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Quantitative single cell determination of ERK phosphorylation and regulation in relapsed and refractory primary acute myeloid leukemia

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We investigated the constitutive activation of the MEK/ERK pathway in acute myelogenous leukemia (AML) via a flow cytometric technique to quantitate expression of phosphorylated ERK (p-ERK). A total of 42 AML samples (16 newly diagnosed, 26 relapsed/refractory) were analyzed. Normal bone marrow CD34$^+$ cells ($n=10$) had little or no expression of p-ERK, while G-CSF-mobilized CD34$^+$ cells exhibited enhanced p-ERK levels. Markedly elevated p-ERK levels were found in 83.3% of the AML samples, with no differences observed between the newly diagnosed and relapsed/refractory samples. Treatment with a MEK inhibitor resulted in significantly decreased p-ERK levels in both the newly diagnosed and relapsed/refractory samples, which was associated with growth arrest, but not apoptosis induction. In summary, we defined conditions for the analysis of MAPK signaling in primary AML samples. Normal CD34$^+$ cells expressed very low levels of p-ERK, and increased p-ERK levels were found in normal G-CSF-stimulated circulating CD34$^+$ cells. Constitutively high p-ERK levels observed in the majority of AML samples suggest deregulation of this pathway that appears to be independent of disease status. The ability of ERK inhibition to promote growth arrest rather than apoptosis suggests that clinical trials of MEK/ERK inhibitors may be more effective when combined with chemotherapy.
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Introduction

Disruption of proapoptotic and cell survival pathways is common in acute myelogenous leukemia (AML), which potentially accounts for the resistance to chemotherapy and treatment failure.[1,2] The Ras/Raf/MEK/ERK pathway is known to control cell proliferation and cell survival, two activities that have a direct impact on tumor promotion and progression.[3,4] We have previously reported that ERK is constitutively activated in more than 50% of primary AMLs but not in normal CD34$^+$ progenitors[5] and that constitutive ERK activation is an independent prognostic factor for survival in AML (Kornblau *et al*, *Blood* 2001; **98**: 716a). These observations prompted us to consider the MEK/ERK pathway as a potential therapeutic target in this disease.

Several small molecule inhibitors that target specific components of the MEK/ERK pathway are available, allowing the opportunity to test the biological contribution of these targets to cell survival. Some of these small molecules are currently under clinical development,[6] following pharmacodynamic and preclinical studies that have established antitumor effects of these inhibitors.[7,8] One such agent, CI-1040, is an orally active small molecule that is noncompetitive with ATP, and a highly selective inhibitor of MEK. CI-1040 is presently undergoing phase II evaluation in cancer patients.[8] We have observed that CI-1040 inhibited cell proliferation and survival in human leukemia cell lines. No studies have been conducted to examine the effects of CI-1040 in relapsed/refractory AML cells.

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Materials and methods

Cell lines and primary samples

Human AML cell lines (OCI-AML3, NB4) were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 1 mM L-glutamine and 50 μg/ml penicillin/streptomycin (Gemini Bio-Products, Woodland, CA, USA). Normal bone marrow (NBM) samples were obtained by aspiration from the posterior iliac crest of 12 healthy donors. Peripheral blood CD34$^+$ cells (PB CD34$^+$) were collected by leukapheresis procedures from four normal volunteers who had received rhG-CSF. PB and BM aspirate samples were obtained from 42 AML patients. The study comprised 26 male and 16 female subjects, 16 patients were studied at diagnosis and 26 had relapsed/refractory leukemias. (see Supplementary Information Table 1) Donors and patients gave informed consent according to institutional guidelines at The University of Texas MD Anderson Cancer Center.

Cell culture and primary cell isolation

For all *in vitro* experiments, cells were harvested in log-phase growth, washed and seeded at the appropriate concentration in the presence of CI-1040 (Pfizer Global Research and Development, Ann Arbor, MI, USA) or in an equal volume of the vehicle DMSO. Mononuclear cells (MNCs) were separated from PB and BM samples obtained from healthy donors and AML patients, by differential Ficoll–Hypaque (Sigma Chemical Co. St Louis, MO, USA) centrifugation.

CD34$^+$ cells were highly purified, by Mini Macs high-gradient magnetic separation column (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. Assessment of cell numbers and viability was made by Trypan blue exclusion and counting using a hemacytometer. Cells used for *in vitro* studies were resuspended in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin–streptomycin. The numbers were adjusted to a starting concentration of 1.0×10^6/ml, and cultured at 37°C in an atmosphere of 5% CO_2 with CI-1040 at 3 μM or with vehicle (DMSO).

p-ERK activation

To examine the efficacy of CI-1040, we investigated its ability to block ERK activation after stimulation with phorbol-12 myristate 13-acetate (PMA) (Sigma). An aliquot of cells, for each cell line, at different time points and for each culture condition, was treated with 15 μM PMA for 3 min at 37°C and used as positive control of p-ERK activation. Similarly, according to cell availability, PMA-induced ERK activation was performed on aliquots of primary cells from AML samples collected at time 0 and after liquid culture with and without CI-1040 by exposure to 15 μM PMA for 3 min at 37°C.

Measurement of p-ERK expression by flow cytometry (FCM)

p-ERK expression was evaluated by FCM using a previously described procedure[9] with modifications. Briefly, PMA-stimulated and unstimulated cells were fixed using 2% formaldehyde (Polysciences Inc., Warrington, PA, USA) at 37°C for 10 min. Following fixation, the samples were resuspended in 90% ice-cold methanol (Fisher Scientific, Fair Lawn, NJ, USA) and kept on ice for 30 min. Cells suspensions were centrifuged and washed once with PBS containing 4% bovine serum albumin (BSA) and then labeled with the primary monoclonal antibody (mAb) specific for p-ERK (clone E10, Cell Signaling Technology Inc., Beverly, MA, USA) for 30 min at 4°C. An irrelevant mouse antibody of the appropriate subclass was used as a negative control to determine background fluorescence. After one wash, cells were incubated with a goat antibody fragment anti-mouse Ig conjugated with phycoerythrin (PE) (Biosource, Camarillo, CA, USA) for 30 min at 4°C, according to the manufacturer's recommendation and samples were measured immediately. p-ERK expression was measured in selected subpopulations of PMA-stimulated or unstimulated cells with mAb directed against surface antigens according to the procedure described above.

Western blot analysis

p-ERK expression was verified by Western blot analysis as described previously.[5] Briefly, PMA-stimulated and unstimulated cells were washed twice and lysed for 30 min on ice in a solution containing 10 mM NaF, 1 mM Na_3VO_4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% NaN_3, 10 mM iodoacetamide, 3 mM PMSF, and 1% Triton X-100 supplemented with protease inhibitor cocktail (Roche Diagnostic Corp., Indianapolis, IN, USA). Proteins were subjected to SDS–PAGE, transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Little Chalfont, England), and immunoblotted with the same p-ERK-specific mAb used for FCM analysis. Membranes were then probed with an HRP-conjugated secondary antibody and reacted with ECL reagent (Amersham). Signals were detected by phosphoimager (Storm 860, Molecular Dynamics Version 4.0; Molecular Dynamics, Sunnyvale, CA, USA) and quantified by Scion Image software (Scion Corp., Frederick, MD, USA). In order to normalize the levels of p-ERK, samples were probed with a mAb recognizing $p42^{MAPK}$ (clone D-2, Santa Cruz Biotechnology Inc., CA, USA).

Apoptosis and cell-cycle analysis

Cells were examined over a culture period of 24–96 h using Annexin V to detect phosphatidylserine externalization on the plasma membrane. Cells were washed twice with PBS and resuspended in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$; Sigma Chemical Co.). FITC-conjugated Annexin V (Annexin V-FITC) (Roche) was added at a final concentration of 1 μg/ml. The mixture was incubated at room temperature for 15 min in the dark prior to flow cytometric analysis. Membrane integrity was simultaneously assessed by propidium iodide (PI) (0.25 μg/ml) exclusion.

PI staining for cell-cycle distribution and DNA degradation analysis: cells were fixed in ice-cold ethanol (70% v/v) for 1 h at 4°C and stained with PI (25 μg/ml PI, 180 U/ml RNase, 0.1% Triton X-100, and 30 mg/ml polyethylene glycol in 4 mM citrate buffer, pH 7.8; Sigma).

FCM and FACS analysis

FCM analysis was conducted using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) operated at 488 nm, which detects green (FITC/CD surface antigens, Ps/AxV), red (PE/pERK1/2 and PI), and with the >670 nm band pass filter (APC/CD surface antigens) fluorescence. Data acquisition and analysis (10 000–20 000 events) was performed with the CellQuestPro software (Becton Dickinson). Cell-cycle distribution was analyzed using the ModFit LT software (Verity Software House, Topsham, ME, USA).

Statistical analysis

p-ERK expression evaluated by FCM was analyzed using the Kolmogorov–Smirnov statistic test (D), which allows the objective and accurate identification of small differences in fluorescence intensity. Samples with a $D>0.1$ were considered positive.

The two-sided Student's t-test was used to evaluate the significance of differences between groups. Results are expressed as the mean $\pm$ standard error of the mean (s.e.m.).

Results

Evaluation of p-ERK levels in normal $CD34^+$ cells

The levels of p-ERK were evaluated in highly purified normal $CD34^+$ cells harvested from 16 donors. BM $CD34^+$ cells were studied under steady-state conditions (10 samples) and after G-CSF-mobilization (two samples). Steady-state BM $CD34^+$ cells did not or only weakly expressed p-ERK ($D=0.07\pm0.035$) (Figure 1). G-CSF-mobilized BM $CD34^+$ cells showed an approximately 4.4-fold increase ($D=0.31\pm0.08$) and G-CSF mobilized PB $CD34^+$ cells ($n=4$) even higher levels of p-ERK ($D=0.56\pm0.038$) ($P=0.02$, PB $CD34^+$ compared to BM $CD34^+$ cells), suggesting that the effects on p-ERK levels were not only due to G-CSF stimulation, but also vary in different compartments (Figure 1).

p-ERK levels in primary AML samples

We investigated the constitutive p-ERK levels in primary blast cells taken from AML patients. For these experiments, cells were separated and analyzed immediately after harvesting. We found constitutive ERK phosphorylation in 83.3% (35/42) cases, with a mean D value of 0.32 ± 0.04, confirming that the majority of AML shows enhanced activity of this pathway. Seven cases

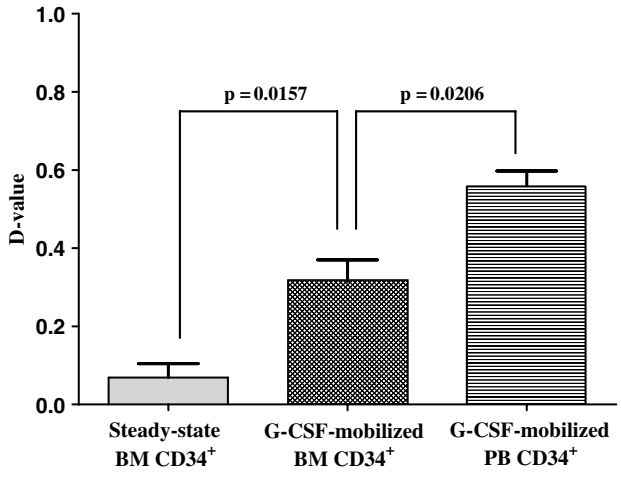

Figure 1 Normal CD34$^+$ cells from different sources show distinct ERK activation status. p-ERK expression in normal CD34$^+$ was analyzed by FCM as described in Materials and methods. MACS-sorted CD34$^+$ progenitor cells from steady-state BM CD34$^+$ cells were found to have little or no expression of p-ERK. G-CSF induced ERK activation in both BM and PB CD34$^+$ cells. The results are expressed as the mean$\pm$s.e.m. of *D* values obtained using Kolmogorov–Smirnov analysis.

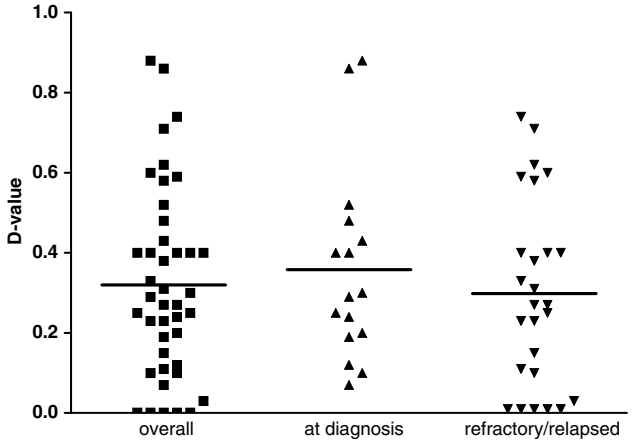

Figure 2 Constitutive p-ERK expression in primary AML samples. p-ERK expression in primary AML samples was analyzed by FCM as described in Materials and methods. Out of the 42 samples analyzed, 35 showed constitutive expression of p-ERK. There was no difference between samples from newly diagnosed and relapsed/refractory AML samples.

showed undetectable or very low levels of p-ERK (Figure 2). PMA activation induced a significant increase of p-ERK in all but one AML sample studied (from $D=0.32\pm0.04$ to $D=0.61\pm0.04$ ($P<0.0001$), data not shown). The detection of p-ERK levels by FCM was confirmed by Western blot analysis (Figure 3). No differences between diagnostic and relapsed/refractory samples were observed: *D* values were 0.36 ± 0.06 and 0.30 ± 0.05, respectively ($P=$NS) (Figure 2).

Effects of CI-1040 on p-ERK levels in AML cells

We then investigated the effects of CI-1040 on AML cell lines. CI-1040 completely inhibited ERK phosphorylation in NB4 cells, which exhibited constitutive ERK phosphorylation, after 24 h of exposure (Figure 4). Similar results were obtained in OCI-AML-3 cells (data not shown). Next, we examined the effects of CI-1040 on 27 AML samples. In DMSO-treated controls, a significant increase of p-ERK was observed at 24 h compared to time 0 (from $D=0.36\pm0.05$ to $D=0.46\pm0.04$, $P=0.03$) (Figure 5a). A 24 h incubation with CI-1040 led to a statistically significant decrease in p-ERK levels (from $D=0.46\pm0.04$ to $D=0.31\pm0.04$, $P=0.00027$). More than 50% inhibition was observed in 7/27 samples, with two samples showing complete abrogation of ERK phosphorylation (100% of inhibition), and $>25\%$ inhibition in additional seven samples. CI-1040 caused a $>20\%$ p-ERK decrease in 52% of samples. In the remaining samples, there appeared to be little or no change in p-ERK levels after 24 h exposure to CI-1040.

CI-1040 was also able to inhibit PMA-induced ERK activation reducing p-ERK levels from $D=0.60\pm0.08$ with vehicle alone to $D=0.31\pm0.07$ (Figure 5b).

We then compared the effects of CI-1040 on p-ERK at different stages of disease. A significant reduction in p-ERK was observed in both diagnostic ($P=0.0075$) and relapsed/refractory ($P=0.0031$) AML samples (Figure 6).

Effects of the specific MEK inhibitor CI-1040 on apoptosis

Treatment of OCI-AML3 cells with CI-1040 induced a time-dependent increase of apoptotic cells as demonstrated by hypodiploid DNA content, which appeared to require at least 48 h (Figure 7).

However, in primary patient samples, an increase in apoptotic cells was observed in only one sample obtained from a chemotherapy refractory patient. Annexin V+ cells increased after 24 h in the presence of inhibitor (11.86% in DMSO *vs* 31.62% in CI-1040), and increased 6.2-fold after 72 h (8.58 *vs* 53.36%) (data not shown). Notably, in the same patients, we observed strong inhibition of p-ERK levels at 3 μM CI-1040 ($>75\%$ inhibition by flow cytometry, and complete inhibition by Western blotting analysis). However, as noted, all other samples did not enter apoptosis following MEK inhibition with CI-1040.

Discussion

We report the activation of ERK in primary cells from patients with AML at diagnosis or with refractory or relapsed disease by FCM and demonstrate that a small molecule inhibitor of MEK/ERK, CI-1040, profoundly decreased the expression of p-ERK without inducing apoptosis.

Several groups have described the use of phospho-specific antibodies to detect phospho-proteins by FCM.[9–14] We describe here optimized conditions for p-ERK quantitation in primary patients cells by FCM (see Supplemental Information).

The observed lack of constitutive ERK activation in steady-state normal hematopoietic progenitor supports the notion that therapeutic strategies targeting MEK/ERK signaling pathway would selectively affect leukemic cells.[5,15,16] Previous studies suggested that circulating progenitor cells differ from their BM counterparts with respect to cell-cycle characteristics and proliferative response to cytokines,[17–20] which is also reflected in our finding of differential ERK phosphorylation and may reflect effects mediated by the microenvironment.

The finding that a high percentage (83.3%) of AML samples in our study contains activated ERK is remarkable. In comparison

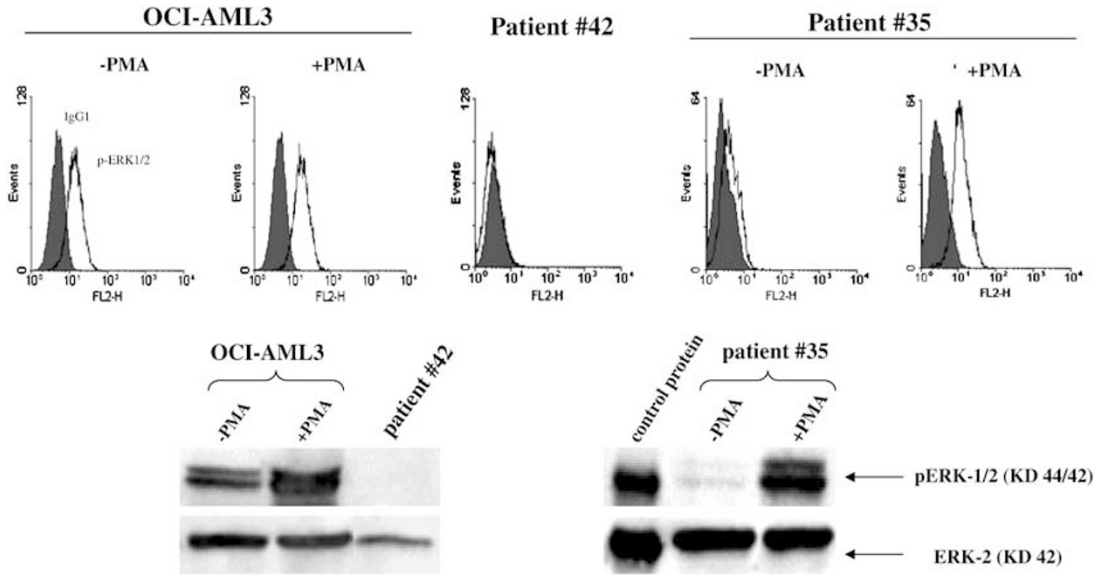

Figure 3 Correlation between FCM and Western blot analysis of p-ERK in OCI-AML3 and primary AML cells. Cells were processed for FCM and Western blot analysis as described in Materials and methods. Results obtained with both techniques were comparable.

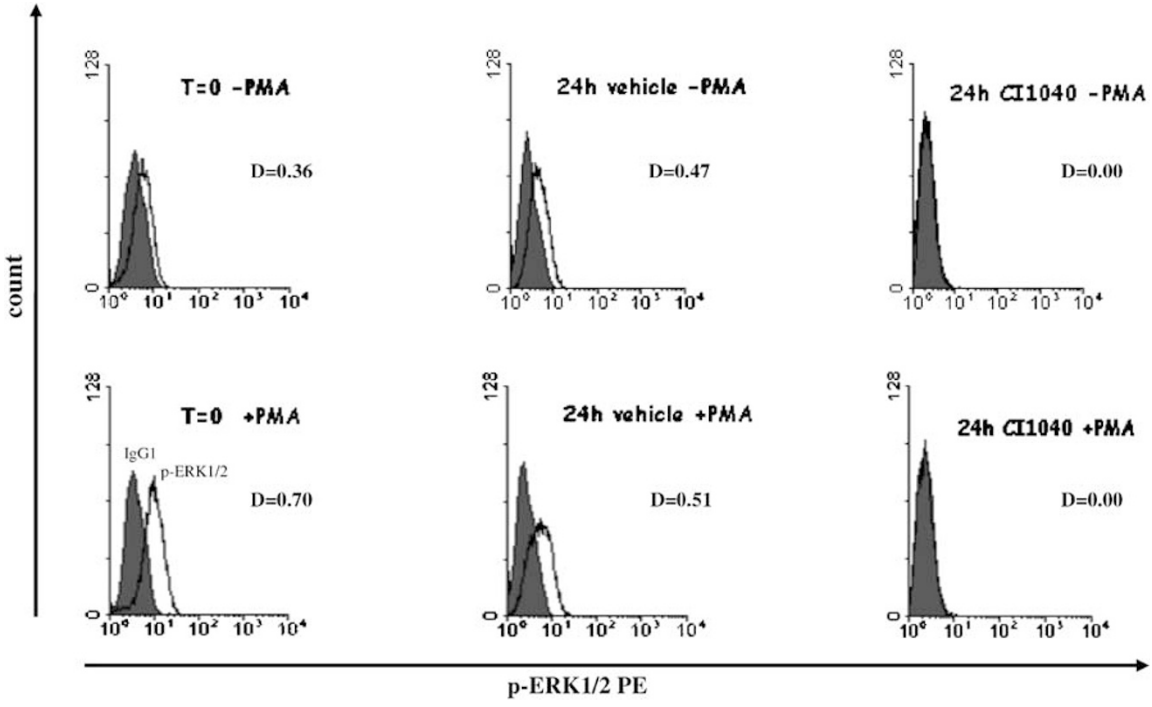

Figure 4 MEK blockade inhibits p-ERK expression in human leukemia cell line. Histograms show a representative experiment of constitutive p-ERK expression and pharmacodynamic inhibition in NB4 cells. Filled plot indicates isotype control. Cells were seeded at a starting concentration of 3×10^5 cells/ml, and cultured in the presence of vehicle control (DMSO) or CI-1040 at the indicated concentrations for up to 24 h. Viable cells were counted by Trypan blue dye exclusion at the indicated time points and processed for FCM analysis as described in Materials and methods. CI-1040 inhibited p-ERK expression after 24 h of liquid culture.

with previous reports[5,21,22] using Western blotting for analysis of p-ERK, we examined here, for the first time, clinical samples by FCM. FCM allows to better identify and quantify phosphoproteins in subpopulations of cells with undetectable or very low expression levels as compared to other methods, because of its ability to measure single cells and not just population averages. This ability is further maximized by the use of Kolmogorov–Smirnov analysis, which is suited to identify quantitatively such small subpopulations. No correlation between constitutive p-ERK expression and stage of disease, or between CI-1040-induced p-ERK inhibition and disease status was found. Results therefore suggest that prior therapy does not select clones with increased levels of p-ERK.

We noted that inhibition of ERK phosphorylation in AML cell lines was associated with inhibition of cell growth.[5] Consistent with the role of RAS/MAPK signaling in the G_1/S transition[3]

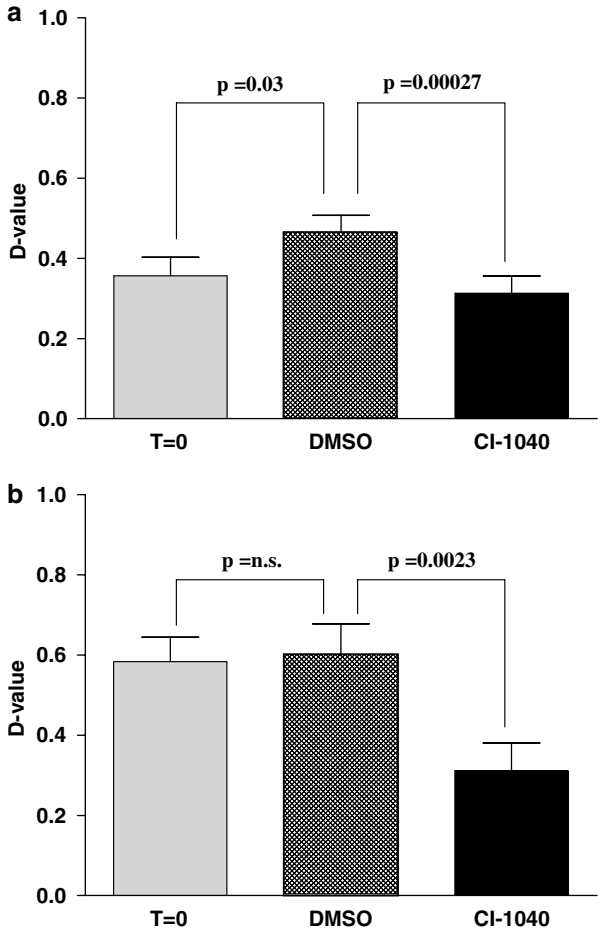

Figure 5 CI-1040 inhibits p-ERK expression in primary AML cells. There was a significant decrease in p-ERK in both (a) unstimulated ($P=0.00027$) and (b) PMA-stimulated ($P=0.0023$) AML samples incubated for 24 h in 10% FCS with 3 μM CI-1040. Results are expressed as mean $\pm$ s.e.m.

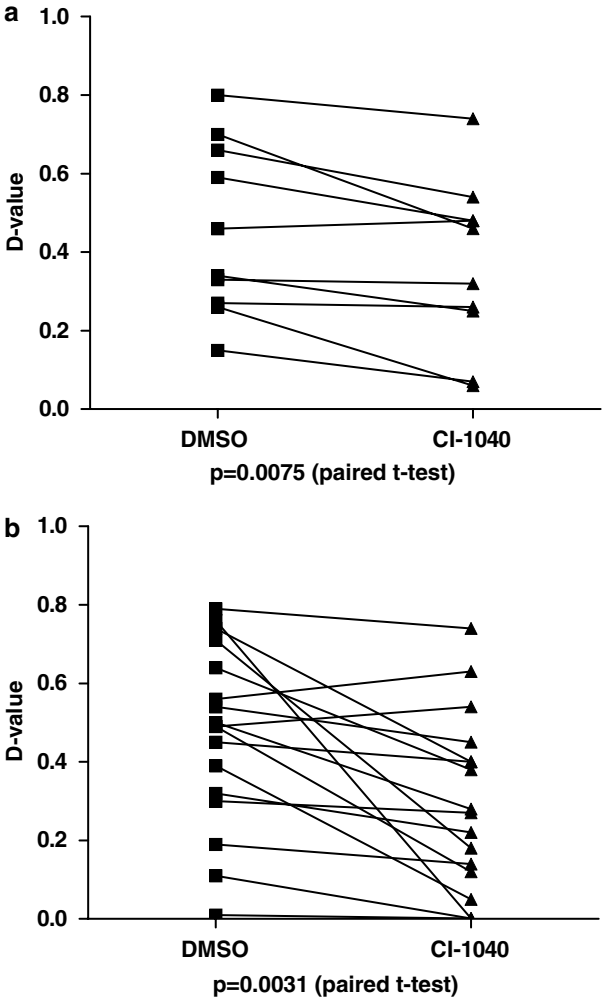

Figure 6 p-ERK inhibition in individual AML samples grouped according to disease status. Diagnostic (a) or relapsed/refractory (b) AML samples were equally sensitive to inhibition by CI-1040.

through post-translational regulation of $p27^{Kip1}$,[23,24] CI-1040 induced G_1 cell-cycle arrest in AML cell lines, associated with induction of apoptosis. In contrast, in primary AML, CI-1040 decreased p-ERK expression without effects on cell survival. Previous studies using the MEK inhibitor PD98059 reported that inhibition of p-ERK was coupled with the induction of apoptosis.[5,22] We failed to demonstrate apoptosis induction after 24 h and even after extended incubation with 3 μM CI-1040. Only in a single sample, p-ERK inhibition was associated with induction of apoptosis.

Preclinical studies demonstrated antitumor activity of CI-1040 in breast, pancreas, and colon tumor models and this activity has been shown to correlate with its inhibition of p-ERK[8] (Mitchell *et al*, *Proc Am Soc Clin Oncol* 2002; **21**: 8). CI-1040 was able to inhibit ERK1 and ERK2 phosphorylation by 99 and 92%, respectively, in MDA-MB-231 cells at a concentration of 1 μM. Other results from phase I clinical trials demonstrated that, although well tolerated, CI-1040 was active only in few patients, although inhibition of p-ERK between 46 and 100% was obtained in all but one sample[8] (Lorusso *et al*, *Proc Am Soc Clin Oncol* 2002; **21**: 81a). Our study of AML samples *in vitro* is consistent with these Phase I clinical results.

Consistent with the role of ERK signaling in growth factor-stimulated cell proliferation, the ability of MEK/ERK inhibitors PD98059 and U0126 to antagonize cell-cycle progression was reported.[25,26] However, Kamakura *et al*[27] suggested that these two inhibitors also prevent activation of MEK5/ERK5, raising the possibility that their antiproliferative effects are due to inhibition of both ERK1/2 and ERK5 signaling. Compared with the other kinase inhibitors, PD185342 (CI-1040) appears to be highly specific for MEK1/2, with very low activity against MEK5,[28,29] which may explain the lack of apoptosis induction. Other signaling pathways could also contribute to sustained AML blast survival. Xu *et al*[30] and Zhao *et al*[31] demonstrated that the PI3K pathway is deregulated in primary AML cells and that PI3K inhibition resulted in decreased survival, raising the possibility of signaling redundancy.[30]

With the exception of STI571, there is little evidence that the inhibition of any kinase alone, although effective *in vitro*, leads to useful antitumor effects in the clinical setting. However, several preclinical studies have already demonstrated synergistic effects of kinase inhibitors in combination with standard chemotherapy,[6,32,33] while others have reported apparent failure of combination strategies.[34] This raises the question of how to optimize the design of combination studies and when in the

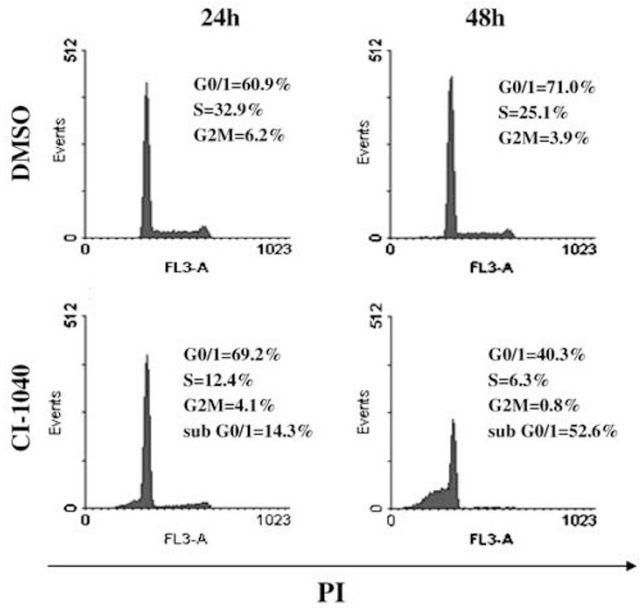

Figure 7 CI-1040 induces time-dependent apoptosis in OCI-AML3 cells. OCI-AML3 cells growing exponentially were cultured in the presence of vehicle control (DMSO) or CI-1040. Cells were harvested at different time points, washed, fixed in 70% ethanol, and stained for DNA content by PI. Apoptosis was evaluated as percentage of cells with hypodiploid DNA content. Results are expressed as net apoptosis induction.

course of tumor evolution it is most likely to have a measurable impact.[6]

The integration of different signal transduction pathways is decisive for cell survival.[35] As a consequence, combinations of inhibitors that target crosstalking survival pathways may be more effective than single agents. Activated PI3K/Akt and Raf/MEK/ERK pathways can synergize in the abrogation of cytokine dependence of hematopoietic cells.[36] Simultaneous inhibition of Bcl-2 and MEK/ERK signaling (by CI-1040) resulted in highly synergistic reduction of cell growth and induction of apoptosis in AML cell lines with constitutive ERK activation[37,38] and may be a strategy to convert the cytostatic effects of MAPK inhibition into apoptotic responses.

In conclusion, our data suggest that pharmacological inhibition of a single molecular target in AML might not result in antitumor activity. More likely, targeting additional signaling pathways that act in parallel or downstream of one another could be more effective, and predictive pharmacodynamic assays aimed at selecting potentially sensitive patient populations may be critical for developing targeted combination therapies.[38]

Acknowledgements

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Supplementary Information

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu).

References

1 Ketley NJ, Allen PD, Kelsey SM, Newland AC. Mechanisms of resistance to apoptosis in human AML blasts: the role of differentiation-induced perturbations of cell-cycle checkpoints. *Leukemia* 2000; **14**: 620–628.
2 Del Poeta G, Venditti A, Del Principe MI, Maurillo L, Buccisano F, Tamburini A *et al.* Amount of spontaneous apoptosis detected by Bax/Bcl-2 ratio predicts outcome in acute myeloid leukemia (AML). *Blood* 2003; **101**: 2125–2131.
3 Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL *et al.* Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* 2003; **17**: 1263–1293.
4 Platanias LC. Map kinase signaling pathways and hematologic malignancies. *Blood* 2003; **101**: 4667–4679.
5 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.
6 Dancey J, Sausville EA. Issues and progress with protein kinase inhibitors for cancer treatment. *Nat Rev Drug Discov* 2003; **2**: 296–313.
7 Sebolt-Leopold JS. Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene* 2000; **19**: 6594–6599.
8 Allen LF, Sebolt-Leopold J, Meyer MB. CI-1040 (PD184352), a targeted signal transduction inhibitor of MEK (MAPKK). *Semin Oncol* 2003; **30**: 105–116.
9 Chow S, Patel H, Hedley DW. Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors. *Cytometry* 2001; **46**: 72–78.
10 Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry* 2003; **55A**: 61–70.
11 Perez OD, Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. *Nat Biotechnol* 2002; **20**: 155–162.
12 Tazzari PL, Cappellini A, Bortul R, Ricci F, Billi AM, Tabellini G *et al.* Flow cytometric detection of total and serine 473 phosphorylated Akt. *J Cell Biochem* 2002; **86**: 704–715.
13 Fleisher TA, Dorman SE, Anderson JA, Vail M, Brown MR, Holland SM. Detection of intracellular phosphorylated STAT-1 by flow cytometry. *Clin Immunol* 1999; **90**: 425–430.
14 Zell T, Khoruts A, Ingulli E, Bonnevier JL, Mueller DL, Jenkins MK. Single-cell analysis of signal transduction in CD4T cells stimulated by antigen *in vivo*. *Proc Natl Acad Sci USA* 2001; **98**: 10805–10810.
15 Birkenkamp KU, Esselink MT, Kruijer W, Vellenga E. An inhibitor of PI3-K differentially affects proliferation and IL-6 protein secretion in normal and leukemic myeloid cells depending on the stage of differentiation. *Exp Hematol* 2000; **28**: 1239–1249.
16 Fichelson S, Freyssinier JM, Picard F, Fontenay-Roupie M, Guesnu M, Cherai M *et al.* Megakaryocyte growth and development factor-induced proliferation and differentiation are regulated by the mitogen-activated protein kinase pathway in primitive cord blood hematopoietic progenitors. *Blood* 1999; **94**: 1601–1613.
17 Lemoli RM, Tafuri A, Fortuna A, Petrucci MT, Ricciardi MR, Catani L *et al.* Cycling status of CD34+ cells mobilized into peripheral blood of healthy donors by recombinant human granulocyte colony-stimulating factor. *Blood* 1997; **89**: 1189–1196.
18 Verfaillie CM, Almeida-Porada G, Wissink S, Zanjani ED. Kinetics of engraftment of CD34(−) and CD34(+) cells from mobilized blood differs from that of CD34(−) and CD34(+) cells from bone marrow. *Exp Hematol* 2000; **28**: 1071–1079.
19 Steidl U, Kronenwett R, Rohr UP, Fenk R, Kliszewski S, Maercker C *et al.* Gene expression profiling identifies significant differences between the molecular phenotypes of bone marrow-derived and circulating human CD34+ hematopoietic stem cells. *Blood* 2002; **99**: 2037–2044.
20 Lemoli RM, Bertolini F, Petrucci MT, Gregorj C, Ricciardi MR, Fogli M *et al.* Functional and kinetic characterization of

granulocyte colony-stimulating factor-primed CD34− human stem cells. *Br J Haematol* 2003; **123**: 720–729.
21 Towatari M, Iida H, Tanimoto M, Iwata H, Hamaguchi M, Saito H. Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia* 1997; **11**: 479–484.
22 Lunghi P, Tabilio A, Dall'Aglio PP, Ridolo E, Carlo-Stella C, Pelicci PG *et al*. Downmodulation of ERK activity inhibits the proliferation and induces the apoptosis of primary acute myelogenous leukemia blasts. *Leukemia* 2003; **17**: 1783–1793.
23 Cheng M, Sexl V, Sherr CJ, Roussel MF. Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proc Natl Acad Sci USA* 1998; **95**: 1091–1096.
24 Hoshino R, Tanimura S, Watanabe K, Kataoka T, Kohno M. Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: upregulation of p27(Kip1). *J Biol Chem* 2001; **276**: 2686–2692.
25 Balmanno K, Cook SJ. Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells. *Oncogene* 1999; **18**: 3085–3097.
26 Lobenhofer EK, Huper G, Iglehart JD, Marks JR. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. *Cell Growth Differ* 2000; **11**: 99–110.
27 Kamakura S, Moriguchi T, Nishida E. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. *J Biol Chem* 1999; **274**: 26563–26571.
28 Mody N, Leitch J, Armstrong C, Dixon J, Cohen P. Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. *FEBS Lett* 2001; **502**: 21–24.
29 Squires MS, Nixon PM, Cook SJ. Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1. *Biochem J* 2002; **366**: 673–680.
30 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.
31 Zhao S, Konopleva M, Cabreira-Hansen M, Xie Z, Hu W, Milella M *et al*. Inhibition of phosphatidylinositol 3-kinase (PI3K) dephosphorylates BAD and promotes apoptosis in myeloid leukemias. *Leukemia* 2004; **18**: 267–275.
32 Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000; **6**: 4885–4892.
33 MacKeigan JP, Taxman DJ, Hunter D, Earp III HS, Graves LM, Ting JP. Inactivation of the antiapoptotic phosphatidylinositol 3-kinase–Akt pathway by the combined treatment of taxol and mitogen-activated protein kinase kinase inhibition. *Clin Cancer Res* 2002; **8**: 2091–2099.
34 Wilkinson E. Surprise phase III failure for ZD1839. *Lancet Oncol* 2002; **3**: 583.
35 Blalock WL, Navolanic PM, Steelman LS, Shelton JG, Moye PW, Lee JT *et al*. Requirement for the PI3K/Akt pathway in MEK1-mediated growth and prevention of apoptosis: identification of an Achilles heel in leukemia. *Leukemia* 2003; **17**: 1058–1067.
36 Shelton JG, Steelman LS, Lee JT, Knapp SL, Blalock WL, Moye PW *et al*. Effects of the RAF/MEK/ERK and PI3K/AKT signal transduction pathways on the abrogation of cytokine-dependence and prevention of apoptosis in hematopoietic cells. *Oncogene* 2003; **22**: 2478–2492.
37 Milella M, Estrov Z, Kornblau SM, Carter BZ, Konopleva M, Tari A *et al*. Synergistic induction of apoptosis by simultaneous disruption of the Bcl-2 and MEK/MAPK pathways in acute myelogenous leukemia. *Blood* 2002; **99**: 3461–3464.
38 Milella M, Precupanu C, Gregorj C, Ricciardi M, Petrucci M, Kornblau SM *et al*. Beyond single pathway inhibition: MEK inhibitors as a platform for the development of pharmacological combinations with synergistic anti-leukemic effects. *Curr Pharm Des*, in press.