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PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kB, MAPkinase and p53 pathways

VL Grandage[1], RE Gale[1], DC Linch[1] and A Khwaja[1]

[1]*Royal Free and University College London Medical School, Department of Haematology, 98 Chenies Mews, London, UK*

The phosphoinositide 3-kinase (PI3-kinase) signalling pathway plays a key role in the regulation of cell survival and proliferation. We show that the PI3-kinase/Akt pathway is constitutively active in primary acute myeloid leukaemia (AML) cells and that blockade by the selective inhibitor LY294002 reduces survival of the total blast population (mean 52%). The ERK/MAPK module is also constitutively active and treatment with the MAPKK inhibitor U0126 reduces cell survival by 22%. In 10 of 18 samples, PI3-kinase contributes to MAPK activation as incubation with LY294002 leads to a marked reduction in its phosphorylation. PI3-kinase inhibition reduces survival of the CD34+38− AML progenitor subset by 44%, whereas MAPKK inhibition has little effect. Reporter assays in primary AML cells show that blocking PI3-kinase leads to a marked reduction of constitutive NF-kB activity and promotes p53-mediated transcription. This is associated with a synergistic interaction between LY294002 and Ara-C. An inducible activated form of Akt protects normal myeloid cells from Ara-C and etoposide-mediated apoptosis. These results show that blocking PI3-kinase has direct antileukaemic effects and potentiates the response to conventional cytotoxics via a number of targets including NF-kB, p53 and MAPK. Inhibitors of PI3-kinase and Akt may be useful in the treatment of AML.
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Introduction

The class 1 phosphoinositide 3 kinases are a ubiquitously expressed family of proteins that are central to cell survival and proliferation. Akt, a serine/threonine kinase, is a key mediator of phosphoinositide 3-kinase (PI3-kinase) signalling and is activated by phosphorylation of threonine 308 and serine 473[1] Activated Akt is then responsible for the phosphorylation of numerous downstream targets, regulating apoptosis and survival, cell growth and the cell cycle.[2] There is now increasing evidence for PI3-kinase/Akt dysregulation in human malignancy,[2] but the role of PI3-kinase activation in acute myeloid leukaemia (AML) is less clear. Recently, constitutive phosphorylation of Akt has been described in a significant proportion of patients with AML.[3–5] The method of Akt activation is not clear but does appear to be important for leukaemia cell survival.[3,4] The presence of constitutive Akt phosphorylation has also been linked to a shorter overall survival in AML.[3] In this study, we have investigated further the role of PI3-kinase in AML cell survival and proliferation. Our main aims have been to identify the effect of PI3-kinase inhibition on survival of bulk AML blasts and on leukaemic progenitors; to examine the potential for combining PI3-kinase inhibition with conventional cytotoxics and to identify the key downstream effector molecules that mediate the prosurvival effect of PI3-kinase in AML.

Correspondence: Dr VL Grandage, Department of Haematology, University College London, 98 Chenies Mews, London WC1E 6HX, UK; Fax: +44 207 679 6222; E-mail: vlg@talk21.com
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Materials and methods

Cell culture

Patient samples were obtained from patients presenting to University College Hospital, London, at presentation or relapse of AML. Informed consent was obtained from all patients prior to obtaining the sample. All patients had circulating leukaemic blasts in the peripheral blood and these were isolated by ficoll gradient centrifugation. Mononuclear cells were frozen in RPMI 1640 with L-glutamine (Invitrogen, Paisley, Scotland), foetal calf serum (FCS) and dimethyl sulphoxide (DMSO) and stored in liquid nitrogen. Of the samples analysed, 70% were from frozen cells and the remainder were analysed fresh. Fresh or frozen/thawed cells were cultured in RPMI and 10% FCS (RPMI/10% FCS). No exogenous cytokines were added. CD34+ cells as controls were isolated and cultured according to the methods of Watts *et al*.[6]

MTS assay

Cells were resuspended at 1×10^6 cells/ml in RPMI/10% FCS. The inhibitor under investigation was added at the appropriate concentration and the cells were incubated for 48 h at 37°C 5% CO_2. LY294002 and U0126 were purchased from LC Labs (Woburn, MA, USA) and used at 25 and 10 μM, respectively, unless otherwise stated. Cytosine Arabinoside (Ara C) was obtained from David Bull Laboratories, Victoria, Australia.

Western blotting

This was carried out as previously described.[7]

Antibodies

Phospho-p42/44 (Thr 202/Tyr204), Phospho-Akt (Ser 473), Phospho-Akt (Thr 308), Phospho-p70S6Kinase (Thr 389), Phospho-GSK 3β (Ser 9) were all from Cell Signalling Technology; p27 (C-19) PTEN (N-19), Stat 5b (C-17), Bax (N) from Santa Cruz Biotechnology Inc; Bcl-X, Bcl-2 both from BD Transduction labs and phospho-FKHRL1(Thr 32) was from Upstate Biotechnology.

Annexin V

This was carried out as previously described.[7]

Luciferase reporter assay

The NF-kB and P53 luciferase reporter plasmids and the renilla luciferase plasmid (pRL-CMV) were obtained from Promega. Between 1 and 5×10^6 AML blasts/CD34 + cells were nucleofected (Amaxa, CD34 kit) with 2 μg of either NF-kB or P53 reporter plus 0.5 μg of pRL-CMV. Transfected cells were incubated overnight (with or without LY294002) in RPMI/10% FCS. Cells were lysed and induced NF-kB or p53 reporter activity was corrected for the constitutive renilla luciferase expression using a Dual Luciferase Kit (Promega). Results were compared to the background reading obtained with the backbone pGL2 luciferase vector (Promega) that lacks eucaryotic promoter and enhancer sequences. Readings above background were considered positive.

Cell sorting

Leukaemic blasts were labelled with CD38 FITC clone AT13/5, (DAKO) and CD34 Phycoerythrin BD (Biosciences Pharmingen), according to the manufacturer's guidelines. They were then electronically sorted on an EPICS, Beckman–Coulter flow cytometer.

Mutational analysis

ITDs in the juxtamembrane region of the FLT3 gene were detected using polymerase chain reaction (PCR) amplification of exons 14 and 15 (previously 11 and 12) and the intervening intron as previously described.[8] Mutation in the activation loop of FLT3 (D835) were detected by PCR and restriction enzyme digestion of exon 20 as described by Kottaridis *et al*.[9] and Yamamoto *et al*.[10] N-ras mutations were detected using heteroduplex analysis. DNA was amplified using Optimase Polymerase™ (Transgenomic Limited, Crewe, UK) according to the manufacturer's specifications with 32 cycles of amplification, each 30 s at 95°C, 30 s at 63°C and 30 s at 72°C. Primers for N-Ras exon 1 covering codons 12 and 13 (1F 5′-GC TCGCCAATTAACCCTGATTAC-3′, 1R 5′-TGGGTAAAGAT GATCCGACAAGTGA-3′) gave a product of 227 bps, and for exon 2 covering codon 61 (2F 5′-ACACCCCCAGGATTCTTA CAGA-3′, 2R 5′-TCTTCCCTAGTGTGGTAACCTC-3′) a product of 273 bps. PCR products were denatured and analysed using denaturing HPLC on a Transgenomic WAVE™ DNA fragment analysis system at 60.5°C for exon 1, 59.2°C for exon 2. Patterns from patient samples were compared with those from DNA of the HL60 myeloid cell line (exon 2 mutation, wild-type exon 1). PCR products from samples with altered patterns were directly sequenced using the CEQ™ DTCS Quick Start kit and a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, USA).

32D stable myrAkt-ER/A2myrAkt-ER cell lines

32D murine myeloid cells were grown in RPMI/10% FCS and WEHI conditioned medium. The myristolated Akt Δ4–129 and the control A2myrAkt Δ4–129 are fused to an oestrogen receptor in a PWZL retrovirus vector containing a neomycin-resistance gene. These plasmids were kindly donated by Dr R Roth, Stanford University.[11] Stable pools of transfected cells were generated by electroporation.

To assess the effect of Akt activation on chemotherapeutic effect, the cells were incubated overnight with or without tamoxifen (0.5 μM), in the presence of murine IL3 (mIL3) 10 ng/ml (peprotech EC Ltd, London). The following day, the cells were washed and resuspended, at a concentration of 4×10^5/ml, in RPMI/10% FCS with or without 10 ng/ml mIL3, in the presence or absence of tamoxifen. Ara-C (0–1 μg/ml) or etoposide (0–50 μM) was then added, mixed and incubated for 48 h, then an MTS assay was carried out. Results were normalised to the 10 ng/ml mIL3 cells (no cytotoxics) with or with out tamoxifen.

Results and discussion

Akt is constitutively active in primary AML cells

To determine if Akt is activated in primary AML cells, AML blasts were utilised from patients at presentation or relapse (see Table 1 for patient details). Both fresh and frozen samples were analysed (30% of samples were analysed fresh). Akt phosphorylation was detected by Western blot, using an antibody directed against phosphorylated Akt at serine 473. Samples from 20 patients examined had phosphorylated Akt indicating that the PI3-kinase pathway is activated in AML blasts. This concurs with the findings of Min *et al*[3] who reported that 72% of their patient samples showed phosphorylation of Akt (ser 473). It is possible that there may have been a selection bias with our patients as only those with a significant level of circulating blasts were chosen.

Overnight incubation with the selective PI3-kinase inhibitor, LY294002 reduced or abolished Akt phosphorylation (Figure 1a). This was also seen after incubations as short as 4 h (Figure 1b). Using scanning densitometry, the mean reduction was $53 \pm 4\%$ ($n = 20$ $P = 0.0001$ by Wilcoxon's test). To illustrate that this was constitutive activation, Akt phosphorylation in the AML blasts was compared to that found in normal CD34 + cells grown in myeloid culture in the presence of SCF, IL-3 and G-CSF (day 7) (Figure 1c). Akt phosphorylation was not detected in the normal myeloid cells. Akt phosphorylation at serine (ser) 473 is thought to occur after initial phosphorylation at threonine (thr) 308, phosphorylation at both residues is required for full activation of Akt. We therefore looked at phosphorylation at thr 308. Figure 1d shows eight patient samples with constitutive phosphorylation of Akt at thr 308. Incubation with LY294002 leads to a reduction in phosphorylation at this residue as is seen with phosphorylation of Akt at ser 473 (Figure 1e). Constitutive Akt phosphorylation could be due to downregulation of one of the negative regulators of PI3-kinase, such as PTEN. PTEN expression in 12 patients with AML was at a similar level to a mononuclear cell fraction from a normal donor (Figure 1f) and there appeared to be no correlation with pAkt expression (data not shown). It is possible that activating mutations of Flt3 and or Ras may lead to constitutive activation of PI3-kinase. Mutational analysis revealed that three out of 16 patients tested had an FLT3 ITD and five had N-ras mutations (Table 1). One patient had both an FLT3 ITD and N-*ras* mutation. Of the 16 patients examined, no patients were found with an FLT3 D835 mutation. No correlation was seen between the presence of an activating Flt3 or Ras mutation and the degree of Akt phosphorylation seen.

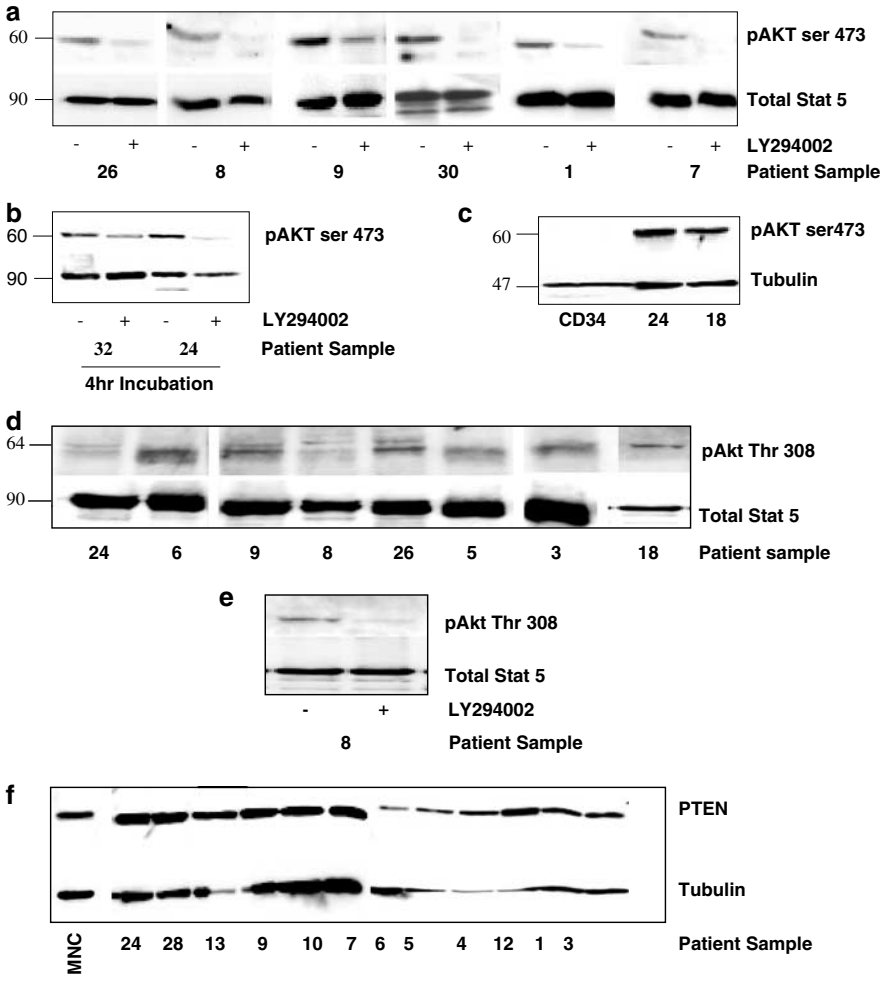

Figure 1 Akt is constitutively activated in AML but not normal CD34+ cells, and PI3-kinase blockade leads to a reduction in phosphorylation. (a) Primary AML cells were incubated for 24 h with or without 25 μM LY294002 and total cell lysates made. In all, 4×10^5 cell equivalents were loaded per lane and resolved by SDS–PAGE. The blot was probed with phospho-Akt ser 473 and total stat 5 to check for protein loading. The results from six patients are shown here. (b) A further blot is shown to illustrate that this effect is evident with incubations as short as 4 h. (c) Western blot to show activation of Akt in two patient samples, but not in nonleukaemic myeloid cells (CD34+ cells in myeloid culture, day 7). Tubulin is shown as a loading control. (d, e) Western blot illustrating that Akt is also constitutively phosphorylated at threonine (thr) 308 (eight examples shown here) and that incubation with LY294002 reduces this phosphorylation. (f) Western blot showing PTEN protein in 12 patients with AML and a normal mononuclear cell preparation. The blot was reprobed with an anti-tubulin antibody to check protein loading.

Effect of PI3-kinase blockade on leukaemic cell survival

To investigate the role of PI3-kinase in AML survival, cells were incubated with 25 μM LY294002 and an MTS assay was carried out at 48 h. This time point was chosen to allow maximal differential in cell number between control and treated cells. Cell number was reduced by an average of $52 \pm 3.4\%$ (range 6–85%, $n = 30$ patient samples, $P = 0.0001$ by Wilcoxon's test) (Figure 2a). This could be due to a reduction in proliferation or an increase in apoptosis or both. The level of apoptosis was therefore examined after incubation with LY294002 using a flow-cytometric Annexin V assay to detect the percentage of apoptotic cells. After 24 h, $19 \pm 4\%$ of control cells and $43 \pm 5\%$ of LY294002-treated cells were annexin positive (range 21–63%, $n = 9$, $P = 0.01$ by Wilcoxon's test) (Figure 2b), suggesting that the reduction in MTS activity was at least partly due to increased apoptosis and that PI3-kinase activation is important for leukaemic cell survival. A dose response for LY294002 was carried out in seven patients. AML blasts were incubated with increasing doses of LY294002 (0–50 μM) and an MTS assay carried out at 48 h. The mean IC_{50}, 7 μM LY294002 (Figure 2c) is in keeping with the *in vitro* IC_{50} of PI3-kinase activity by LY294002 reported as 10 μM by Davies *et al*.[12]

PI3-kinase blockade reduces MAPK activation in some patients with AML

The ERK MAP-Kinase (MAPK) pathway is another key regulator of cellular processes including differentiation, proliferation and apoptosis. We found that all of 18 AML samples investigated had constitutively active MAPK when examined by Western blotting using a phosphospecific MAPK p42/44 antibody. When these cells were incubated with 25 μM LY294002 for a minimum of 4 h, MAPK phosphorylation was reduced in 10/18 patients (Figure 2d). Quantification by scanning densitometry showed a decrease to $36 \pm 6\%$ (mean $\pm$ s.e.m.) of control. This suggests

Table 1 Patient characteristics and outcome

	FAB type	*Pres. WCC*	*Cytogenetics*	*Age at pres.*	*Flt 3/RAS*	*Length of first CR*	*Current status*	*Survival*	*Outcome*
1[a,b]	M5b	52	46xx	66	WT/WT	1° refractory	Dead	6 weeks	Leukaemia
2[a,b]	M2	117	Del(9) q12q34	37	WT/EX2	Transplant in first CR	Dead	11 months	TRM
3[b]+	N/A				WT/WT				
4	M4	67	Constitutional 45x	67	ITD/WT	Not applicable	Dead	2 days	Leukaemia
5	M2	47	No dividing cells	49	WT/WT	5 years	Alive	5 years	CR1
6[a,b]	M5a	162	46xy del(10)	40	WT/EX1+2	1° refractory	Dead	1 month	Haemorrhage
7[a,b]	M1	401	46xx	28	ITD/WT	Mini allo in CR1	Dead	7 months	TRM
8[a,b]	M5	257	46xy	45	ITD/WT	N/A	N/A		
9[a,b]	M5a	89	46xx	64	ITD/EX1	1° refractory	Dead	2 weeks	Leukaemia
10[a,b]	2°MDS	38.1	N/A	64	WT/WT	N/A	Dead	8 months	Regenerative failure
11	N/A				WT/NA				
12[a]	M2	47	Trisomy 21	43	ITD/D835/WT		Alive	4 years	CR2
13[a]	M1	140	46xy	62	ITD/WT	12 months	Alive	4 years	CR4
14	M1	33	46xy	38	ITD/WT	46 months	Alive	46 months	CR1
15[a]	M2	10	Trisomy 11	43	ITD/WT	7 months	Alive	46 months	CR2
16[a,b]	M5b	188	46xx	57	WT/WT	39 months	Alive	39 months	CR1
17[a]	M2	42	t(8;22)	27	WT/WT	Transplant in CR1	Alive	37 months	CR1
18[b]	M1	34	N/A	72	WT/WT	Treated palliatively	Dead	1 month	Leukaemia
19	relapsed	36	46xx,r(7)(?p?q)t(9;22)(q34;q11)	61	WT/WT	1° refractory	Dead	6 weeks	Leukaemia
20[a]	M1	14	46xx	43	N/A/NA	Transplant in CR1	Alive	30 months	CR1
21[a,b]	M2	35	46xy	69	WT/EX1	Lost to FU			
22[a]	N/A		46xx	>60	WT/WT				
23[a,b]	M4	50	Trisomy 21	37	N/A/NA	Transplant in CR1	Dead	16 months	Relapse
24	M4	233	Inv16(p13q22)	56	WT/EX2	27 months	Alive	28 months	CR1
25[a,b]	M0	131	5q-	39	N/A/NA	Transplant in CR1	Alive	27 months	CR1
26[a,b]	Trans ET	146	Complex	81	WT/WT	Treated non intensively	Dead	1 month	Leukaemia
27[a,b]	M5	69	Complex	41	WT/WT		Dead	9 months	Leukaemia (CNS)
28	M1	80	46xx	51	WT/EX1	16 months	Dead	19 months	Leukaemia
29[a]	M2	8	45xyadd(8)(q22)add(15)(p1)add(21)(p1)	69	WT/EX2	23 months	Dead	40 months	Leukaemia
30[a,b]	M2	29	t(8;21) +Y	55	WT/NA	15 months	Dead	19 months	Leukaemia
31[b]	M4eo	42	Variant inv 16	48	N/A/NA	11 months	Alive	12 months	CR1
32[a,b]	M4	180	Complex inv 16+9+22	43	N/A/NA	9 months	Alive	10 months	CR1
33[a,b]	M5 relapse	3	N/A	55	N/A/NA	N/A	Dead	2 months	Leukaemia
34[a]	M4	100	46xx	51	N/A N/A	Transplant in CR1	Alive	44 months	CR1
35[a]	M2	10	7q-	14	N/A N/A	Transplant in CR1	Dead	10 months	Leukaemia
36[a]	M1	43	Abn haplo t(3;13)	61	N/A N/A	7 months	Dead	13 months	Leukaemia
37[a]	M2	10	t(8;21)	58	N/A N/A	9 months	Dead	15 months	Sepsis
38[a]	M4	120	46xy	26	N/A N/A	Transplant in CR1	Alive	39 months	CR1
39[a]	N/A								
40[a]	M4	541	t(10;11)	12	WT/N/A	Transplant in CR1	Dead	14 months	Leukaemia

[a]MTS assay.
[b]Western data.

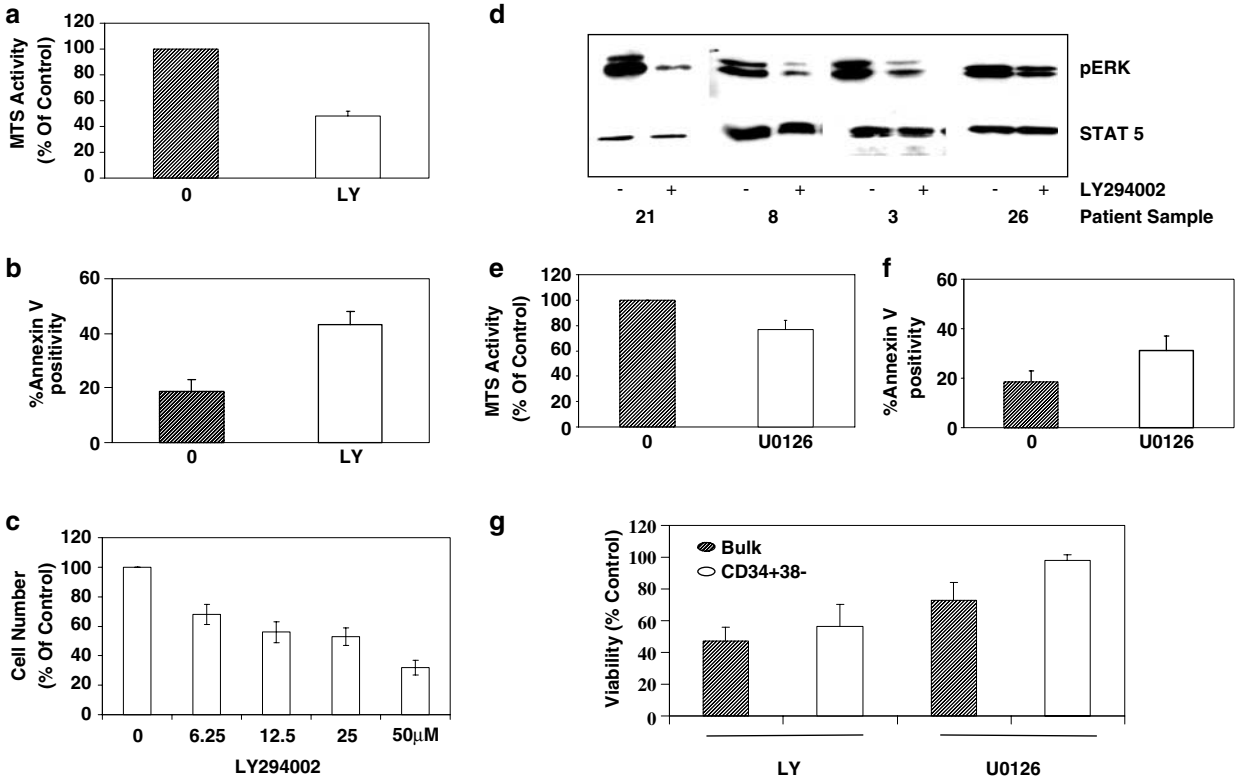

Figure 2 Incubation of primary AML blasts with LY294002 leads to a reduction in MTS activity and an increase in apoptosis. PI3-kinase regulates MAPK activity in primary AML samples and the effect of MAPK blockade on proliferation and apoptosis is examined. (a, b) Primary AML cells were incubated with and without 25 μM LY294002 and an MTS assay was carried out at 48 h. The cell number was reduced after incubation with LY294002 by an average of $52 \pm 3.4\%$ (range 6–85%, $n=30$). Annexin V staining was carried out at 24 h to assess the level of apoptosis. Apoptosis was increased to $43 \pm 5\%$ (range 21–63%) after incubation LY294002 compared to a control of $19 \pm 4\%$ ($n=9$). (c) Primary leukaemic cells were incubated with increasing doses of LY294002 and an MTS assay was carried out at 48 h. The mean results for each concentration are shown $\pm$s.e.m. ($n=7$). The IC_{50} for these results is 7 μM LY294002. (d) Western blot showing ERK activation in four primary AML samples and reduction in phosphorylation after 24 h incubation with 25 μM LY294002. In all, 4×10^5 cell equivalents loaded per lane and resolved by SDS-PAGE. The blot was probed initially for Phospho ERK 1/2 and then reprobed with total Stat 5 to check for protein loading. (e) Cells were incubated with 10 μM U0126 for 48 h and an MTS assay was carried out. Results were expressed as a percentage of control. The mean reduction in MTS activity was $23 \pm 4.8\%$ (range 0–69%, $n=29$). (f) Cells were incubated in 10 μM U0126 at 37°C for 24 h. Apoptosis was measured by annexin V staining and flow cytometry. Apoptosis was $31 \pm 6\%$ (range14–61%) compared to a control level of $19 \pm 4\%$ ($n=9$). (g) CD34 + CD38− cells were isolated by sterile sorting and incubated with either 25 μM LY294002 or 10 μM U0126 (MEK1 inhibitor) for 24–48 h. Either an MTS assay or annexin V staining was carried out to assess cell viability and expressed as a percentage of control. Incubation with LY294002 reduced cell viability to $56 \pm 14\%$ ($n=5$) in the stem cell population compared to $47 \pm 9\%$ for the bulk (unsorted) population. MAPK blockade reduced bulk blast viability to $73 \pm 11\%$ but did not affect the leukaemic stem cell subset, $97 \pm 4\%$ ($n=4$).

that PI3-kinase activity is upstream of MAPK, and raises the possibility that some of the effects of PI3-kinase inhibition on leukaemic cell survival may be due to reduced MAPK activity. To assess this further, the effect of U0126, a selective MEK inhibitor, on cell proliferation and apoptosis was examined. MTS assay at 48 h showed a reduction in cell number of $23 \pm 4.8\%$ (range 0–69%, $n=29$, $P=0.005$ by Wilcoxon's test) when compared to control cells (cf LY 52%) (Figure 2e). Annexin V assay, at 24 h, showed apoptosis levels of $31 \pm 6\%$ (range 14–61%, $n=9$, $P=0.15$ by Wilcoxon's test) compared to control $19 \pm 4\%$ (Figure 2f). Milela *et al*[13] found constitutive activation of MAPK in 74% of their patients with AML and showed that MEK inhibition led to a net increase in apoptosis of 13% (10–23%), this is very similar to our data (12% increase). Although MAPK can be regulated by PI3-kinase, this is unlikely to be the major antiapoptotic pathway in AML because the reduction in cell number after MAPK inhibition was considerably less than after PI3-kinase blockade (23 *vs* 52%, respectively), and PI3-kinase blockade had effects in cells where it was not involved in MAPK activation. Several possible mechanisms have been described for PI3-kinase regulation of MAPK,[14–17] but it is not yet evident which, if any, of these mechanisms is relevant in AML.

Effect of PI3-kinase inhibition on viability of CD34 + 38− AML cells

In five separate experiments, AML blasts were sterile sorted to isolate the CD34 + 38− fraction in which the leukaemic stem cell is thought to reside. Cell viability was assessed after 24–48 h by annexin V binding or MTS assay and compared with results for the bulk (unsorted) blast population. Figure 2g shows that PI3-kinase inhibition reduces the viability of the CD34 + 38− fraction to $56 \pm 14\%$ of control (cf bulk $47 \pm 9\%$). Xu *et al*[4] treated leukaemic stem cells with LY294002 for 16 h prior to injecting them into NOD/SCID mice. They found reduced

engraftment after LY294002 treatment compared to control cells. This suggests that the PI3-kinase pathway is equally important for survival in both the stem cell subset and bulk cell populations. The effect of MAPK inhibition was compared with that of PI3-kinase blockade. MAPK blockade with U0126 reduced bulk blast viability to 73±11% but in contrast to PI3-kinase blockade, did not affect the CD34+38− cells (97±4%). These results suggest that inhibiting a given pathway in the bulk leukaemic blast population does not necessarily predict what would happen when the same pathway is inhibited in individual subpopulations.

PI3-kinase regulates NF-kB and p53 activity in AML cells

Having found that PI3-kinase/Akt is important for AML survival, we wished to identify downstream effector molecules that could mediate this process. NF-kB is a ubiquitously expressed transcription factor and increased activity is associated with antiapoptotic effects. NF-kB has been reported to be constitutively active in a number of malignancies including AML,[18] and may be regulated by PI3-kinase. Using a novel technique for high level transfection into normal CD34+ cells and primary AML blasts ('Nucleofection'), we measured reporter gene activity using an NF-kB reporter plasmid. The transfection efficiency using a GFP expression plasmid was 54±9 for CD34+ cells and 39±6% for AML blasts. Reporter activity was at background levels in normal CD34+ cells and could be appropriately increased by stimulation with TNFα, a known activator of NF-kB. Constitutive NF-kB activity, above levels found in unstimulated CD34+ cells, was seen in all patients examined (Figure 3a). Incubation with LY294002 led to a significant reduction of constitutive NF-kB activity in 9/10 patients to 34±7% of control ($n=9$, $P=0.01$ by Wilcoxon's test) (Figure 3b). Our findings are consistent with those of Guzman *et al* who found NF-kB activation in all the AML specimens they examined ($n=11$). NF-kB was constitutively active in leukaemic (CD34+, CD38−, CD123+) stem cells but not normal stem cells, and leukaemic stem cells underwent apoptosis with the proteosomal inhibitor MG132 (which reduces NF-kB activity by increasing IkB) whereas normal stem cells did not.[18]

p53 is a central mediator of the cells response to DNA damage and other stresses. Mutations in p53 are a common occurrence in many malignant disorders and may confer a degree of chemoresistance. p53 is regulated by mdm 2, an E3 ubiquitin ligase, which is itself regulated by PI3-kinase/Akt.[19] We wanted to see what effect if any, blockade of the PI3-kinase pathway had on p53 activity in primary AML cells. Using a p53 reporter assay, it was found that blockade of PI3-kinase activity with LY294002 led to a more than two-fold increase in p53 activity in four out of nine patients tested (Figures 3c). The mean increase was 192% with an s.e.m. of 47% ($n=9$, $P=0.02$ by Wilcoxon test). Genes trans-activated by p53 can either induce cell cycle arrest or promote apoptosis. Low p53 activity due to mutation can lead to aggressive tumour behaviour and reduced therapeutic sensitivity but mutations are uncommon in AML (5–15%).[20–22] This pathway could be a mechanism for overactive PI3-kinase to promote resistance to cytotoxic agents – blockade of PI3-kinase/Akt could lead to increased chemosensitivity by increasing p53 levels. Regulation of both NF-kB and p53 by PI3-kinase/Akt in AML cells could reduce the apoptotic response to cytotoxic chemotherapy.

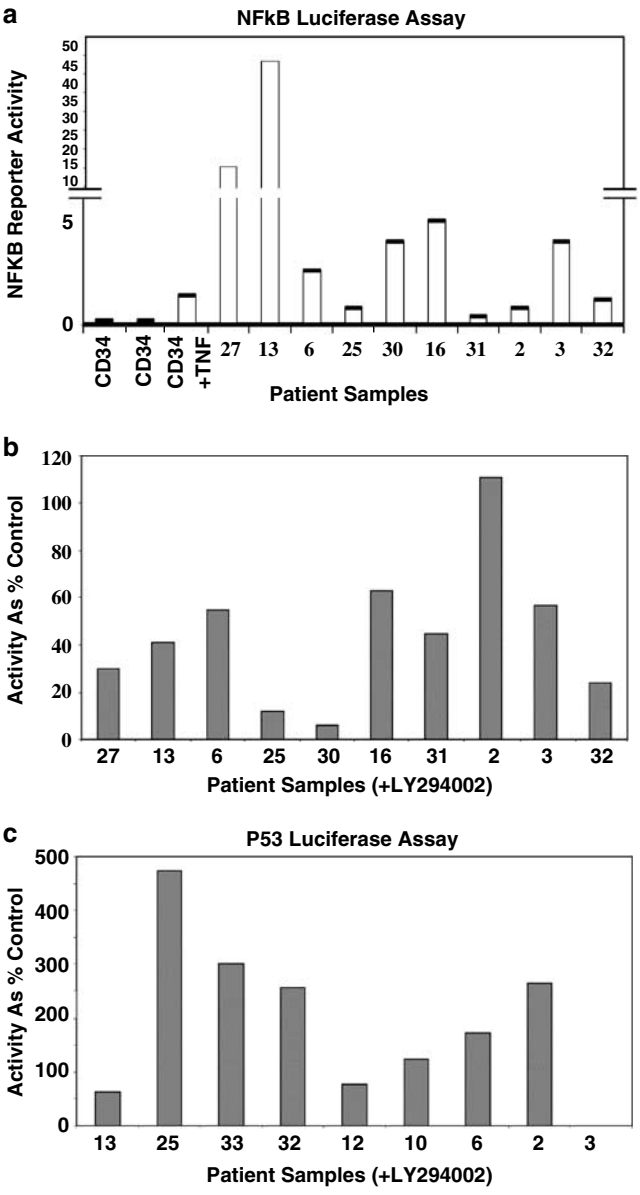

Figure 3 The effect of PI3-kinase blockade on NF-kB and p53 reporter activity. (a) Primary AML cells or normal CD34+ cells were transfected with an NF-kB reporter gene The transfection efficiency for the CD34+ cells was 54±9% and for AML blasts was 39±6%. All patients had constitutive activation of NF-kB, above levels seen in unstimulated CD34+ cells. (b) Incubation with 25 μM LY294002 led to a reduction in NF-kB activity in 9/10 patients. The activity was reduced to a mean of 34±7% of control ($n=9$). NF-kB reporter activity as a percentage of untreated control cells is shown for each individual. (c) The cells were transfected with a p53 reporter gene and incubated overnight with 25 μM LY294002. PI3-kinase inhibition led to a more than two-fold increase in p53 activity in 4/9 patients examined. p53 reporter assay readings are shown after incubation with 25 μM LY294002 as a percentage of untreated control cells, for individual patients. Patient number 3 had undetectable reporter activity.

PI3-kinase inhibition can enhance the cytotoxic effect of Ara-C

The enhancement of antiapoptotic mechanisms by constitutively activated PI3-kinase/Akt may reduce the effectiveness of cytotoxic agents. Therefore, the effect of blocking the PI3-kinase

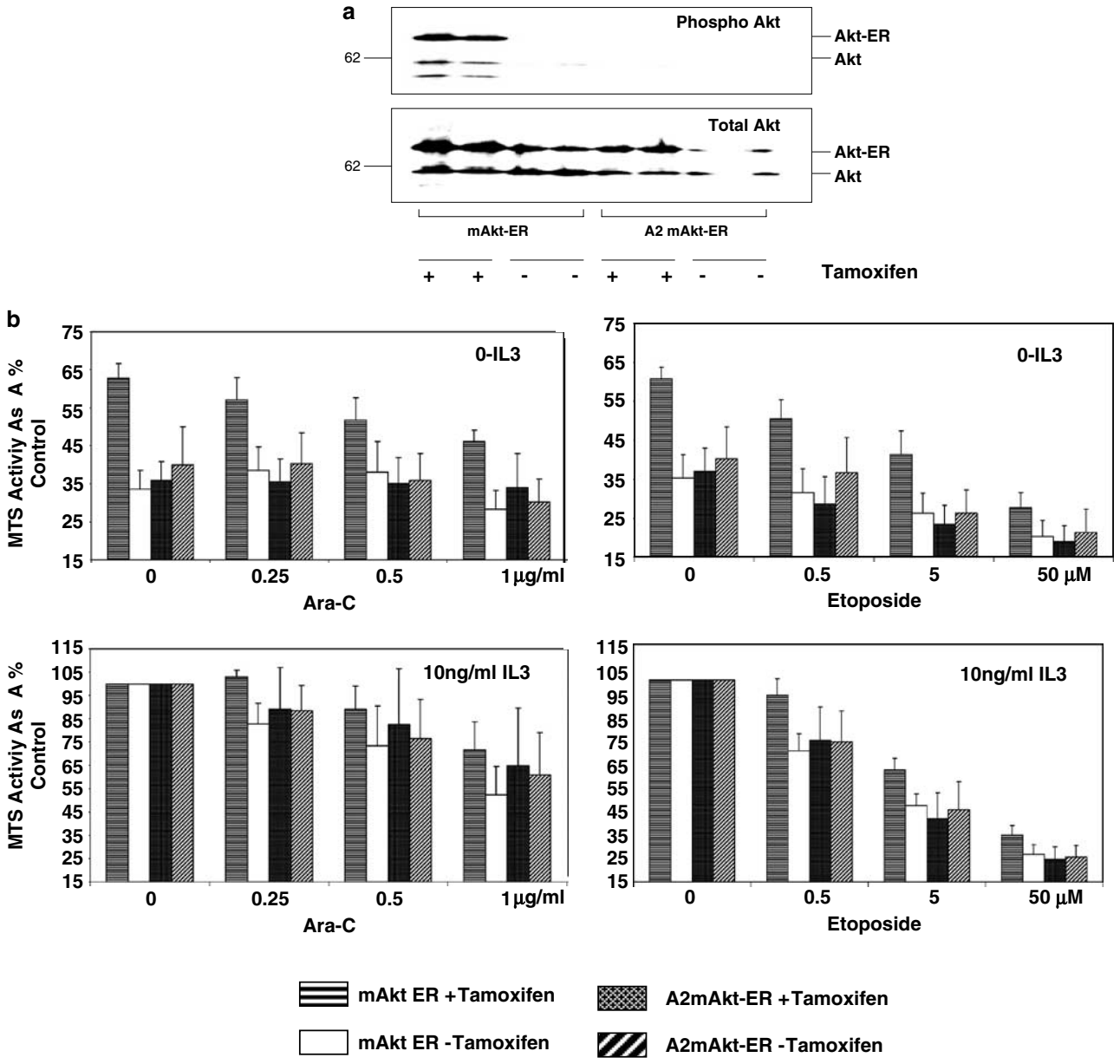

Figure 4 Akt activation is sufficient to protect haemopoietic cells from the cytotoxic effects of Ara-C and etoposide. 32D cells stably expressing either the active mAkt-ER or control A2mAkt-ER construct were incubated with or without tamoxifen (0.5 μM) overnight. (a) Western blot to show that incubation with tamoxifen leads to phosphorylation of mAkt in the mAkt-ER cells but not the A2mAkt-ER-expressing cells. The blot was then reprobed with total Akt to check for protein loading. (b) Cells were incubated overnight with or without tamoxifen. The following day they were washed and resuspended in RPMI/10% FCS with or without 10 ng/ml murine IL3, in the presence or absence of tamoxifen. Ara-C (0–1 μg/ml) or etoposide (0–50 μM) were added and the cells incubated at 37°C. An MTS assay was carried out at 48 h. Results were normalised to cells stimulated with 10 ng/ml IL3, in the absence of cytotoxics. Tamoxifen-induced activation of Akt led to a significant reduction in apoptosis, in the presence or absence of IL3, for Ara-C $P = 0.009$ and 0.002, respectively, and etoposide $P = 0.05$ and 0.01 by paired *t*-test ($n = 3$).

pathway on sensitivity to Ara-C, a central cytotoxic component of many AML treatment regimens, was examined. As Ara-C exerts its cytotoxic activity at least partly in a cell cycle-dependent manner, it was possible that PI3-kinase blockade could reduce cell proliferation and have a deleterious effect on the Ara-C response. AML cells were coincubated with varying concentrations of LY294002 (0–50 μM) and Ara-C (0–2 μg/ml) and an MTS assay carried out at 48 h. PI3-kinase blockade did not have an adverse impact on the cell kill obtained with maximal effective Ara-C concentration for each patient tested. Using the Calcusyn® program for calculating the amount of synergy between different drug combinations, the combination indices for Ara-C and LY294002 were calculated for each patient. A combination index of <1 represents a synergistic interaction, of $=1$ signifies an additive interaction and >1 represents an antagonistic interaction. One patient out of the eight patients examined was not sensitive to Ara-C and therefore synergy could not be calculated. Of the remaining patients, five out of seven exhibited synergy (values at ED_{50} of 0.2, 0.7, 0.6, 0.5, 0.9), one showed an additive interaction (ED_{50} value 1) and

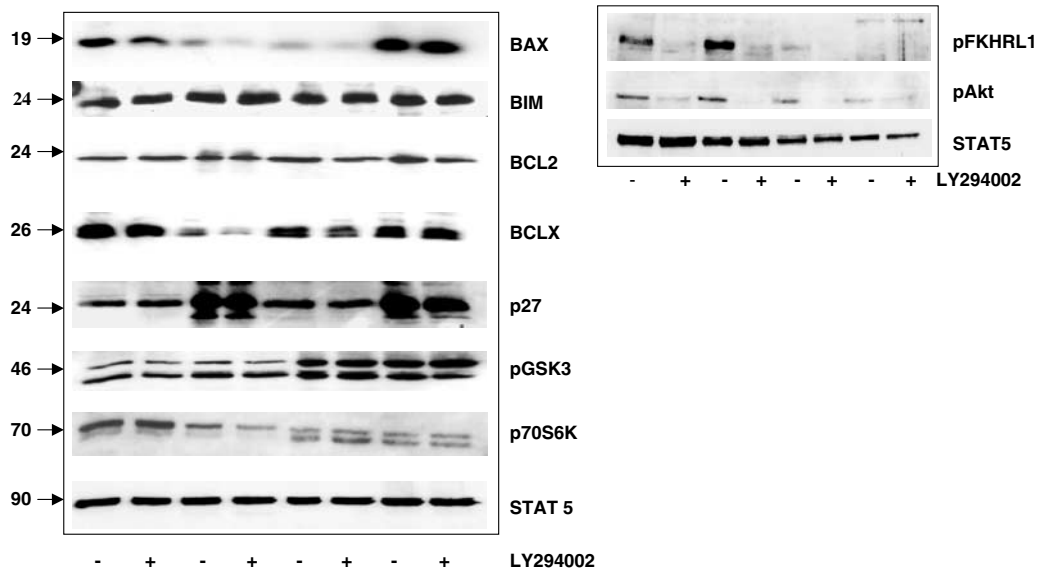

Figure 5 The effect of PI3-kinase blockade on downstream targets. Cells were incubated with and with out 25 μM LY294002 overnight and total cell lysates made. In all, 4×10^5 cell equivalents were resolved by SDS-PAGE. The blot was probed with primary antibody annotated on the right and reprobed with total stat 5 to assess protein loading (four examples shown).

one a moderately antagonistic interaction (ED_{50} value 1.4). Other groups have shown that PI3-kinase blockade potentiates the response to several chemotherapeutic agents including paclitaxel, vincristine, doxorubicin, trastuzumab and etoposide.[23–25]

The effect of activating Akt on chemotherapeutic effect

The results with primary AML cells indicate that PI3-kinase is involved in cell survival and implicated in resistance to chemotherapy-induced apoptosis. PI3-kinase has a number of downstream targets of which Akt is one of the most important in survival signalling. To investigate if activation of Akt alone is sufficient to protect haemopoietic cells from cytotoxic effects of chemotherapy, an inducible activated Akt vector system was used. Myristolation of Akt targets it to the membrane, making it constitutively active. By fusing myristolated Akt to a mutant murine oestrogen receptor hormone-binding domain (mAkt-ER), the activation can be turned on and off by incubation with tamoxifen. A construct in which the membrane targeting ability of the myristolation sequence was abrogated, by mutation of glycine to alanine at the second amino-acid position, was used as a control (A2mAkt-ER).[11] An IL3-dependent myeloid cell line, 32D, was used to stably express these constructs and showed appropriate activation of Akt in response to tamoxifen. (Figure 4a). Both cell lines were exposed to varying concentrations of etoposide or Ara-C in the presence or absence of 10 ng/ml murine IL3. MTS assays were carried out 48 h later. Activation of Akt by incubation with tamoxifen was sufficient to protect these cells from factor withdrawal and led to a significant reduction in cell kill in the presence or absence of IL3, for Ara-C ($P=0.009$ and $P=0.002$, respectively, by paired *t*-test) and for etoposide ($P=0.05$ and 0.01) (Figure 4b). Incubation of 32D cells expressing the inactive control plasmid (A2mAkt-ER) with tamoxifen did not alter the cytotoxic effects of Ara-C and etoposide ($P>0.05$ for all conditions), indicating that Akt activation was responsible for the results seen. In contrast to the transient activation of Akt following IL3 stimulation, with a return to baseline after 2–3 h (data not shown), the 32D cells expressing the inducible activated Akt-ER construct have sustained Akt activation. This indicates that persistent Akt activity as seen in primary AML cells has enhanced anti-apoptotic effect and may make a significant contribution to chemoresistance.

The effect of PI3-kinase blockade on other downstream signalling pathways

So far we have shown that PI3-kinase potentially regulates survival by influences on MAPK, NF-kB and p53 pathways. Akt is also known to regulate survival and proliferation by phosphorylation of a number of other downstream signalling proteins. We looked at several of these to see which, if any, were affected by PI3-kinase blockade in AML cells. Bcl xl and BIM levels can be regulated by PI3-kinase,[2,26] but we found no evidence for this in the AML cells tested, four examples of which are shown in Figure 5. We found GSK 3 and P70S6 Kinase, which can be targets of the PI3-kinase/Akt pathway,[2] to be constitutively phosphorylated in AML cells. However, inhibition of PI3-kinase by LY294002 did not appear to alter this phosphorylation. This suggests that alternative signalling pathways are responsible for this phosphorylation and we have found this to be the case in several leukaemic lines tested (data not shown). In some AML cases, constitutive phosphorylation of the Forkhead family member FKHRL1 was detected and found to be regulated in a PI3-kinase-dependent manner. Addition of LY294002 appeared to have no significant effect on the total levels of the cell cycle regulators p21 and p27.

In conclusion, we have found that the PI3-kinase pathway is activated in all the AML samples we tested. We have shown that inhibition of this pathway leads to an increase in apoptosis in the leukaemic stem cell pool, unlike MAPK blockade, and that it potentiates the response to cytotoxic chemotherapy. We have demonstrated that PI3-kinase/Akt positively regulates the

antiapoptotic NF-kB pathway and negatively regulates the P53 pathway in AML blasts. These results identify key pathways downstream of PI3-kinase in AML and show the potential of PI3-kinase inhibitors in the treatment of AML as a single agent and also in combination with conventional cytotoxics.

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