Leukemia (2008) 22, 1728–1736

www.nature.com/leu

ORIGINAL ARTICLE

The dual PI3 kinase/mTOR inhibitor PI-103 prevents p53 induction by Mdm2 inhibition but enhances p53-mediated mitochondrial apoptosis in p53 wild-type AML

K Kojima[1], M Shimanuki[1], M Shikami[2], IJ Samudio[3], V Ruvolo[3], P Corn[4], N Hanaoka[1], M Konopleva[3], M Andreeff[3] and H Nakakuma[1]

[1]Department of Hematology/Oncology, Wakayama Medical University, Wakayama, Japan; [2]Department of Hematology, Aichi Medical University School of Medicine, Aichi, Japan; [3]Department of Stem Cell Transplantation and Cellular Therapy, MD Anderson Cancer Center, The University of Texas, Houston, TX, USA and [4]Genitourinary Medical Oncology, MD Anderson Cancer Center, The University of Texas, Houston, TX, USA

Activation of the phosphatidylinositol-3 kinase/Akt/mammalian target of the rapamycin (PI3K/Akt/mTOR) pathway and inactivation of wild-type p53 by murine double minute 2 homologue (Mdm2) overexpression are frequent molecular events in acute myeloid leukemia (AML). We investigated the interaction of PI3K/Akt/mTOR and p53 pathways after their simultaneous blockade using the dual PI3K/mTOR inhibitor PI-103 and the Mdm2 inhibitor Nutlin-3. We found that PI-103, which itself has modest apoptogenic activity, acts synergistically with Nutlin-3 to induce apoptosis in a wild-type p53-dependent fashion. PI-103 synergized with Nutlin-3 to induce Bax conformational change and caspase-3 activation, despite its inhibitory effect on p53 induction. The PI-103/Nutlin-3 combination caused profound dephosphorylation of 4E-BP1 and decreased expression of many proteins including Mdm2, p21, Noxa, Bcl-2 and survivin, which can affect mitochondrial stability. We suggest that PI-103 actively enhances downstream p53 signaling and that a combination strategy aimed at inhibiting PI3K/Akt/mTOR signaling and activating p53 signaling is potentially effective in AML, where *TP53* mutations are rare and downstream p53 signaling is intact.
Leukemia (2008) **22,** 1728–1736; doi:10.1038/leu.2008.158; published online 12 June 2008
Keywords: PI3K/Akt/mTOR signaling; p53; Mdm2; apoptosis; AML

Introduction

Signaling via the phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) is crucial for divergent physiological processes including transcription, translation, cell-cycle progression and apoptosis.[1–3] Activation of the PI3K/Akt/mTOR pathway results in disrupted proliferation control and apoptosis, resulting in a competitive growth advantage for cancer cells.[3–5] Studies have shown that the PI3K/Akt/mTOR pathway is constitutively activated in 50–70% of acute myeloid leukemia (AML) cases, suggesting that this pathway could constitute a therapeutic target.[6–11]

p53 is the most frequently inactivated protein in human cancer, and more than 50% of all solid tumors carry p53 mutations in the *TP53* gene that abrogate its DNA binding and transactivation activity.[12] Although *TP53* mutations rarely occur in AML, it has been suggested that inactivation of wild-type p53 frequently occurs through binding to its principal cellular regulator murine double minute 2 homologue (Mdm2).[13–15] Mdm2 is a p53-specific E3 ubiquitin ligase, and mediates the ubiquitin-dependent degradation of p53.[16] Mdm2 has been found to be overexpressed in approximately 50% of AML cases,[13–15] a process that can actively enhance tumorigenic potential and resistance to apoptosis.

As many pathway components are frequently affected in AML, effective targeted therapies need to be developed that synergize through inhibition of multiple targets.[10,17–20] Here we report the interactions of PI3K/Akt/mTOR and Mdm2/p53 pathways following simultaneous inhibition of PI3K/mTOR signaling utilizing the dual inhibitor PI-103 and activation of p53 signaling by Mdm2 inhibitor Nutlin-3.[21–23] We found that PI-103 enhanced Nutlin-induced, p53-mediated Bax conformational change and mitochondrial apoptosis of AML cells. However, inhibition of PI3K/Akt/mTOR signaling negatively regulated basal and Nutlin-induced p53 levels. The reduced p53 induction appeared attributable to mTOR inactivation, which attenuates protein synthesis of p53 at the mRNA translational level. The synergistic apoptotic effects of PI-103 and Nutlin-3 or PI-103 and the DNA damaging agent doxorubicin depended on wild-type p53 status. These findings suggest that PI-103 actively enhances downstream p53 signaling and that a combination strategy aimed at inhibiting PI3K/Akt/mTOR signaling and activating p53 signaling is potentially effective in AML, where *TP53* mutations are rare and downstream p53 signaling is intact.[23]

Materials and methods

Reagents

The dual PI3K/mTOR inhibitor PI-103, the PI3K inhibitor LY294002, the mTOR inhibitor rapamycin and the selective small-molecule antagonist of Mdm2, Nutlin-3, were purchased from Axxora (San Diego, CA, USA), dissolved in dimethyl sulfoxide (DMSO) and kept frozen at −20 °C. In some experiments, cells were cultured with 50 μM Z-VAD-FMK (Axxora). Z-VAD-FMK was added to the cells 1 h before drug administration. The final DMSO concentration in the medium did not exceed 0.1% (v/v). At this concentration, DMSO itself had no effect for up to 72 h on cell growth or viability of AML cells used in this study.

Cell lines, primary samples and cultures

AML cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS). OCI-AML-3 and

Correspondence: Dr K Kojima, Department of Hematology/Oncology, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8510, Japan.
E-mail: k-koji@wakayama-med.ac.jp
Received 22 January 2008; revised 17 May 2008; accepted 20 May 2008; published online 12 June 2008

MOLM-13 cells have wild-type p53. In HL-60, p53 is disabled by a large deletion of the *TP53*. OCI-AML-3 cells were transduced with retroviruses encoding either p53-specific shRNA (nucleotides 611–629, GenBank accession no. NM000546) or scrambled shRNA and stable shRNA-expressing cells were generated.[24] The corresponding oligonucleotides were annealed and cloned under the control of the H1 promoter into a self-inactivating lentiviral vector. The vector was also designed to carry the green fluorescent protein reporter gene under control of the human ubiquitin-C promoter to monitor infection efficiency. Lentiviral infections were done essentially as previously described,[24] and the potency and specificity of each construct were determined by protein immunoblotting. Cell lines were harvested in log-phase growth, seeded at a density of 2×10^5 cells per ml and exposed to PI-103 and/or Nutlin-3 simultaneously. Heparinized bone marrow and peripheral blood samples with more than 70% leukemia cells were obtained from previously untreated AML patients after informed consent, according to institutional guidelines per Declaration of Helsinki. Mononuclear cells were purified by Ficoll-Hypaque (Sigma Chemical Co., St Louis, MO, USA) density-gradient centrifugation and nonadherent cells were resuspended in RPMI 1640 medium supplemented with 10% FCS at a density of 5×10^5 cells per ml. Cell viability was evaluated by triplicate counts of trypan blue dye-excluding cells.

Apoptosis analysis

Evaluation of apoptosis by the Annexin V-propidium iodide (PI) binding assay was performed as described.[23] To measure mitochondrial membrane potential ($\Delta\psi_m$), cells were loaded with MitoTracker Red CMXRos (300 nM) and MitoTracker Green (100 μM, both from Molecular Probes, Eugene, OR, USA) for 1 h at 37 °C. The ψ_m was then assessed by measuring CMXRos retention (red fluorescence) while simultaneously adjusting for mitochondrial mass (green fluorescence). All experiments were conducted in triplicate.

Western blot analysis

Western blot analysis was performed as previously described.[23] The following antibodies were used: rabbit polyclonal anti-p53 (FL-393; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-Mdm2 (D-12; Santa Cruz Biotechnology); rabbit polyclonal anti-phospho-Mdm2 (Ser^{166}); mouse monoclonal anti-p21 (Ab-1; EMD Biosciences, San Diego, CA, USA); rabbit polyclonal anti-Puma (Ab-1; EMD Biosciences); mouse monoclonal anti-Noxa (114C307; EMD Biosciences); rabbit polyclonal anti-Bax (Cell Signaling Technology, Beverly, MA, USA); mouse monoclonal anti-Bcl-2 (Dako Cytomation, Carpinteria, CA, USA); rabbit polyclonal anti-survivin (R&D Systems, Minneapolis, MN, USA); rabbit polyclonal anti-caspase 3 (Cell Signaling Technology); rabbit polyclonal anti-Akt (Cell Signaling Technology); rabbit monoclonal anti-phospho-Akt (Ser^{473}; Cell Signaling Technology); rabbit monoclonal anti-p70 S6 kinase (Cell Signaling Technology); rabbit polyclonal anti-phospho-p70 S6 kinase (Thr^{389}; Cell Signaling Technology); rabbit polyclonal anti-4E-BP1 (Cell Signaling Technology); rabbit monoclonal anti-phospho-4E-BP1 ($Thr^{37/46}$; Cell Signaling Technology) and mouse monoclonal anti-β-actin (AC-74; Sigma Chemical Co.).

Cell cycle analysis

Cells were permeabilized in 70% ice-cold ethanol, incubated overnight with PI solution (25 μg/ml PI) and analyzed as described previously.[23] Data were gated on the FL2-area versus FL2-width cytogram to exclude doublets and aggregates, and a minimum of 3×10^4 gated cells was analyzed per sample.

Quantitation of intracellular proteins by flow cytometry

For intracellular p53 detection, cells were fixed with 2% paraformaldehyde, permeabilized with 100% ice-cold methanol and incubated for 1 h at 4 °C with antibody against p53 or its isotypic control (BD Biosciences, San Jose, CA, USA).[25] Involvement of Bax conformational change was analyzed by means of an antibody directed against the NH_2-terminal region of Bax (YTH-6A7; Trevigen, Gaithersburg, MD, USA).[25] Cellular fixation, permeabilization and staining with primary antibody or an isotypic control were performed using the Dako IntraStain kit (Dako Cytomation), according to the manufacturer's instructions. After washing, cells were incubated with Alexa Fluor 488 chicken anti-mouse secondary antibodies (Molecular Probes) for 30 min at 4 °C. Total Bax levels were determined by using polyclonal anti-Bax antibodies (Cell Signaling Technology) and Alexa Fluor 488 chicken anti-rabbit secondary antibodies (Molecular Probes).

Real-time quantitative PCR

OCI-AML-3 cells were treated with 5 μM PI-103 and/or 5 μM Nutlin-3a for up to 4 h. RNA was prepared from cells using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and first-strand cDNA was generated using random hexamers (SuperScript III First-Strand Synthesis SuperMix; Invitrogen, Carlsbad, CA, USA) from 1 μg total RNA. The mRNA expression levels of p53 and 18S were quantified using *Taq*Man gene expression assays (p53: 4319446F, 18S: 4319413E, Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7000 Sequence Detection System, as described previously.[18]

RT-PCR and DNA sequencing of p53

Total RNAs were extracted from bone marrow and peripheral blood mononuclear cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed with oligo(dT) as primer (Superscript II System; Invitrogen). PCR for p53 gene expressions followed by direct sequencing were performed as described previously.[23]

Statistical analysis

The statistical analysis was performed using the 2-tailed Student's *t*-test and the Pearson's correlation coefficient. Statistical significance was considered when $P<0.05$. Average values were expressed as mean ± s.d. Synergism, additive effects and antagonism were assessed as previously described.[23] The combination index (CI), a numerical description of combination effects, was calculated using the more stringent statistical assumption of mutually nonexclusive modes of action. When $CI = 1$, this equation represents the conservation isobologram and indicates additive effects. CI values less than 1.0 indicate a more than expected additive effect (synergism), whereas CI values more than 1.0 indicate antagonism between the two drugs.

Results

PI3K/mTOR inhibition by PI-103 enhances Nutlin-induced, p53-mediated apoptosis in AML cell lines

We first examined the effect of the dual PI3K/mTOR inhibitor PI-103 on the growth and viability of cultured AML cell lines. OCI-AML-3 and MOLM-13 cells had wild-type p53 and HL-60 cells deleted p53. OCI-AML-3 and MOLM-13 cells constitutively expressed phosphorylated Akt and 4E-BP1 (Figure 1a). HL-60 cells expressed phosphorylated 4E-BP1 but lacked phosphorylated Akt. OCI-AML-3 cells were treated with increasing concentration of PI-103 for 24 h, and the activity was measured by monitoring phosphorylation of Akt and 4E-BP1. PI-103 almost completely (>95%) blocked the constitutive phosphorylation of Akt at 5 μM in OCI-AML-3 cells (Figure 1b). PI-103 partially inhibited phosphorylation of 4E-BP1, with 50% inhibition at 6.3 μM. PI-103 inhibited the proliferation of AML cells by inducing G_1-phase cell cycle arrest (Table 1) whereas it exhibited modest cytotoxic activity associated with induction of apoptosis (Figure 1c). These results are consistent with previously published data.[21] To clarify if PI-103 enhances p53-dependent apoptosis, we next combined PI-103 and Nutlin-3. As shown in Figure 1c, PI-103 augmented Nutlin-induced phosphatidylserine externalization in wild-type p53 cells. The interaction study showed synergistic effects in OCI-AML-3 and MOLM-13 cells. The CI values were 0.29 for ED50, 0.29 for ED75 and 0.31 for ED90 in OCI-AML-3 cells, and were 0.54, 0.49 and 0.45, respectively, in MOLM-13 cells. The averaged CI values calculated from the values for ED50, ED75 and ED90, were 0.29 in OCI-AML-3 cells and 0.48 in MOLM-13 cells. These findings suggest that PI-103 synergizes with Nutlin-3 to induce apoptosis in AML cells with wild-type p53. Such potentiation effect was not seen in Nutlin-resistant HL-60 cells that harbor mutant p53 (Figure 1c). To confirm that the synergism depends on p53, we investigated the combination effect of PI-103 and Nutlin-3 on apoptosis induction in OCI-AML-3 cells infected with retroviruses encoding either scrambled shRNA or p53-specific shRNA. Knockdown of p53 in OCI-AML3 cells not only rendered this cell line resistant to Nutlin-induced apoptosis but also attenuated apoptosis induction by the PI-103 and Nutlin-3 combination (Figures 1d and e). As PI-103 potentiated p53-mediated apoptosis at concentration as low as 0.5 μM in OCI-AML-3 cells (Figure 1f), it is unlikely that off-target effect of PI-103 plays a central role in the synergistic apoptotic effect between PI-103 and Nutlin-3.

PI-103 enhances p53-mediated Bax activation

Because PI-103 itself had a modest apoptogenic effect, we hypothesized that PI3K/mTOR inhibition could actively enhance p53 signaling. p53-mediated apoptosis pathways converge upon interaction between anti- and proapoptotic Bcl-2 family proteins, resulting in activation and conformational change of the proapoptotic Bax.[26] Involvement of Bax conformational change was analyzed by an antibody directed against the NH2-terminal region of Bax (clone YTH-6A7). The epitope-specific antibody can react only with Bax in active conformation because the NH2-terminal region is occluded in unstressed intact cells.[27] Cells were preincubated for 1 h in the presence of 50 μM Z-VAD-FMK before the addition of PI-103, Nutlin-3 or both to inhibit caspase activation-mediated Bax cleavage. As shown in Figure 2a, few control cells were stained with this antibody. Irrespective of p53, PI-103 induced conformational change of Bax. As reported, an increase in the

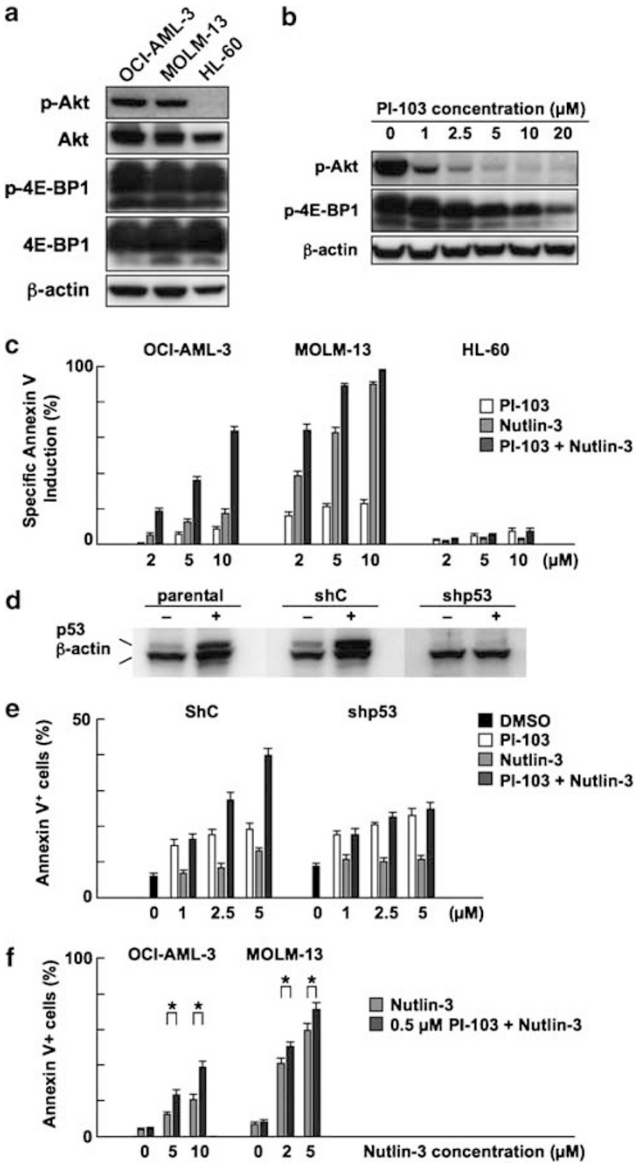

Figure 1 Inhibition of phosphatidylinositol-3 kinase (PI3K)/mammalian target of the rapamycin (mTOR) enhances p53-dependent apoptosis in acute myeloid leukemia (AML) cells. (**a**) Expression of phosphorylated Akt and 4E-BP1 in AML cell lines. OCI-AML-3 and MOLM-13 cells constitutively expressed phosphorylated Akt. (**b**) Expression of phosphorylated Akt and 4E-BP1 in OCI-AML-3 cells after 24-h treatment with the indicated concentrations of PI-103. β-Actin was used to confirm equal loading of proteins. (**c**) AML cells were incubated with the indicated concentrations of PI-103 or Nutlin-3 for 24 h, and the Annexin V-positive fractions were measured by flow cytometry. Results are expressed as mean ± s.d. PI-103 enhanced Nutlin-induced apoptosis in p53 wild-type OCI-AML-3 and MOLM-13 cells but not in mutant p53 HL-60 cells. (**d**) Parental OCI-AML-3 cells and those transduced with retroviruses encoding either scrambled shRNA (shC) or p53-specific shRNA (shp53) were treated with 5 μM Nutlin-3 for 4 h, and p53 expression was determined by western blot analysis. (**e**) OCI-AML-3 cells were transduced with retroviruses encoding either scrambled shRNA (shC) or p53-specific shRNA (shp53) and stable shRNA-expressing cells were generated. The cells were incubated with the indicated concentrations of PI-103 or Nutlin-3 for 24 h, and the Annexin V-positive fractions were measured by flow cytometry. Results are expressed as mean ± s.d. (**f**) OCI-AML-3 cells were treated for 24 h with the indicated concentrations of Nutlin-3 in the absence or presence of 0.5 μM PI-103, and the Annexin V-positive fractions were measured by flow cytometry. Results are expressed as mean ± s.d. *indicates significance at $P<0.05$.

percentage of active Bax-positive cells following incubation with Nutlin-3 was only seen in p53 wild-type OCI-AML-3 and MOLM-13 cells.[18,23,28] Interestingly, PI-103 considerably enhanced Bax conformational change by Nutlin-3 in the p53 wild-type cells, suggesting that PI3K/mTOR inhibition actively enhances p53-mediated mitochondrial apoptotic pathway. When Bax antibodies that detect total Bax were used, no differences in the fluorescence pattern between control and drug-treated cells were observed (not shown).

Table 1 Antiproliferative activity of PI-103

Cells	*Compound*	*Cell cycle distribution (%)*		
		G_1	S	G_2/M
OCI-AML-3	DMSO	51 ± 3	35 ± 4	14 ± 1
	PI-103	76 ± 3	19 ± 2	5 ± 1
MOLM-13	DMSO	48 ± 4	37 ± 4	15 ± 2
	PI-103	83 ± 3	10 ± 1	7 ± 2
HL-60	DMSO	46 ± 4	33 ± 4	21 ± 1
	PI-103	77 ± 2	17 ± 2	6 ± 1

Abbreviations: AML, acute myeloid leukemia; DMSO, dimethyl sulfoxide; PI, propidium iodide.
AML cells were treated with 5 μM PI-103 for 24 h and examined by flow cytometry.

PI-103 reduces basal and Nutlin-induced p53 levels

To examine if the synergistic apoptotic effect between PI-103 and Nutlin-3 is associated with p53 induction, changes in p53 levels were assessed after 12-h treatment. Unexpectedly, PI-103 treatment of p53 wild-type AML cells reduced basal p53 levels and inhibited p53 induction by Nutlin-3 (Figures 2b–d), suggesting that PI-103 actively enhances p53-mediated apoptosis downstream of p53.

PI-103 inhibits protein synthesis of p53 at the mRNA translational level via mTOR inhibition

To monitor the fate of previously stabilized pools of p53, OCI-AML-3 cells were preincubated for 1 h with 70 μM cycloheximide, and 5 μM PI-103 was added. Cycloheximide did not affect the rate of decrease in p53 levels after PI-103 treatment (Figure 3a), suggesting that PI-103 would reduce p53 levels via the blockage of new protein synthesis. We next investigated if the reduction of p53 levels is prevented by the proteasome inhibitor MG132. After 1 h preincubation with 0.2 μM MG132, OCI-AML-3 cells were treated with 5 μM PI-103. MG132 did not block p53 reduction after PI-103 treatment (Figure 3a), indicating that the mechanism(s) of p53 decrease is independent of ubiquitilation. We treated OCI-AML-3 cells with PI-103 or Nutlin-3 for 4 h and monitored the expression of p53 mRNA by

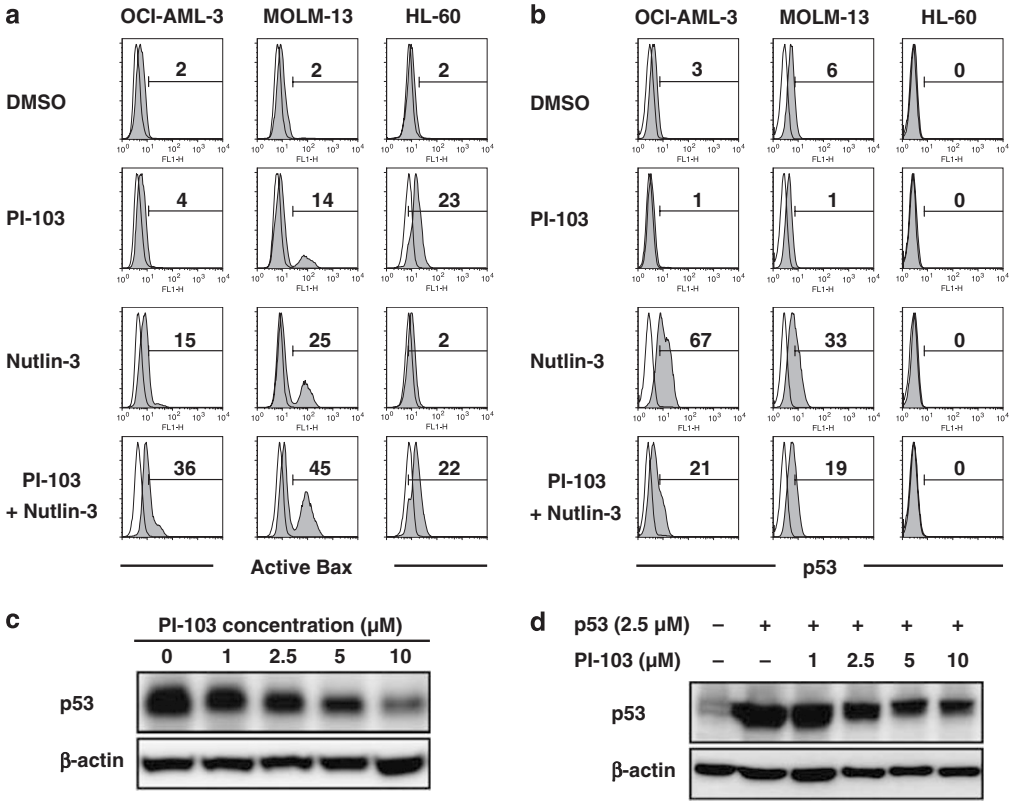

Figure 2 Phosphatidylinositol-3 kinase (PI3K)/mammalian target of the rapamycin (mTOR) inhibition enhances p53-mediated Bax activation whereas reducing basal and Nutlin-induced p53 levels. (**a**, **b**) Cells were treated for 12 h with 5 μM PI-103 or 5 μM Nutlin-3 (2 μM in MOLM-13 cells) either as individual agents or in combination. (**a**) Bax conformational change was determined by staining with the active conformation-specific anti-Bax antibody YTH-6A7 (shaded histogram) or a corresponding isotype control (open histogram). To block caspase activation-mediated conformational change of Bax, cells were preincubated for 1 h with 50 μM Z-VAD-FMK. (**b**) p53 levels were evaluated by staining with the anti-p53 antibody (shaded histogram) or a corresponding isotype control (open histogram). Results are representative of three independent experiments. (**c**) Expression of p53 in OCI-AML-3 cells after 24-h treatment with the indicated concentrations of PI-103. β-Actin was used as a loading control. (**d**) Expression of p53 in OCI-AML-3 cells after 24-h treatment with the indicated concentrations of PI-103 in the absence or presence of 2.5 μM Nutlin-3. β-actin was used as a loading control.

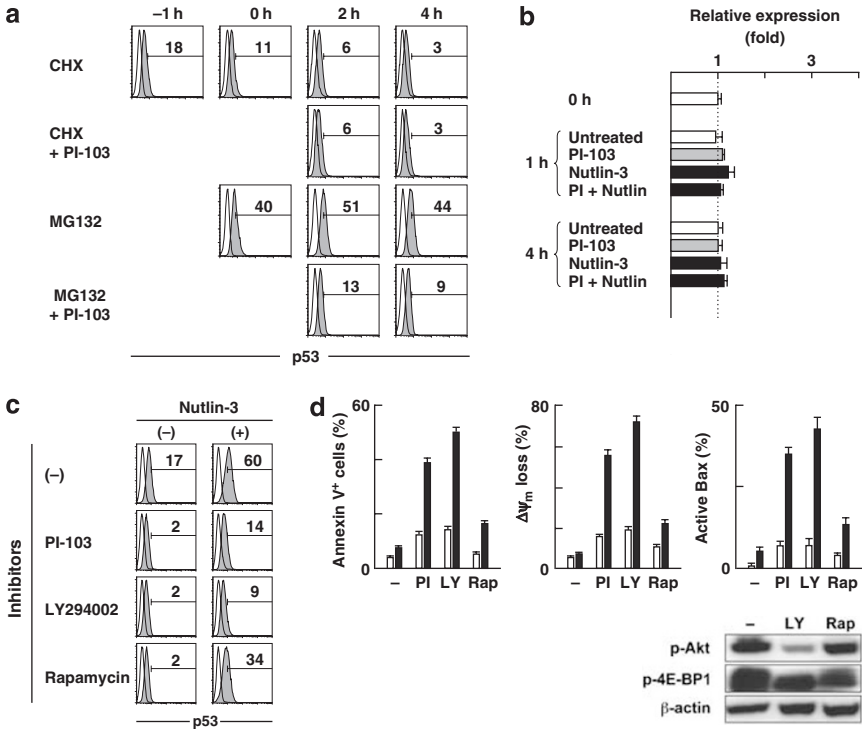

Figure 3 Phosphatidylinositol-3 kinase (PI3K)/mammalian target of the rapamycin (mTOR) inhibition prevents p53 synthesis. (**a**) OCI-AML-3 cells were preincubated for 1 h with 70 μM cycloheximide (CHX) or 0.2 μM MG132, and p53 levels after the addition of 5 μM PI-103 were monitored. Results are representative of three independent experiments. (**b**) OCI-AML-3 cells were treated with 5 μM PI-103 and 5 μM Nutlin-3 for 4 h, either as individual agents or in combination (PI + Nutlin), and p53 transcripts were quantitated by real-time RT-PCR. Each real-time PCR was performed in triplicate, and the average fold induction relative to time 0 is shown with s.d. (**c**) OCI-AML-3 cells were treated for 12 h with 5 μM PI-103, 25 μM LY294002 or 0.02 μM rapamycin in the absence or presence of 5 μM Nutlin-3, and p53 levels were evaluated by flow cytometry. Dimethyl sulfoxide (DMSO)-treated cells served as the control. Results are representative of three independent experiments. (**d**) OCI-AML-3 cells were treated for 12 h with 5 μM PI-103 (PI), 25 μM LY294002 (LY) or 0.02 μM rapamycin (Rap) in the absence or presence of 5 μM Nutlin-3, and their apoptotic effects were investigated. PI3K/mTOR inhibitors reinforced induction of p53-mediated apoptosis. Comparable results were obtained in two other independent experiments. LY294002 blocked phosphorylation of Akt and 4E-BP1 that signals downstream of PI3K, whereas the mTOR inhibitor rapamycin interfered only with the phosphorylation status of 4E-BP1.

real-time PCR. In these conditions, PI-103 did not significantly affect the p53 mRNA levels (Figure 3b). These findings suggest that PI-103 could inhibit p53 synthesis at the translational level. PI-103 is a dual PI3K/mTOR inhibitor and we next investigated if single inhibition of PI3K or mTOR could reduce p53 levels. OCI-AML-3 cells were treated with the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin, and p53 levels were determined. As shown in Figure 3c, both LY294002 and rapamycin reduced basal and Nutlin-induced p53 levels. As mTOR activation is downstream of PI3K signaling, mTOR inhibition appeared to be a component that contributes to p53 inactivation. We also examined the effect of LY294002 and rapamycin on Nutlin-induced apoptosis. Interestingly, despite their inhibitory effect on p53 induction, both LY294002 and rapamycin synergize with Nutlin-3 to induce phosphatidylserine externalization, Bax conformational change and mitochondrial membrane permeabilization (Figure 3d).

PI3K/mTOR inhibition results in decreased expression of p53-related pro- and antiapoptotic Bcl-2 family proteins and activation of caspases in AML cells

To investigate the molecular events that contribute to apoptosis after PI-103/Nutlin-3 treatment, OCI-AML-3 cells were treated for 12 h with 5 μM PI-103 or 5 μM Nutlin-3, either as individual agents or in combination, and p53-related pro- and antiapoptotic protein levels were investigated. In accordance with the results of flow cytometry, PI-103 reduced basal and Nutlin-induced p53 levels (Figure 4a). Interestingly, PI-103 treatment resulted in decreased expression of many pro- and antiapoptotic proteins (Figure 4a). Levels of downstream transcriptional targets of p53 including Mdm2, p21 and Noxa were decreased. Noxa is a major p53-induced proapoptotic Bcl-2 family protein. Levels of the antiapoptotic proteins Bcl-2 and survivin were also decreased. Puma, Bax and β-actin levels did not change significantly. Nutlin-3 induced proapoptotic Noxa and Bax as well as Mdm2 and p21 (Figure 4a). Although extended p53 activation reduces Bcl-2 and survivin levels,[28] their decrease was not evident after a 12-h exposure to Nutlin-3. The PI-103/Nutlin-3 combination disrupted the balance of anti- and proapoptotic Bcl-2 family proteins, resulting in relatively decreased levels of antiapoptotic Bcl-2 and survivin levels (Figure 4a). Bax, Puma, Bcl-2 and survivin were detectable in p53-deleted HL-60 cells, and their levels were decreased after PI-103 treatment (Figure 4b), suggesting that PI-103 reduces various p53-related proteins in a p53-independent fashion. PI-103 induced cleavage of caspase 3, which was enhanced by Nutlin-3 cotreatment in p53 wild-type OCI-AML-3 cells (Figure 4c). Akt has been known to induce phosphorylation of Mdm2 at Ser^{166}, allowing Mdm2-mediated ubiquitination and degradation of p53.[29] The possibility that PI-103 paradoxically phosphorylates Mdm2 was excluded because PI-103 inhibited phosphorylation of Akt and Mdm2 (Figures 4a and 5).

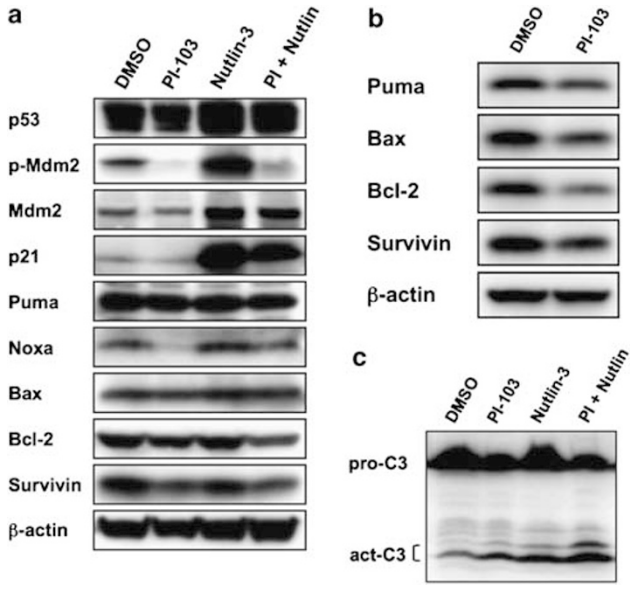

Figure 4 PI-103 reduces levels of p53-related pro- and antiapoptotic Bcl-2 family proteins and induces caspase activation. (**a**) Expression of p53-related proteins in OCI-AML-3 cells, which were treated for 12 h with 5 μM PI-103 or 5 μM Nutlin-3 either as individual agents or in combination. β-Actin was used to confirm equal loading of proteins. (**b**) Expression of Puma, Bax, Bcl-2 and survivin in p53-deleted HL-60 cells after 12-h treatment with 5 μM PI-103. (**c**) PI-103 induced caspase-3 activation in OCI-AML-3 cells after 16 h. Cleavage of caspase-3 was most evident when cells were treated with PI-103 and Nutlin-3 in combination.

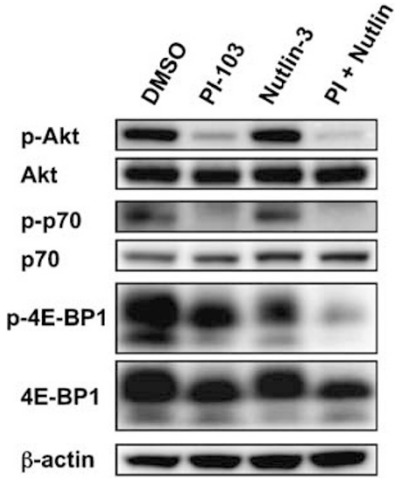

Figure 5 PI-103 cooperated with Nutlin-3 to block phosphorylation of 4E-BP1. Activities of PI-103 and Nutlin-3 against the phosphatidylinositol-3 kinase (PI3K)/mammalian target of the rapamycin (mTOR) pathway were assessed in OCI-AML-3 cells after 12-h treatment with 5 μM PI-103 or 5 μM Nutlin-3. Dimethyl sulfoxide (DMSO)-treated cells served as the control. Results are representative of three independent experiments.

PI-103/Nutlin-3 combination abrogates phosphorylation of 4E-BP1

We investigated the effect of PI-103/Nutlin-3 on PI3K/mTOR signaling. PI-103 was effective in blocking phosphorylation of Akt that signals downstream of PI3K (Figure 5). Nutlin-3 did not interfere with the phosphorylation status of Akt. PI-103 also blocked the phosphorylation of p70 S6 kinase and 4E-BP1, downstream markers of mTOR signaling (Figure 5). Interestingly, Nutlin-3 treatment resulted in decreased levels of phosphorylated 4E-BP1. Furthermore, the PI-103/Nutlin-3 combination almost completely abrogates phosphorylation of 4E-BP1 in the absence of apoptosis of this timepoint. Translation repressor protein 4E-BP1 inhibits cap-dependent translation by binding to the eIF4E translation initiation factor and dephosphorylation of 4E-BP1 results in inactivation of translation.[30] Thus, cooperative dephosphorylation of 4E-BP1 by PI-103 and Nutlin-3 might contribute to decreased expression of a wide range of proteins.

PI-103 synergizes with doxorubicin to induce apoptosis in AML cells

Anthracyclines are one of the most active chemotherapeutic agents for the therapy of AML and they remain the backbone of induction and consolidation regimens. We investigated if PI3K/mTOR inhibition potentiates the apoptotic effect of doxorubicin, a DNA damaging agent, which activates p53. AML cells were treated with PI-103 and doxorubicin for 24 h, either as individual agents or in combination. OCI-AML-3 cells were treated with doxorubicin at 0, 100, 250 or 500 nM and MOLM-13 and HL-60 cells at 0, 50, 100 or 250 nM. The concentration ratio of doxorubicin to Nutlin was 1:10. The results indicated a synergistic interaction of PI-103 and doxorubicin in induction of apoptosis in OCI-AML-3 and MOLM-13 cells (Table 2). PI-103 and doxorubicin showed a moderate antagonistic effect on apoptosis induction in HL-60 cells. As doxorubicin has p53-independent mechanisms to induce apoptosis, it remains unclear which mechanism(s) of doxorubicin could antagonize PI-103 to induce apoptosis. To further investigate the role of p53 in the interaction between PI-103 and doxorubicin, we used OCI-AML-3 cells with retroviruses encoding either scrambled shRNA or p53-specific shRNA. The interaction study showed synergistic effects in parental and scrambled OCI-AML-3 cells but not in p53 knockdown cells (Figure 6). The averaged CI values were 0.67 in parental cells (0.70 for ED50, 0.68 for ED75, 0.66 for ED90, 0.64 for ED95), 0.76 in scrambled cells (0.71 for ED50, 0.74 for ED75, 0.79 for ED90, 0.83 for ED95) and 1.56 in p53 knockdown cells (1.21 for ED50, 1.43 for ED75, 1.69 for ED90, 1.91 for ED95). We suggest that the synergistic interaction of PI-103 and doxorubicin appeared to at least partially depend on p53 status (Figure 6).

PI-103 augments p53-mediated apoptosis in primary AML cells

We cultured primary cells from seven AML patients with more than 70% blasts with PI-103 and/or Nutlin-3, and evaluated apoptosis after 48 h. *TP53* mutation was not detected in these cases. The cells expressed phosphorylated Akt and 4E-BP1 with a wide degree of variation (Figure 7a). The protein expression levels relative to an internal control β-actin ranged from 0.4 to 4.5 in phosphorylated Akt and from 1.3 to 3.2 in phosphorylated 4E-BP1. There was no significant correlation between relative levels of phosphorylated Akt and 4E-BP1 ($r=0.64$, $P=0.12$). We then investigated the correlation between their expression levels and degree of apoptosis by PI-103. There was not a significant correlation between relative phosphorylated Akt or 4E-BP1 levels with the percentage of PI-103-induced specific Annexin V induction ($r=0.24$, $P=0.61$ in phosphorylated Akt and $r=0.55$, $P=0.20$ in phosphorylated 4E-BP1). The cooperative

Table 2 Combination index values for apoptotic effects of doxorubicin and Nutlin-3

	ED50	*ED75*	*ED90*	*ED95*	*Averaged CI*[a]
OCI-AML-3	0.71	0.62	0.55	0.50	0.60
MOLM-13	0.75	0.66	0.58	0.53	0.63
HL-60	1.45	1.34	1.26	1.21	1.32

Abbreviations: AML, acute myeloid leukemia; CI, combination index.
[a]The averaged CI values were calculated from the ED_{50}, ED_{75} and ED_{90} and ED_{95}.

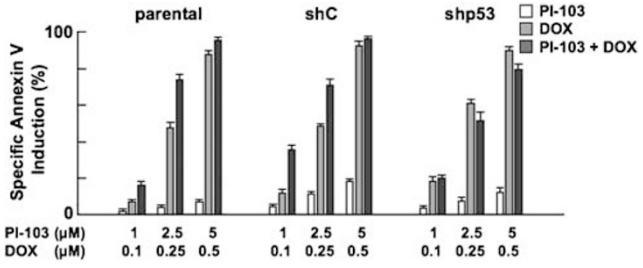

Figure 6 Inhibition of phosphatidylinositol-3 kinase (PI3K)/mammalian target of the rapamycin (mTOR) synergizes with doxorubicin to induce apoptosis in a p53-dependent manner. Parental and retrovially transduced OCI-AML3 cells (virus encoding either scrambled shRNA (shC) or p53-specific shRNA (shp53)) were incubated with the indicated concentrations of PI-103 or doxorubicin for 24 h, and the Annexin V-positive fractions were measured by flow cytometry. Results are expressed as mean ± s.d.

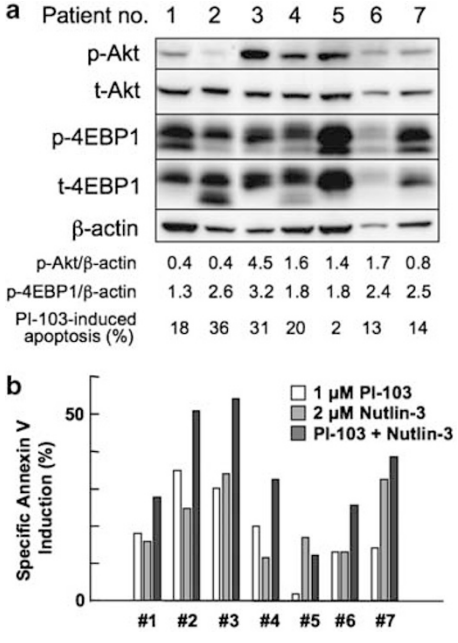

Figure 7 PI-103 augments p53-mediated apoptosis in primary acute myeloid leukemia (AML) cells. (**a**) Expression of phosphorylated Akt and 4E-BP1 in primary AML cells. The expression levels of phosphorylated Akt and 4E-BP1 relative to an internal control β-actin, and the percentage of specific Annexin V induction after 48-h treatment with 1 μM PI-103 are shown. (**b**) Primary AML cells from seven AML patients were incubated for 48 h with 1 μM PI-103 or 2 μM Nutlin-3, either as individual agents or in combination, and the Annexin V-positive fractions were measured by flow cytometry.

nature of the PI-103/Nutlin-3 interaction was seen in six of seven primary samples (Figure 7b).

Discussion

Our data support a mechanistic rationale by which inhibition of PI3K/Akt/mTOR signaling can augment p53-mediated apoptosis. As inhibitors directed at PI3K (LY294002) or mTOR (rapamycin) alone enhanced Nutlin-induced apoptosis, as was seen for the dual PI3K/mTOR inhibitor PI-103, one of the targets for enhancing p53-mediated apoptosis appeared to be mTOR. It has been shown that PI3K/Akt/mTOR signaling includes a negative feedback loop originating from mTOR and targeting an upstream component of the signaling chain.[21,31,32] Although inhibition of mTOR generally leads to a cytostatic cellular response, it has been shown to greatly enhance the proapoptotic effect of cytotoxic agents known to act through different mechanisms.[33–35] Several mechanisms have been described, including downregulation of antiapoptotic Bcl-2 family proteins or inhibition of p21 translation, both of which were found in our PI-103/Nutlin-3 combination.[33–35] Inhibition of Akt is another candidate mechanism for enhancing p53-mediated apoptosis, because synergistic Bax activation was much more prominent in the PI-103/Nutlin-3 or LY294002/Nutlin-3 combination than in the rapamycin/Nutlin-3 combination. Akt is the physiological Bax kinase responsible for abrogation of Bax-mediated proapoptotic activities.[1] Akt inhibits the Bax conformational change, preventing disruption of the mitochondrial membrane potential and caspase-3 activation.[36,37] The dual PI3K/mTOR inhibitor PI-103 has been shown to shut down the negative feedback from mTOR and to efficiently reduce Akt phosphorylation.[21] The inhibitory effect of PI-103 on Akt activation may explain why PI-103 (and LY294002) more efficiently augmented p53-mediated apoptosis than rapamycin.

We found that Nutlin-3 blocks phosphorylation of 4E-BP1, a downstream marker of mTOR. 4E-BP1 is an mRNA translation repressor and its phosphorylation is associated with active mRNA translation.[30] Consistent with our results, using murine leukemia cells expressing a temperature-sensitive p53 or a doxycycline-inducible lung carcinoma cell line model capable of expressing p53, it has been reported that wild-type p53 activation dephosphorylates 4E-BP1 and inhibits translation initiation.[38,39] Cooperative dephosphorylation of 4E-BP1 by PI-103 and Nutlin-3 suggests that mTOR inhibition and p53 activation strongly disrupt mTOR-mediated translational control, resulting in imbalanced expression of pro- and antiapoptotic proteins and unstable mitochondrial membrane potential.

There was no significant correlation between the levels of phosphorylated Akt and those of phosphorylated 4E-BP1 in primary AML samples. Such discrepancy was also seen in HL-60 cells, which showed high levels of phosphorylated 4E-BP1 with undetectable levels of phosphorylated Akt. It has been reported that mTOR activity could be controlled through the Erk pathway.[40–42] As constitutive Erk activation is seen in a majority of clinical AML specimens as well as in HL-60 cells,[43] activation of the Raf/Mek/Erk pathway could mediate 4E-BP1 phosphorylation independent of the status of the PI3K/Akt/mTOR signaling. Identification of predictive biomarkers for PI-103 would allow identification of patients who are most likely to benefit from dual PI3K/mTOR inhibition. In our series of primary samples, however, neither phosphorylated Akt nor phosphorylated 4E-BP1 levels were predictive of apoptotic response to PI-103. This could be attributable to the cellular effects of

PI3K/mTOR inhibition, which is cytostatic rather than cytotoxic.[21] Another possibility is that deregulation of the Raf/Mek/Erk pathway is a determinant in AML cell resistance to PI-103, as recently reported in cancer cell lines to PI3K inhibitors.[44] Further investigation is needed to clarify the predictive biomarkers for apoptosis induced by PI-103 or PI-103/Nutlin-3 combination.

A promising quality of PI-103 is its lack of toxicity in preclinical mouse models.[21] Recent preliminary data suggest that PI-103 effectively inhibits AML progenitor growth with relatively little effect on normal hematopoietic progenitors.[45] Our results support further investigation into the potential of PI-103 in combination with agents like Nutlins or existing p53-inducing therapies in AML.

Acknowledgements

We acknowledge Dr Maria Soengas of Department of Dermatology, University of Michigan, Ann Arbor, MI, USA for kindly providing retrovirus encoding p53-specific shRNA. KK was supported in part by grants from the Kanae Foundation For Life and Socio-Medical Science and Novartis Foundation (Japan) for the Promotion of Science, MA by the Paul and Mary Haas Chair in Genetics and a grant from NIH, CA55164 and CA100632.

References

1 Parcellier A, Tintignac LA, Zhuravleva E, Hemmings BA. PKB and the mitochondria: AKTing on apoptosis. *Cell Signal* 2008; **20**: 21–30.
2 Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. mTOR, translation initiation and cancer. *Oncogene* 2006; **25**: 6416–6422.
3 Martelli AM, Nyåkern M, Tabellini G, Bortul R, Tazzari PL, Evangelisti C *et al.* Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia* 2006; **20**: 911–928.
4 Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. *Curr Opin Oncol* 2006; **18**: 77–82.
5 Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* 2005; **9**: 59–71.
6 Xu Q, Simpson SE, Scialla TJ, Bagg A, Carroll M. Survival of acute myeloid leukemia cells requires PI3 kinase activation. *Blood* 2003; **102**: 972–980.
7 Min YH, Eom JI, Cheong JW, Maeng HO, Kim JY, Jeung HK *et al.* Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. *Leukemia* 2003; **17**: 995–997.
8 Grandage VL, Gale RE, Linch DC, Khwaja A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, MAPkinase and p53 pathways. *Leukemia* 2005; **19**: 586–594.
9 Bardet V, Tamburini J, Ifrah N, Dreyfus F, Mayeux P, Bouscary D *et al.* Single cell analysis of phosphoinositide 3-kinase/Akt and ERK activation in acute myeloid leukemia by flow cytometry. *Haematologica* 2006; **91**: 757–764.
10 Kornblau SM, Womble M, Qiu YH, Jackson CE, Chen W, Konopleva M *et al.* Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood* 2006; **108**: 2358–2365.
11 Tamburini J, Elie C, Bardet V, Chapuis N, Park S, Broët P *et al.* Constitutive phosphoinositide 3-kinase/Akt activation represents a favorable prognostic factor in *de novo* acute myelogenous leukemia patients. *Blood* 2007; **110**: 1025–1028.
12 Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991; **253**: 49–53.
13 Faderl S, Kantarjian HM, Estey E, Manshouri T, Chan CY, Rahman Elsaied A *et al.* The prognostic significance of p16(INK4a)/p14(ARF) locus deletion and MDM-2 protein expression in adult acute myelogenous leukemia. *Cancer* 2000; **89**: 1976–1982.
14 Seliger B, Papadileris S, Vogel D, Hess G, Brendel C, Störkel S *et al.* Analysis of the p53 and MDM-2 gene in acute myeloid leukemia. *Eur J Haematol* 1996; **57**: 230–240.
15 Bueso-Ramos CE, Yang Y, deLeon E, McCown P, Stass SA, Albitar M. The human MDM-2 oncogene is overexpressed in leukemias. *Blood* 1993; **82**: 2617–2623.
16 Moll UM, Petrenko O. The MDM2-p53 interaction. *Mol Cancer Res* 2003; **1**: 1001–1008.
17 Wong S, McLaughlin J, Cheng D, Zhang C, Shokat KM, Witte ON. Sole BCR-ABL inhibition is insufficient to eliminate all myeloproliferative disorder cell populations. *Proc Natl Acad Sci USA* 2004; **101**: 17456–17461.
18 Kojima K, Konopleva M, Samudio IJ, Ruvolo V, Andreeff M. MEK inhibition enhances nuclear proapoptotic function of p53 in AML cells. *Cancer Res* 2007; **67**: 3210–3219.
19 Konopleva M, Contractor R, Tsao T, Samudio I, Ruvolo PP, Kitada S *et al.* Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* 2006; **10**: 375–388.
20 Tamburini J, Chapuis N, Bardet V, Park S, Sujobert P, Willems L *et al.* Mammalian target of rapamycin (mTOR) inhibition activates phosphatidylinositol 3-kinase/Akt by up-regulating insulin-like growth factor-1 receptor signaling in acute myeloid leukemia: rationale for therapeutic inhibition of both pathways. *Blood* 2008; **111**: 379–382.
21 Fan QW, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D *et al.* A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 2006; **9**: 341–349.
22 Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z *et al. In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004; **303**: 844–848.
23 Kojima K, Konopleva M, Samudio IJ, Shikami M, Cabreira-Hansen M, McQueen T *et al.* MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. *Blood* 2005; **106**: 3150–3159.
24 Verhaegen M, Bauer JA, Martín de la Vega C, Wang G, Wolter KG, Brenner JC *et al.* A novel BH3 mimetic reveals a mitogen-activated protein kinase-dependent mechanism of melanoma cell death controlled by p53 and reactive oxygen species. *Cancer Res* 2006; **66**: 11348–11359.
25 Kojima K, Konopleva M, McQueen T, O'brien S, Plunkett W, Andreeff M. Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. *Blood* 2006; **108**: 993–1000.
26 Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR. PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science* 2005; **309**: 1732–1735.
27 Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S *et al.* Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 1999; **144**: 891–901.
28 Kojima K, Konopleva M, Samudio IJ, Schober WD, Bornmann WG, Andreeff M. Concomitant inhibition of MDM2 and Bcl-2 protein function synergistically induce mitochondrial apoptosis in AML. *Cell Cycle* 2006; **5**: 2778–2786.
29 Ashcroft M, Ludwig RL, Woods DB, Copeland TD, Weber HO, MacRae EJ *et al.* Phosphorylation of HDM2 by Akt. *Oncogene* 2002; **21**: 1955–1962.
30 Pause A, Belsham GJ, Gingras AC, Donzé O, Lin TA, Lawrence Jr JC *et al.* Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. *Nature* 1994; **371**: 762–767.
31 Hay N. The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* 2005; **8**: 179–183.
32 Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* 2006; **124**: 471–484.
33 Tirado OM, Mateo-Lozano S, Notario V. Rapamycin induces apoptosis of JN-DSRCT-1 cells by increasing the Bax: Bcl-xL ratio through concurrent mechanisms dependent and independent of its mTOR inhibitory activity. *Oncogene* 2005; **24**: 3348–3357.
34 Vega F, Medeiros LJ, Leventaki V, Atwell C, Cho-Vega JH, Tian L *et al.* Activation of mammalian target of rapamycin signaling pathway contributes to tumor cell survival in anaplastic lymphoma

kinase-positive anaplastic large cell lymphoma. *Cancer Res* 2006; **66**: 6589–6597.
35 Beuvink I, Boulay A, Fumagalli S, Zilbermann F, Ruetz S, O'Reilly T *et al*. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. *Cell* 2005; **120**: 747–759.
36 Yamaguchi H, Wang HG. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* 2001; **20**: 7779–7786.
37 Arokium H, Ouerfelli H, Velours G, Camougrand N, Vallette FM, Manon S. Substitutions of potentially phosphorylatable serine residues of bax reveal how they may regulate its interaction with mitochondria. *J Biol Chem* 2007; **282**: 35104–35112.
38 Horton LE, Bushell M, Barth-Baus D, Tilleray VJ, Clemens MJ, Hensold JO. p53 activation results in rapid dephosphorylation of the eIF4E-binding protein 4E-BP1, inhibition of ribosomal protein S6 kinase and inhibition of translation initiation. *Oncogene* 2002; **21**: 5325–5334.
39 Tilleray V, Constantinou C, Clemens MJ. Regulation of protein synthesis by inducible wild-type p53 in human lung carcinoma cells. *FEBS Lett* 2006; **580**: 1766–1770.
40 Herbert TP, Tee AR, Proud CG. The extracellular signal-regulated kinase pathway regulates the phosphorylation of 4E-BP1 at multiple sites. *J Biol Chem* 2002; **277**: 11591–11596.
41 Chow S, Minden MD, Hedley DW. Constitutive phosphorylation of the S6 ribosomal protein via mTOR and ERK signaling in the peripheral blasts of acute leukemia patients. *Exp Hematol* 2006; **34**: 1183–1191.
42 McCubrey JA, Steelman LS, Abrams SL, Bertrand FE, Ludwig DE, Bäsecke J *et al*. Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy. *Leukemia* 2008; **22**: 708–722.
43 Milella M, Kornblau SM, Estrov Z, Carter BZ, Lapillonne H, Harris D *et al*. Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. *J Clin Invest* 2001; **108**: 851–859.
44 Yu K, Toral-Barza L, Shi C, Zhang WG, Zask A. Response and determinants of cancer cell susceptibility to PI3K inhibitors: combined targeting of PI3K and Mek1 as an effective anticancer strategy. *Cancer Biol Ther* 2007; **7**: 310–318.
45 Park S, Chapuis N, Bardet V, Willems L, Tamburini J, Knight ZA *et al*. PI-103, a dual inhibitor of class I phosphatidylinositide 3-kinase and mTOR, has anti-leukemic activity in acute myeloid leukemia. *Blood* 2007; **110**: 268a. [Abstract #876].