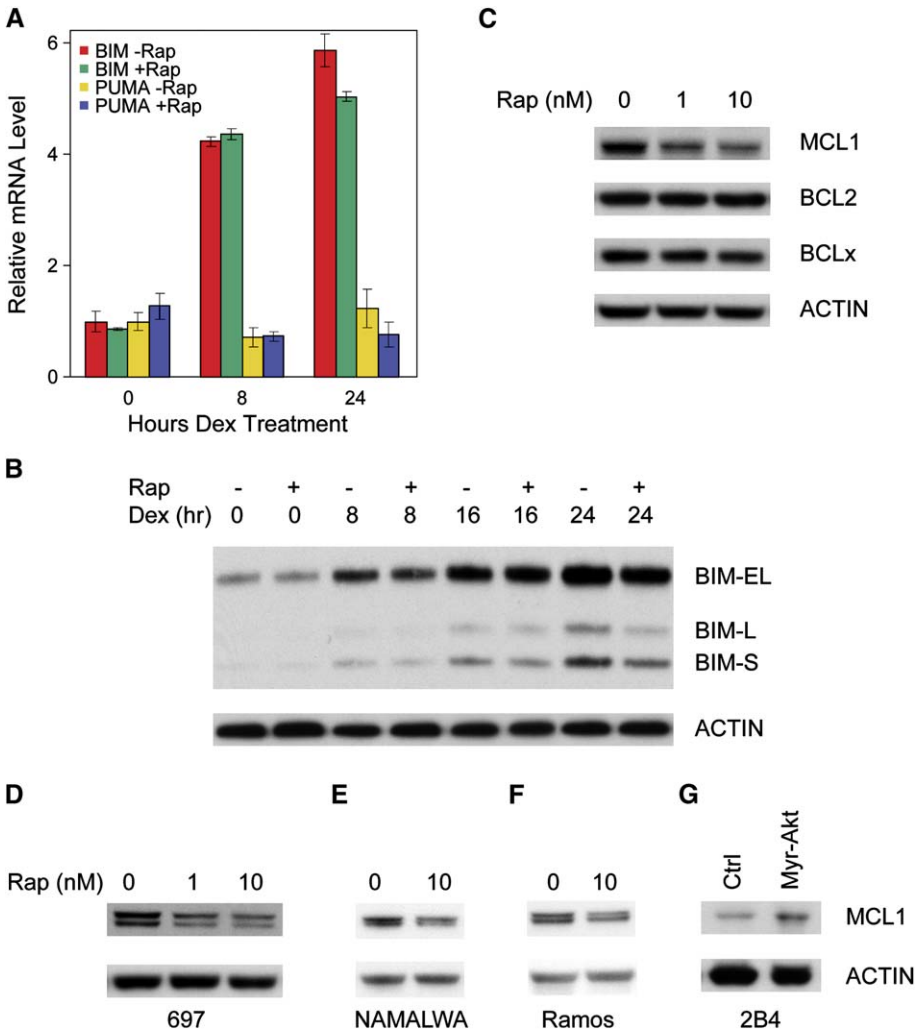

Figure 3. Rapamycin specifically downregulates MCL1 among BCL2 family proteins

A: Dexamethasone-induced expression of BH3-only Bim and Puma was not affected by rapamycin in CEM-c1 cells as determined by quantitative real-time PCR analysis. Error bars represent the mean ± standard deviation of triplicates.

B: CEM-c1 cells were pretreated with or without Rapamycin for 16 hr, followed by 1 μM dexamethasone for the indicated time, and Bim expression was assessed by immunoblot.

C: CEM-c1 cells were treated with rapamycin at the indicated concentration for 16 hr, and antiapoptotic protein expression was assessed by immunoblot.

D–F: 697 cells (**D**), NAMALWA cells (**E**), or Ramos cells (**F**) were treated with rapamycin for 16 hr, and MCL1 levels were assessed by Western blotting. All cell lines demonstrated > 90% viability when they were assessed for MCL1 expression.

G: MCL1 expression was assessed in 2B4 cells expressing constitutively active Akt as compared to vector control.

in the presence or absence of dexamethasone (Figure 3A). Therefore, it appears that modulation of *BIM* and *PUMA* expression is not the mechanism by which rapamycin sensitizes cells to GC-induced apoptosis.

Next, we assessed the levels of antiapoptotic BCL2 family proteins in CEM-c1 cells treated with rapamycin. The level of BCL2 and BCL-X_L did not change, but MCL1, an antiapoptotic protein essential for lymphoid cell survival (Opferman et al., 2003), was reduced (Figure 3C). A similar decrease in MCL1 expression after rapamycin treatment was demonstrated in 697 (Figure 3D), NAMALWA (Figure 3E), and Ramos (Figure 3F) cells. Conversely, MCL1 levels were higher in the GC-resistant 2B4 cells expressing Myr-Akt as compared to control GC-sensitive 2B4 cells (Figure 3G), similar to previous reports demonstrating modulation of MCL1 levels by AKT activation (Liu et al., 2001; Wang et al., 1999). Therefore, activation of AKT may confer GC resistance through augmentation of MCL1 levels, while at least a portion of the rapamycin-mediated sensitization of lymphoid malignancy cells may work via a decrease in MCL1 expression.

Overexpression of MCL1 sequesters BIM and renders cells resistant to GC

Given that MCL1 expression is correlated with GC resistance in ALL (Figure 1) (Holleman et al., 2004), Mcl1 is a critical antiapoptotic factor in murine lymphocyte development and survival (Opferman et al., 2003), and rapamycin modulates MCL1 levels (Figure 3), we hypothesized that MCL1 may be a critical regulator of GC-induced apoptosis and part of the mechanism by which rapamycin sensitizes cells to glucocorticoids. 2B4 cells transduced with a retrovirus encoding MCL1 expressed 4- to 8-fold higher levels than cells infected with a control virus (Figure 4A). After 16 hr of dexamethasone exposure, only 30% of 2B4 cells transduced with a control virus were viable, whereas over 90% of cells overexpressing MCL1 were viable (Figure 4B). As expected, MCL1 overexpression inhibited apoptosis via a number of different stimuli (Figure S5). However, the greatest protection was conferred against GC-induced apoptosis (Figure 4B) (Figure S5). MCL1 expression similarly protected GC-sensitive human 697 cells from GC-induced apoptosis (Figure S6). Next, we expressed a FLAG-tagged MCL1 whose level was not regulated by rapamycin (Figure S7A) and found that 2B4 cells expressing the FLAG-tagged MCL1 were protected against GC-induced apoptosis even if treated with rapamycin and dexamethasone (Figure 4C).

Next, we wanted to further investigate the mechanism of MCL1-mediated protection against GC-induced death. We hypothesized the BH3-only protein BIM induced by GC-treatment (Figure 3) might be sequestered by MCL1 and thus unable to

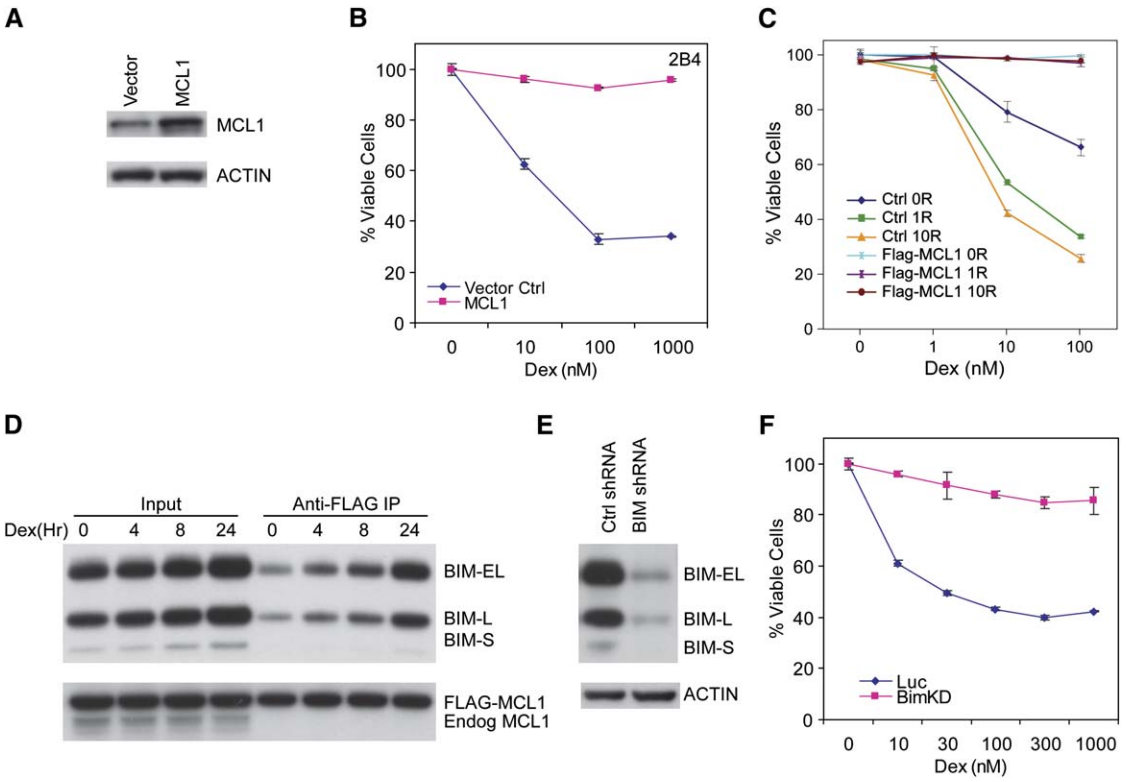

Figure 4. Expression of MCL1 confers GC resistance and sequesters BIM

2B4 cells overexpressing MCL1 are resistant to dexamethasone treatment.

A: MCL1 overexpression in 2B4 cells by retroviral-mediated expression.

B: 2B4 cells transduced with either an MCL1 retrovirus or vector control were treated with increasing concentrations of dexamethasone for 16 hr, followed by FACS analysis for Annexin V staining.

C: 2B4 cells expressing FLAG-MCL1 or control were treated with increasing concentrations of dexamethasone and either 0 nM (0R), 1 nM (1R), or 10 nM (10R) rapamycin for 16 hr, followed by FACS analysis.

D: 2B4 cells expressing FLAG-MCL1 were treated with 100 nM dexamethasone for the indicated time, and immunoprecipitation with an anti-FLAG M2 antibody was performed. Immunoprecipitated BIM and MCL1 were assessed by immunoblot.

E: Bim expression was suppressed by lentivirus-mediated shRNA. Extra-long (EL), long (L), and short (S) isoforms of BIM were assessed by immunoblot.

F: 2B4 cells transduced with the Bim-directed shRNA or a control luciferase-directed shRNA were treated with dexamethasone for 16 hr, followed by FACS analysis. Luc, control luciferase knockdown. BimKD, BIM knockdown.

Error bars represent the mean ± standard deviation.

activate proapoptotic BAX/BAK as a mechanism of resistance. To test this hypothesis, 2B4 cells expressing FLAG-MCL1 were treated with 100 nM dexamethasone for 0, 4, 8, and 24 hr, then subjected to immunoprecipitation using an anti-FLAG antibody. We found progressive accumulation of a BIM/MCL1 complex during the course of the experiment (Figure 4D). Even after BIM expression is induced by dexamethasone, the majority of BIM is bound by MCL1 (Figure 4D and Figure S7B), indicating MCL1 is a major antiapoptotic protein sequestering BIM in these cells. To test the extent to which BIM plays a role in GC sensitivity in this cell line, we knocked down Bim expression in 2B4 cells by lentivirus-mediated short-hairpin RNA (shRNA) expression (Figure 4E). Bim knockdown cells are more resistant to dexamethasone than 2B4 cells transduced with a control lentivirus (Figure 4F), indicating that BIM is a key BH3-only protein in this context. Recently, it has been shown that decreased BIM expression renders 697 cells resistant to GC (Abrams et al., 2004). Our data are consistent with this report and the data from murine models demonstrating an important role for BIM in GC-induced apoptosis (Bouillet et al., 1999; Erlacher et al., 2005). We note that Bim knockdown 2B4 cells are less resistant to GC than 2B4 cells overexpressing MCL1, presumably because MCL1 inhibits other proapoptotic proteins important for GC-induced apoptosis. Therefore, overexpression of MCL1 confers GC resistance in 2B4 cells and works at least in part by sequestration of proapoptotic Bim. Furthermore, MCL1 appears to be a key player in rapamycin-mediated sensitization to GC-induced apoptosis.

Overexpression of MCL1 renders primary lymphocytes resistant to GC-induced apoptosis

To further demonstrate the role of MCL1 in GC resistance, we assessed transgenic mice that express murine *Mcl1* under the control of the major histocompatibility complex class I gene (H2Kb) promoter (Figure 5A) (J.T.O., unpublished data). The H2K promoter is well characterized and has been reported to drive high-level expression in all hematopoietic lineages (Domen et al., 2000). Furthermore, in this model, the cDNA for Mcl1 has had its 3′-UTR and polyA replaced with that of the Moloney MuLV enhancer/polyA to facilitate message stabilization. Western blot analysis revealed approximately 2- to 3-fold elevated levels of MCL1 in the thymocytes or splenocytes of one

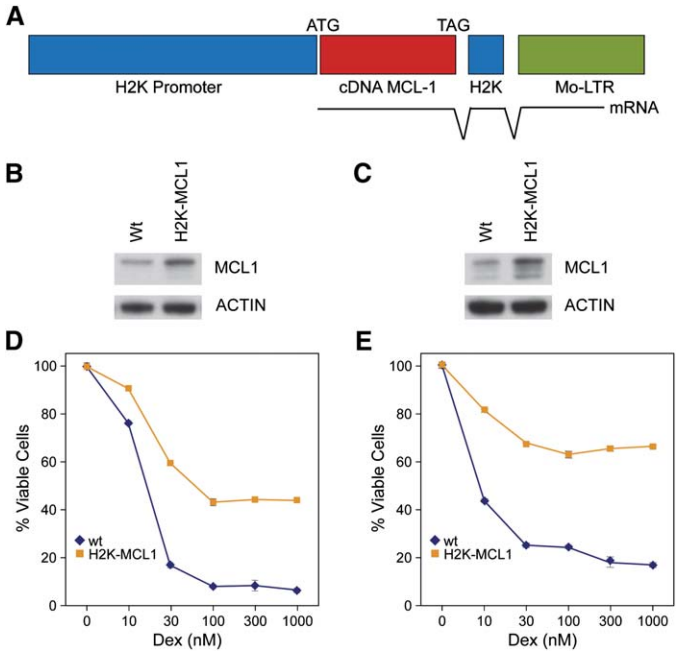

Figure 5. MCL1 confers GC resistance in primary lymphoid cells

A: Illustration of H2K-Mcl1 transgene construct.

B: An immunoblot for MCL1 expression in thymocytes from H2K-Mcl1 transgenic and wild-type mice.

C: Immunoblot for MCL1 expression in splenocytes from H2K-Mcl1 transgenic and wild-type mice.

D: Thymocytes from H2K-Mcl1 transgenic mice and littermate controls were treated with increasing concentrations of dexamethasone for 24 hr followed by FACS analysis for Annexin V−/PI− thymocytes.

E: B220+, Annexin V−/PI− splenic B cells were counted as viable cells and standardized against cells without dexamethasone treatment. Four mice for each genotype were tested. Representative results are shown.

Error bars represent the mean ± standard deviation.

H2K-Mcl1 transgenic line (Figures 5B and 5C). Analysis of splenic B cell and thymic T cell development in this line showed no gross differences in lymphocyte development (J.T.O., unpublished data). In order to examine the contribution of Mcl1 to GC resistance in primary lymphoid cells, thymocytes and splenic B220+ B cells were treated with dexamethasone ex vivo. Both B and T lymphoid cells from mice with the H2K-Mcl1 transgene showed GC resistance as compared to cells from control littermates (Figures 5D and 5E). After 24 hr dexamethasone treatment, fewer than 10% of thymocytes from control littermates were viable (Annexin V/propidium iodide [PI] double-negative) at a concentration of 100 nM dexamethasone, whereas over 40% of thymocytes from H2K-Mcl1 transgenic mice were viable (Figure 5D). Similarly, splenic B cells with the H2K-Mcl1 transgene were relatively resistant to GC-induced apoptosis (Figure 5E). Therefore, overexpression of MCL1 induces GC resistance in primary lymphoid cells.

MCL1 suppression sensitizes cells to GC-induced apoptosis

Several antiapoptotic BCL2 family proteins have been shown to confer GC resistance in ALL cell lines and thymocytes from transgenic mice when overexpressed (Chao et al., 1995; Grillot et al., 1995; Memon et al., 1995; Sentman et al., 1991; Siegel et al., 1992; Strasser et al., 1991). For example, like MCL1, BCL2 or BCL-XL renders 2B4 cells resistant to GC (Memon et al., 1995). However, only MCL1, but not BCL2 or BCL-XL, is overexpressed in GC-resistant ALL cells from primary patients (Figure 1) (Holleman et al., 2004). Furthermore, while rapamycin sensitizes cells to GC, only MCL1, but not BCL2 or BCL-XL, is downregulated in rapamycin-treated cells. Therefore, we hypothesized that MCL1 is a key antiapoptotic BCL2 family protein responsible for GC resistance. To test this hypothesis, MCL1 expression was suppressed by an MCL1-directed shRNA (Figure 6A) in the CEM-c1, 697, and NAMALWA cell lines. All lines transduced with the MCL1 shRNA demonstrated increased sensitivity to GC treatment (Figures 6B, 6E, and 6F). Furthermore, similar to cells treated with rapamycin, the cells treated with the MCL1-directed shRNA were sensitized to GC to a greater extent than any other drug tested (Figures 6C and 6D) (Figure S8). In contrast, when BCL2 expression was suppressed with high efficiency in CEM-c1 cells (Figure 6G), the cells were still resistant to GC-induced apoptosis (Figure 6H). Therefore, it appears that MCL1 is the key antiapoptotic protein governing GC resistance in these cells.

Discussion

We have used an approach to drug discovery based on in silico screening for compounds that reverse a gene expression signature to identify the mTOR inhibitor rapamycin as a potential reversal agent for GC resistance. Given that rapamycin is an FDA-approved drug known to be safe when coadministered with glucocorticoids, and GSEA analysis identified AKT pathway members as highly expressed in resistant samples, we decided to further investigate the potential role for rapamycin as a GC resistance reversal agent in lymphoid malignancy cells. In order to activate the mTOR pathway, we expressed a constitutively active AKT (Myr-Akt) in a cell line previously demonstrated to be sensitive to GC-induced apoptosis. Pretreatment with rapamycin reversed a significant portion of the AKT-mediated GC resistance, demonstrating that mTOR activation can play a role in GC resistance. Furthermore, rapamycin treatment sensitized both GC-resistant and -sensitive human lymphoid malignancy cell lines to GC-induced apoptosis. In order to determine if rapamycin affected the intrinsic apoptotic pathway as a mechanism for reversal of GC resistance, we assessed levels of pro- and antiapoptotic BCL2 family member proteins after rapamycin treatment. Rapamycin specifically reduced the expression of MCL1 but did not affect the level of BCL2 and BCL-XL, or the induction of BIM by dexamethasone. Similar to rapamycin treatment, reduction of MCL1 levels by RNAi sensitized cells to dexamethasone-induced apoptosis, whereas RNAi-mediated reduction of BCL2 had no effect on GC resistance in CEM-c1 cells. These data show the utility of in silico drug screening and prompt further characterization of the role of MCL1 and its inhibition by rapamycin in GC resistance.

Rapamycin and glucocorticoid treatment in ALL

Rapamycin and other mTOR inhibitors are currently in early-phase clinical trials for a number of malignancies based on the importance of the AKT/mTOR pathway in cancer biology. In some tumors where PTEN, a negative regulator of PI3K/AKT/mTOR pathway, is inactivated, cells exhibit enhanced sensitivity to the rapamycin analog CCI-779 (Guertin and Sabatini, 2005). Furthermore, multiple studies have demonstrated synergy

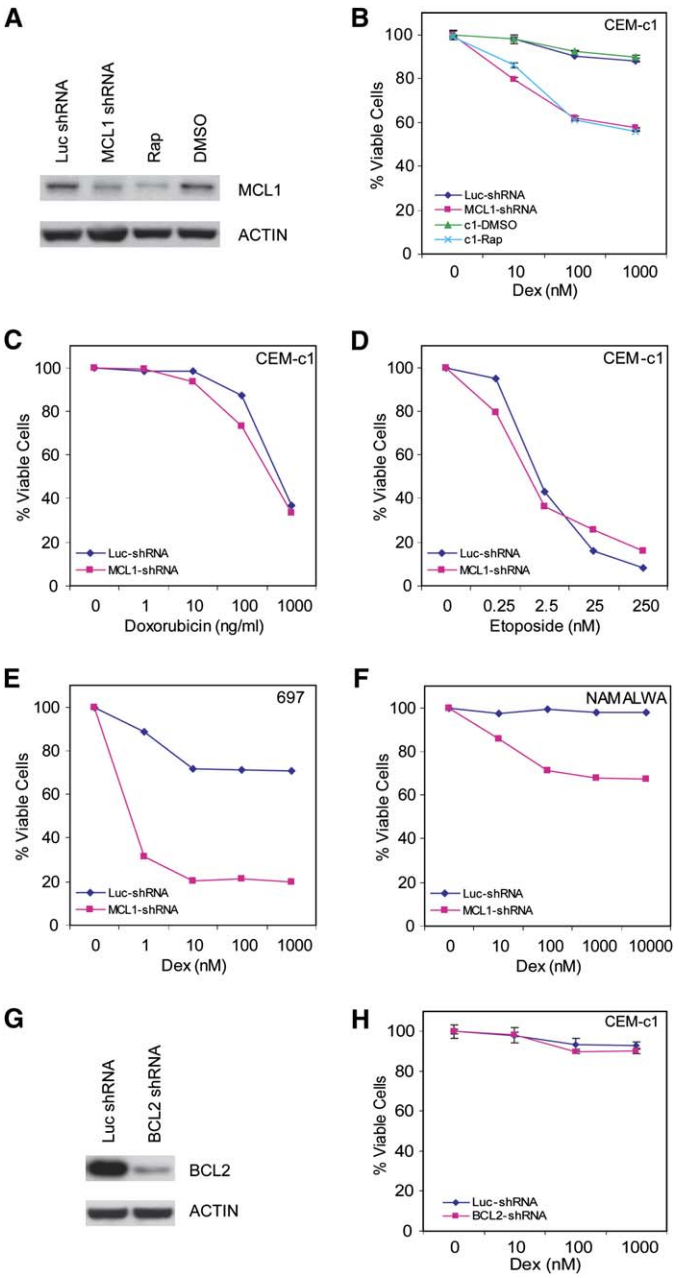

Figure 6. MCL1, but not BCL2, is required for GC resistance in lymphoid malignancy cells

A: MCL1 expression was assessed in CEM-c1 cells transduced with a lentivirus expressing either a luciferase-directed shRNA or an MCL1-directed shRNA, or after treatment with either 10 nM rapamycin or DMSO.

B: CEM-c1 cells treated with either shRNA or rapamycin/DMSO as in **A** were treated with increasing concentrations of dexamethasone. Note that the CEM-c1 cells transduced with the MCL1 shRNA and the rapamycin-treated CEM-c1 cells are equally sensitive to dexamethasone.

C: CEM-c1 cells treated with either control or MCL1-directed shRNAs were treated with increasing concentrations of doxorubicin.

D: CEM-c1 cells treated with either control or MCL1-directed shRNAs were treated with increasing concentrations of etoposide.

E: 697 cells treated with either control or MCL1-directed shRNAs were treated with increasing concentrations of dexamethasone.

F: NAMALWA cells treated with either control or MCL1-directed shRNAs were treated with increasing concentrations of dexamethasone.

G: BCL2 suppression in CEM-c1 cells transduced with a BCL2-directed shRNA.

H: CEM-c1 cells with decreased BCL2 expression are still resistant to dexamethasone-induced apoptosis. Cells were subjected to increasing concentrations of dexamethasone for 24 hr followed by FACS analysis.

Error bars represent the mean ± standard deviation.

between mTOR inhibition and chemotherapy, heightening interest in this approach (Kasukabe et al., 2005; Marimpietri et al., 2005; Mondesire et al., 2004; Wendel et al., 2004). However, it has been difficult to determine where introduction of an mTOR inhibitor might have its greatest clinical impact, perhaps due to the lack of a mechanistic understanding of the observed synergistic effects with chemotherapy, and the fact that it remains unclear to what extent resistance to any particular chemotherapeutic agent is important for outcome in most cancers. As resistance to GC-induced apoptosis is widely recognized as a determinant of poor prognosis in ALL, and based on data presented here, we can now assess inhibition of MCL1 expression as a mechanism for increased sensitivity to glucocorticoids in patients with GC-resistant ALL. Furthermore, as rapamycin is an FDA-approved drug, and glucocorticoids have been safely given in combination to patients post-solid organ transplants, clinical development of such an approach can proceed rapidly. Preliminary studies demonstrate rapamycin sensitization to GC-induced apoptosis in a subset of primary ALL samples. A detailed characterization of such responses is required and will be the focus of future studies. A previous study demonstrated lack of BIM activation as another potential mechanism of GC resistance in ALL (Bachmann et al., 2005), providing support for the idea that the intrinsic apoptotic pathway is critical for GC-induced apoptosis. However, cells unable to activate BIM expression in response to GC might be predicted to be resistant to the GC/rapamycin combination; thus, further study will focus on the identification of samples/patients likely to respond to this drug combination.

An interesting issue is the mechanism by which rapamycin leads to decreased MCL1 and sensitizes to GC-induced apoptosis. Given the broad effects of rapamycin on cellular metabolism, pathways in addition to MCL1 may also contribute. However, as rapamycin and the MCL1 shRNA lead to similar decreases in MCL1 expression and GC sensitization, MCL1 appears to be an important component of rapamycin-mediated sensitization in these studies. MCL1 levels decrease by >50% in rapamycin-treated cells that are sensitized to glucocorticoids. Previous studies using *Mcl1* knockout mice have shown that $Mcl1^{+/-}$ hematopoietic cells are sensitized to some death stimuli (J.T.O., unpublished data). Thus, modest decreases in MCL1 level appear to have a significant effect on the apoptotic threshold to certain stimuli. It is likely that a portion of the rapamycin-mediated MCL1 regulation is via inhibition of mRNA translation. Given that the MCL1 protein is highly regulated and rapidly degraded (Maurer et al., 2006; Nijhawan et al., 2003), inhibition of translation would lead to a rapid decrease in protein level. Whether there is a direct of effect of rapamycin on MCL1 protein degradation is an important area for future investigation.

Specificity of anti- and proapoptotic proteins in cancer drug resistance

Glucocorticoids induce apoptosis via activation of the intrinsic apoptotic program, as demonstrated by resistance to GC-induced apoptosis in BAX/BAK null lymphocytes (Lindsten et al., 2000; Takeuchi et al., 2005). Furthermore, proapoptotic

BIM and PUMA are also necessary for effective GC-induced apoptosis in murine lymphoid cells (Bouillet et al., 1999; Villunger et al., 2003). The demonstration that high-level MCL1 expression is correlated with GC resistance suggests it may play a role in GC resistance (Figure 1) (Holleman et al., 2004). Previous studies demonstrate the importance of Mcl1 in hematopoietic cell survival (Opferman et al., 2003, 2005; Zhou et al., 1998). Our demonstration that overexpression of MCL1 inhibits GC-induced apoptosis and shRNA-mediated inhibition of MCL1 expression sensitizes cells to GC-induced apoptosis provides functional validation of the role of MCL1 in GC-induced apoptosis. It is of interest to note that inhibition of BCL2 expression had no effect on the sensitivity to GC-induced apoptosis in CEM-c1 cells. These data are in keeping with growing evidence that there is functional heterogeneity of the antiapoptotic BCL2 family members (Chen et al., 2005; Kuwana et al., 2005). This has significant implications for the development of combination therapies that might take advantage of small molecules directed to inhibit specific BCL2 family members. For example, potent, specific small molecule inhibitors of BCL2 have recently been reported (Oltersdorf et al., 2005). Our data suggest that such molecules might not be uniformly effective at reversing GC resistance unless they also inhibited MCL1 function. In fact, ABT-737 does not sensitize 2B4 cells expressing MCL1 to dexamethasone-induced apoptosis (Certo et al., 2006). Further characterization of which members of the BCL2 family are involved in the regulation of apoptosis induced by specific therapeutics will help guide more rational, and hopefully more effective, clinical trials.

Gene expression-based drug screening as a tool for drug discovery

An ever-increasing number of gene expression-based genomic analyses are being performed to develop refined signatures of cancer that may be useful for identification of subsets of disease that respond differently to current therapies. However, converting the data contained in these signatures into therapeutic approaches remains difficult. The simplest approach uses the gene expression data to identify a therapeutic target (e.g., a kinase) that is highly expressed in a given tumor (Armstrong et al., 2003). While we have previously used this approach, it requires significant prior knowledge of the importance of the gene of interest or a commitment to fully characterize multiple individual gene products with no guarantee that any will be of relevance. Therefore, we set out to determine if we could identify therapeutic approaches via computational connection of a disease state such as GC resistance to gene expression profiles identified by treatment of cancer cell lines with small molecules. The identification of rapamycin as a GC resistance reversal agent via in silico drug screening provides evidence that such an approach may provide a tool for the development of combination therapies for cancer.

The ability to use gene expression profiles of particular disease states as a tool for drug screening promises to significantly enhance the drug discovery process. We have previously used gene expression profiles as markers for specific cellular phenotypes in high-throughput small molecule screens. This approach, called gene expression-based high-throughput screening, or GE-HTS (Stegmaier et al., 2004), allows one to screen libraries of small molecules for those that reverse (or activate) a signature of interest. The approach described here, the Connectivity Map, is an approach that allows rapid in silico assessment of molecules contained in the database and their ability to reverse signatures associated with specific disease states or drug resistance profiles. Thus, we envision the Connectivity Map as a tool to rapidly assess for potential activity of the thousands of small molecules in the database, and GE-HTS as a complementary approach that is amenable to screening many different libraries of small molecules for a specific activity. Further incorporation of genomic approaches should increase the efficiency of the drug discovery and development process.

Experimental procedures

Microarray analysis of GC-sensitive and GC-resistant ALL samples

Diagnostic bone marrow specimens were characterized as sensitive or resistant to GC-induced apoptosis as previously described (Den Boer et al., 2003; Pieters et al., 1991). Gene expression data were normalized using dChip (Li and Wong, 2001) and filtered with a max-min = 100 and max/min = 4. The probe sets correlated with the sensitive/resistant distinction were determined using a signal-to-noise statistic and permutation testing. The 157 probe sets with $p < 0.0005$ were determined to be part of a GC sensitivity/resistance profile and used for subsequent comparisons. Pediatric ALL samples were obtained either from the German Cooperative ALL (COALL) group or the Erasmus MC, Sophia Children's Hospital. Approval was obtained from the Erasmus MC Institutional Review Board for these studies. Informed consent was obtained according to the Declaration of Helsinki.

Connectivity Map

The current version of the Connectivity Map data set (build01) contains genome-wide expression data for 453 treatment and vehicle control pairs, representing 164 distinct small molecules. A complete description of the data set is provided as Table S1. Enrichment of both the up- and downregulated genes from a given signature in the profiles of each treatment instance were estimated with a metric based on the Kolmogorov-Smirnov statistic, as described (Lamb et al., 2006) and combined to produce a "connectivity score." Connectivity score was set to zero ("null") where the enrichment scores for the up- and downregulated gene sets were of the same sign. Instances were rank ordered in descending order of connectivity score. We used the Kolmogorov-Smirnov statistic to estimate the significance of the concentration of rapamycin instances when ranked by the connectivity score. For 100,000 trials, ten instances were selected at random from the set of 453, and the number of times the statistic for the set of rapamycin instances was equaled or exceeded was recorded. The frequency of this event can be taken as a p value and was found to be zero. All data for the connectivity map can be found at http://www.broad.mit.edu/cmap/ and in GEO.

Cell culture and viability assays

CEM-c1, MOLT4, Raji, Ramos, REH, and NAMALWA cells were purchased from ATCC (Manassas, VA). SU-DHL-1 and SUP-M2 were purchased from DSMZ. All cells were maintained in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum, 2 mM L-glutamine, 1× MEM nonessential amino acids, and 100 μM β-mercaptoethanol. 2B4 cells infected by MIG-Myr-Akt and MIG vector control virus were sorted based on GFP expression. Thymocytes and splenic cells were prepared as described (Opferman et al., 2003) and cultured in 24-well dishes in IMEM media (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and the same supplements as the above RPMI medium. MTT assay was carried out according to the manufacturer's instruction using an MTT kit (Promega). Annexin V (BioVision, Mountain View, CA) and PI staining and FACS analysis of apoptotic cells were performed as described (Opferman and Korsmeyer, 2003). The percentage of viable cells was determined by dividing the number of Annexin V−/PI− cell in the treated sample by the number of Annexin V−/PI− cells in the untreated sample for each time point or concentration. Error bars represent the mean ± standard deviation from duplicate or triplicates.

Mcl1 transgenic mice

A minigene containing the H2K promoter/enhancer and the Moloney MuLV enhancer/poly(A) site driving the expression of mouse Mcl1 cDNA was

injected into zygotes obtained from crosses between F1 (C57BL/6 × C3H) mice. Resulting progeny were tested by Southern blotting, and positive mice were backcrossed to C57BL/6 mice. Four independent founder lines were generated with different expression levels as tested by Western blot. The transgenic mice were generated, housed, and bred in the Dana-Farber Cancer Institute (DFCI) animal facility. All animal experiments were approved by the Dana-Farber Cancer Institute IACUC.

Coimmunoprecipitation

2B4 cells expressing FLAG-MCL1 were treated with 100 nM dexamethasone for 0–24 hr, then lysed in lysis buffer (50 mM Tris-Cl [pH 7.5], NaCl 150 mM, 1% CHAPS, proteinase inhibitors, 1 mM $NaVO_4$, 2 mM NaF) at 4°C for 30 min with rotation. After centrifugation, the supernatant was incubated with beads conjugated with anti-FLAG M2 antibody (Sigma, St. Louis, MO) at 4°C overnight.

Real-time PCR

Real-time PCR was carried out as described using Sybr green (Wei et al., 2004). For each gene, the value was adjusted by ribosomal protein L4. Primers for real-time PCR are as follows: *BIM*: forward, AGACCACCCACG AATGGTTA, reverse, GTGCTGGGTCTTGTTGGTTT; *PUMA*: forward, GACG ACCTCAACGCACAGTA, reverse, CACCTAATTGGGCTCCATCT; human ribosome protein *L4*: forward, CGTTTCTGCATTTGGACTGA, reverse, TCTT GTGCATGGGAAGATTG.

RNAi

Lentiviral shRNA vectors for MCL1, BCL2, Bim, and Luciferase control were kindly provided by Harvard/MIT Broad Institute RNA consortium. The target sequences used for knockdown of the following genes are *MCL1* mRNA (NM_021960), nucleotides 3125–2145 (GCTGTGTTAAACCTCAGAGTT); *BCL2* mRNA (NM_000633), nucleotides 1019–1939 (TGGATGACTGAGTA CCTGAAC); Bim mRNA (NM_009754), nucleotides 35–55 (GTTGGAGCTCT GCGGTCCTTGC).

Microarray data

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession numbers GSE5258, GSE5820, GSE5821, and GSE5822.

Supplemental data

The Supplemental Data include eight supplemental figures and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/4/331/DC1/.

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Accession numbers

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession numbers GSE5258, GSE5820, GSE5821, and GSE5822.