J Mol Med (2012) 90:1133–1144
DOI 10.1007/s00109-012-0886-z

ORIGINAL ARTICLE

Therapeutic potential of MEK inhibition in acute myelogenous leukemia: rationale for "vertical" and "lateral" combination strategies

Maria Rosaria Ricciardi · Maria Cristina Scerpa · Paola Bergamo · Ludovica Ciuffreda · Maria Teresa Petrucci · Sabina Chiaretti · Simona Tavolaro · Maria Grazia Mascolo · Stephen L. Abrams · Linda S. Steelman · Twee Tsao · Antonio Marchetti · Marina Konopleva · Donatella Del Bufalo · Francesco Cognetti · Robin Foà · Michael Andreeff · James A. McCubrey · Agostino Tafuri · Michele Milella

Received: 1 July 2011 / Revised: 8 February 2012 / Accepted: 14 February 2012 / Published online: 8 March 2012

Abstract In hematological malignancies, constitutive activation of the RAF/MEK/ERK pathway is frequently observed, conveys a poor prognosis, and constitutes a promising target for therapeutic intervention. Here, we investigated the molecular and functional effects of pharmacological MEK inhibition in cell line models of acute myeloid leukemia (AML) and freshly isolated primary AML samples. The small-molecule, ATP-non-competitive, MEK inhibitor PD0325901 markedly inhibited ERK phosphorylation and growth of several AML cell lines and approximately 70 % of primary AML samples. Growth inhibition was due to G_1-phase arrest and induction of apoptosis. Transformation by constitutively active upstream

Electronic supplementary material The online version of this article (doi:10.1007/s00109-012-0886-z) contains supplementary material, which is available to authorized users.

M. R. Ricciardi · M. C. Scerpa · M. T. Petrucci · S. Chiaretti · S. Tavolaro · M. G. Mascolo · R. Foà · A. Tafuri
Division of Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University of Rome,
Via Benevento 6,
00161 Rome, Italy

P. Bergamo · L. Ciuffreda · F. Cognetti · M. Milella (✉)
Division of Medical Oncology A,
Regina Elena National Cancer Institute,
Via Elio Chianesi 53,
00144 Rome, Italy
e-mail: milella@ifo.it

M. Milella
e-mail: michelemilella@hotmail.com

S. L. Abrams · L. S. Steelman · J. A. McCubrey
Department of Microbiology and Immunology,
Brody School of Medicine, East Carolina University,
600 Moye Blvd,
27834, Greenville, NC, USA

T. Tsao · M. Konopleva · M. Andreeff
Section of Molecular Hematology and Therapy,
University of Texas M. D. Anderson Cancer Center,
1515 Holcombe Blvd,
77030, Houston, TX, USA

A. Marchetti
Clinical Research Center, Center of Excellence on Aging,
University-Foundation,
Via dei Vestini 31,
66100 Chieti, Italy

D. Del Bufalo
Laboratory of Experimental Preclinical Chemotherapy,
Regina Elena National Cancer Institute,
Via delle Messi D'Oro 156,
00158 Rome, Italy

pathway elements (HRAS, RAF-1, and MEK) rendered FDC-P1 cells exquisitely prone to PD0325901-induced apoptosis. Gene and protein expression profiling revealed a selective effect of PD0325901 on ERK phosphorylation and compensatory upregulation of the RAF/MEK and AKT/$p70^{S6K}$ kinase modules, potentially mediating resistance to drug-induced growth inhibition. Consequently, in appropriate cellular contexts, both "vertical" (i.e., inhibition of RAF and MEK along the MAPK pathway) and "lateral" (i.e., simultaneous inhibition of the MEK/ERK and mTOR pathways) combination strategies may result in synergistic anti-leukemic effects. Overall, MEK inhibition exerts potent growth inhibitory and proapoptotic activity in preclinical models of AML, particularly in combination with other pathway inhibitors. Deeper understanding of the molecular mechanisms of action of MEK inhibitors will likely translate into more effective targeted strategies for the treatment of AML.

Keywords MAPK · Hematological malignancies · Sensitivity · Resistance · Combinations · Synergism

Introduction

The mitogen-activated protein kinase (MAPK) pathway is a key integration point along the signal transduction cascades that emanate from tyrosine kinases and links diverse extracellular stimuli to an array of physiological responses, including proliferation, differentiation, and survival [1]. Constitutive activation of either MAPK kinase (MEK) or extracellular-signal activated kinase (ERK) is sufficient to transform mammalian cells, and many upstream-acting cellular oncogenes critically rely on activation of the MEK/ERK pathway to induce a transformed phenotype. As a result, constitutive MEK/ERK activation is detected in a significant proportion of a variety of human tumors and has recently emerged as a potential target for anticancer therapies [2].

In hematological malignancies, particularly acute leukemias, MAPK is frequently dysregulated and portends an aggressive, therapy-resistant clinical behavior [3–5]. Our group and others have provided compelling evidence that pharmacological blockade of MEK-to-ERK signaling profoundly impairs leukemic, but not normal, cell proliferation, and clonogenic growth, and synergizes with a variety of cytotoxics and biological agents in inducing apoptosis [6–11]. These properties make the MEK/ERK signaling module a prime target for the molecular therapy of hematological malignancies, particularly acute myelogenous leukemia (AML), and support the preclinical/clinical development of MEK inhibitors as therapeutic agents in this disease [5, 12, 13].

Several MEK inhibitors have shown promising preclinical activity in vitro and in vivo against a broad spectrum of solid tumors and are currently in clinical testing [14]. Here, we investigated the molecular and functional effects of the MEK inhibitor PD0325901 [2], recently shown effective in controlling KRAS-driven myeloproliferative disease in vivo in transgenic mouse models [15], in preclinical models of AML, acute lymphoblastic leukemia (ALL), and multiple myeloma (MM). We found that PD0325901 effectively blocks signaling through the MEK/ERK module and exerts potent growth inhibitory and proapoptotic activity, particularly in AML. Analysis of signaling perturbations induced by PD0325901 revealed that, under certain conditions, MEK inhibition leads to compensatory upstream hyperactivation of RAF and/or parallel signaling through the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, both of which may function as "escape" pathways. Consequently, in appropriate cellular contexts, combined MEK/RAF and MEK/mTOR inhibition may result in synergistic inhibition of leukemic cell growth, paving the way for the design of rational, mechanism-based combination strategies.

Experimental procedures

Pathway inhibitors

The MEK inhibitor PD0325901 [*N*-((*R*)-2,3-dihydroxypropoxy)-3,4-difluoro-2-(2-fluoro-4-iodo-phenylamino)-benzamide] [2] was kindly provided by Pfizer Global Research & Development, Ann Arbor, MI, USA. The RAF/multikinase inhibitor Sorafenib was kindly provided by Bayer Pharmaceuticals Co, West Haven, CT, USA. The mTOR inhibitor Temsirolimus was kindly provided by Wyeth-Ayerst Research.

Cell lines and primary samples

Human myeloid, lymphoid, and MM cell lines were maintained in RPMI 1640 medium containing 10 % heat-inactivated fetal calf serum (FCS), 1 mM L-glutamine, and 50 μg/ml penicillin/streptomycin (Gibco, Milan), in a humidified atmosphere containing 5 % CO_2 at 37°C. Cell lines were harvested in log-phase growth for all experiments, washed and seeded at the appropriate concentration in the presence of PD0325901 (0.1–1,000 nM) or a matched concentration of vehicle (DMSO). For combination experiments, synergism, additive effects, and antagonism were assessed using the Chou–Talalay method [16] and the Calcusyn software (Biosoft, Ferguson, MO, USA).

Oncogene-transformed FDC-P1 clones were obtained as previously described [17]. Differential sensitivity of oncogene-transformed FDC-P1 cells to the proapoptotic effects of PD0325901 was determined by plating 10^6 cells/well in six-well plates in RPMI1640 medium supplemented with 10 % fetal bovine serum +/− IL-3 and serial 10-fold dilutions (n=6 dilutions). Annexin V/PI analysis was then performed after 72 h of incubation.

BM aspirates were obtained from 26 AML patients (Table S2), who provided written informed consent, in accordance with regulations and protocols sanctioned by the Human Subjects Committee of Helsinki and approved by the Institutional Review Board of the Sapienza University of Rome. Mononuclear cells (MNC) were separated from AML samples by differential Ficoll–Hypaque centrifugation (1.077 g/ml, Sigma). Cell number and viability was assessed by triplicate trypan blue exclusion counting. Cells were resuspended in complete medium, and cell number was adjusted to a starting concentration of 1.0×10^6/ml, before culture in the presence of either 10 or 100 nM PD0325901 or a matched concentration of vehicle. AML blast progenitor colony assays were performed as previously described: Briefly, 1×10^5 MNC from AML patients were plated in 0.8 % methylcellulose in Iscove's modified Dulbecco's medium (Gibco-BRL) supplemented with 30 % FCS, 5×10^{-5} M β-mercaptoethanol, and 10 % of 5637 cell line supernatant. Cultures were incubated in 35-mm Petri dishes in duplicate for 10 days at 37°C and 5 % CO_2 and evaluated for the number of clusters (8–10 cells) and colonies (>20 cells).

Cell cycle and apoptosis analysis

Cell cycle distribution changes were evaluated using the Acridine Orange (AO) technique, as previously described. Cell-cycle distribution was determined by measuring simultaneously the DNA and RNA total cellular content and analyzed using the ModFit LT software (Verity Software House, Topsham, ME, USA). The percentage of apoptotic cells was measured based on the decreased stainability of apoptotic elements in DNA green fluorescence (sub-G_1 peak) coupled with a higher RNA red fluorescence. Induction of apoptosis was also assessed by measuring annexin V binding to externalized phosphatidylserine.

Western blotting and phospho-proteomic analysis

Protein expression was evaluated by Western blotting as previously described [7]. Briefly, cells were washed twice with a solution containing 10 mM NaF, 0.1 mM Na_3VO_4, 0.1 % NaN_3, 4 mM EDTA, 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-X100 supplemented with protease inhibitor cocktail (Roche Diagnostic Corp., Indianapolis, IN, USA). Equal amount of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA), transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckingamshire, UK), and immunoblotted with antibodies specific for p-Raf-1, p-MEK, MEK, p-ERK, ERK2, p-AKT (S473, unless otherwise specified), AKT (Cell Signaling Technology, Inc., Beverly, MA, USA), p27 and cyclin E (PharMingen, San Diego, CA, USA), Cyclin D1, Cyclin D3, Cdc25a, and Cdc6 (Santa Cruz). Membranes were probed with a horseradish peroxidase-conjugated secondary antibody and reacted with ECL reagent (Amersham). Semiquantitative phospho-proteomic analysis was performed after 1 and 6 h of treatment with either DMSO or PD0325901 (10 nM). The phosphorylation status of 18 different proteins was assessed using a custom Kinexus KCPS-1.0 Phospho-Protein Screen (Kinexus, Vancouver, BC, Canada). Proteomic analysis was carried out using a high throughput Kinex™ Antibody Microarray (Kinexus).

Oligonucleotide arrays

RNA was extracted from either OCI-AML3 or U937 exposed to PD0325901 (10–1,000 nM) or to vehicle (DMSO) for up to 24 h. Total RNA was extracted using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) and further purified using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. For oligonucleotide array analysis, the HGU133 2.0 arrays were used. The detailed protocol for sample preparation and microarray processing is available from Affymetrix (www.affymetrix.com). Experiments were run in duplicate. For statistical analysis, Affymetrix gene expression data were processed with dChip software, (www.dchip.org), which uses an invariant set normalization method. The array with the median overall intensity was chosen as the baseline for normalization. Model-based expressions were computed for each array and probe set, using only *perfect match* probes. For unsupervised analysis, the following non-specific filtering criteria were used: (1) Gene expression level was required to be higher than 100 in at least 10 % of the samples; (2) the ratio of the standard deviation to the mean expression across all samples was required to be between 0.5 and 1,000. Supervised analyses were performed to compare DMSO-treated cells with PD0325901-treated cells at 6 and 24 h. For these comparisons,

a *t* test was used: Only the genes with a $p \leq 0.05$ and a fold change ≥2 were retained.

Results

PD0325901 inhibits ERK phosphorylation and cell growth in leukemic cell lines

PD0325901 dose dependently inhibited ERK phosphorylation, without affecting total ERK protein levels, in OCI-AML3 cells, (Fig. 1a, b). PD0325901 also potently (IC_{50}, 5–100 nM) inhibited the growth of several AML cell lines; the APL cell line NB4 and MM cell lines displayed intermediate sensitivity (IC_{50}, 150–800 nM), while the myeloid cell lines U937 and KG-1 and all of the lymphoid cell lines tested proved resistant, with IC_{50} >1,000 nM (Table S1). PD0325901-sensitive cell lines displayed significantly higher levels of unstimulated ERK phosphorylation (p= 0.049, Fig. S1A). In sensitive cell lines, PD0325901-induced growth inhibition (Fig. 1c) was a consequence of inhibition of cell cycle progression and induction of apoptosis (Fig. S2).

PD0325901 inhibits the growth of primary AML cells

PD0325901 also induced a >30 % reduction in the number of viable cells in 12 of 18 ex vivo-cultured primary AML samples, with a significant (p=0.01) reduction in the percentage of cells in the S-phase of the cell cycle and a significant (p=0.019) increase in the number of apoptotic cells (Table S2 and Fig. S3A-C). As shown for cell lines, PD0325901-sensitive primary AML samples displayed significantly higher levels of unstimulated ERK phosphorylation (p=0.032, Fig. S1B). A representative experiment demonstrating the effect of PD0325901 in a "sensitive" primary AML sample is shown in Fig. S3D. No obvious correlation was observed between sensitivity/resistance to PD0325901 and cytogenetic/molecular profile in the patient samples analyzed (Table S2). PD0325901 also dose dependently inhibited clonogenic growth in six of the eight samples tested (Fig. 1d).

Oncogene-transformed hematopoietic cells are differentially sensitive to MEK inhibition by PD0325901

IL-3-dependent FDC-P1 cells transformed to cytokine independency by gene transduction [17] with a panel of ten different oncogenes (Table 1) were examined for their sensitivity to the pro-apoptotic effects of PD0325901. Parental FDC-P1 cells grown in IL-3 were highly resistant to PD0325901-induced apoptosis (ED_{50} for apoptosis induction >5,000 nM); however, cytokine-independent, transformed, FDC-P1 clones all became sensitive to MEK inhibition by PD0325901, although to variable degrees (ED_{50}, 1–100 nM). In particular, IGF-1R-, v-Fms-, v-Ha-Ras-, ΔRaf-1:ER-, and ΔMEK:ER-transformed FDC-P1 clones were exquisitely sensitive to the proapoptotic action of PD0325901, with an ED_{50} of approximately 1 nM. Overall, these results suggest that the activation of oncogenes that impinge directly on the MAPK pathway may result in hypersensitivity to MEK blockade by PD0325901.

Gene and protein expression profiling

In OCI-AML3, a total of 80 genes were either up- (32 genes) or downregulated (48 genes) by 10 nM PD0325901 after 24 h, with a remarkable over-representation of genes involved in DNA replication initiation and regulation of the G_1/S checkpoint among downmodulated genes (Fig. 2a and Table S3). Conversely, only three genes were significantly downmodulated upon treatment with 10 nM PD0325901 in functionally resistant U937 cells, with no overlap with OCI-AML3 cells (data not shown). Even after exposure of U937 cells to a 100-fold higher drug concentration (1,000 nM), most of the PD0325901-modulated genes were related to stress response (Fig. S4 and Table S4), and only six genes (DUSP6, MCM4, RFC3, CCNE2, STIP1, and YPEL5) were concordantly modulated, as compared with OCI-AML3 cells. Comparative proteomic analysis of OCI-AML3 and U937 cells demonstrated only minimal overlap in the pattern of proteins whose phosphorylation and/or expression levels were significantly modulated by PD0325901 (Fig. 2b). The modulation of CCNE2, Cdc25A, and Cdc6 was confirmed by Western blot analysis in OCI-AML3, and cyclin D3 was also found to be profoundly downregulated at the protein level (Fig. S5).

PD0325901 effects on the phosphorylation status of selected signaling proteins

To better understand the molecular mechanisms of action of PD0325901, the phosphorylation status of different signaling elements was tested in OCI-AML3 cells exposed to PD0325901 (10 nM) for 1 and 6 h (Fig. 3). PD0325901 reduced ERK1/2 phosphorylation by >80 %; interestingly, PD0325901 also transiently increased the phosphorylation of the upstream kinases PKCα (+121 % at 1 h), Raf-1 (+80 to +160 % at 1 h), and MEK1/2 (+140 % at 6 h), as well as AKT (+120 % at 1 h) and $p70^{S6K}$ (+180 % at 1 h) phosphorylation (Fig. 3). Overall, these data suggest that PD0325901 may lead to compensatory upregulation of both upstream and parallel signaling pathways.

Simultaneous inhibition of RAF and MEK results in highly synergistic induction of apoptosis

Western blot analysis of OCI-AML3 cells exposed to increasing PD0325901 concentrations demonstrated dose-

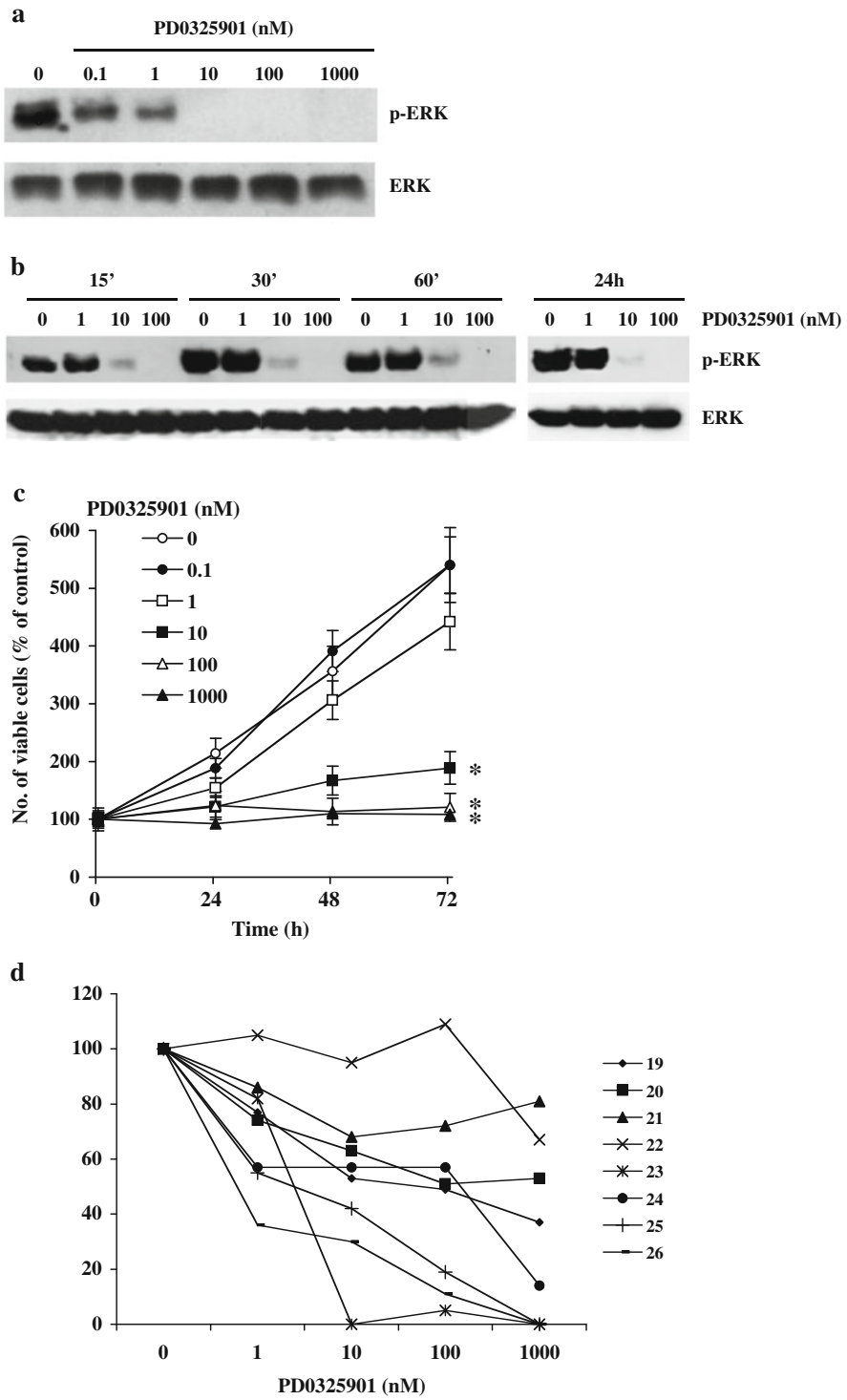


Fig. 1 PD0325901 effects in AML cell lines and primary samples. **a** Dose–response and **b** time course: OCI-AML3 cells were exposed to the indicated concentrations of PD0325901 or vehicle control for up to 24 h; protein lysates were then analyzed by Western blotting for doubly phosphorylated ERK-1/2 (p-ERK) and total ERK-2 (ERK). Results from one experiment representative of at least three independent experiments performed with superimposable results are shown. **c** Dose–response growth curves: OCI-AML3 cells were exposed to increasing concentrations of PD0325901 for the indicated periods of time. Cell counts and viability were then assessed by trypan blue exclusion counting. Results are expressed as percentage of cells plated at the beginning of the experiment (time 0) and represent the average±SD of seven independent experiments (*$p<0.05$ by two-tailed Student's t test for the comparison between PD0325901- and vehicle control-treated cells at the 72-h time point). **d** Clonogenic assay: primary AML samples (patients 19–26 in Table S2) were subjected to semi-solid AML blast colony assays, as described in "Experimental procedures," in the presence of PD0325901 at the indicated concentrations. Results are expressed as percentage of AML colonies in vehicle control-treated samples and represent the average of experimental triplicates. SD were routinely <10 % and were therefore omitted for clarity

dependent phosphorylation of both Raf-1 (S259) and MEK (S217/S221) (Fig. 4a). Thus, we tested the hypothesis that a "vertical" combination strategy, employing RAF and MEK inhibitors, might enhance apoptosis induction. To this purpose, OCI-AML3 cells were exposed to PD0325901 and the RAF inhibitor sorafenib at their approximate IC_{50} (15 and 1,500 nM, respectively) for up to 72 h, alone or in combination; both agents partially inhibited OCI-AML3 growth when used alone, while the combination completely suppressed cell growth (Fig. 4b). From a mechanistic standpoint, the combination of PD0325901 and sorafenib more efficiently suppressed cell cycle progression and strikingly enhanced apoptosis induction (>60 % at 48 h), as compared to each agent alone (Fig. 4c).

Table 1 Differential sensitivity of single oncogene-transformed FDC-P1 cells to MEK inhibition by PD0325901

Cell line	PD0325901 ED_{50} for apoptosis (nM)
FDC-P1[a]	5,000
FD/IGF-1R[b]	1
FD/v-Fms[c]	1
FD/v-ErbB:ER[d]	8
FD/BCR-ABL	20
FD/v-Src	100
FD/v-Ha-Ras	1
FD/ΔB-Raf:ER	6
FD/ΔRaf-1:ER	1
FD/ΔA-Raf:ER	10
FD/ΔMEK1:ER	1

[a] FDC-P1 cells were grown in IL-3

[b] FD/IGF-1R cells were grown in medium supplemented with 10 nM IGF-1

[c] FD/v-Fms, FD/BCR-ABL, FD/v-Src, and FD/v-Ha-Ras were grown in the absence of IL-3

[d] FD/v-ErbB:ER, FD/ΔB-Raf:ER, FD/ΔRaf-1:ER, FD/ΔA-Raf:ER, and FD/ΔMEK1:ER cells were grown in medium containing β-estradiol

When analyzed by conservative isobologram analysis, the growth inhibitory interaction between PD0325901 and sorafenib at a fixed ratio of 1:100 was highly synergistic (Fig. 4d), with an average combination index (CI) at the ED_{50}, ED_{75}, and ED_{90} of 0.3±0.1. Similar results, in terms of both rebound phosphorylation of Raf-1/MEK and synergistic interactions between PD0325901 and sorafenib, were obtained in the MOLM-13 cell line (average CI: 0.4±0.1), when the optimal PD0325901/sorafenib ratio of 10:1 was used (data not shown).

Impaired ERK inhibition and increased AKT activation correlate with resistance to PD0325901

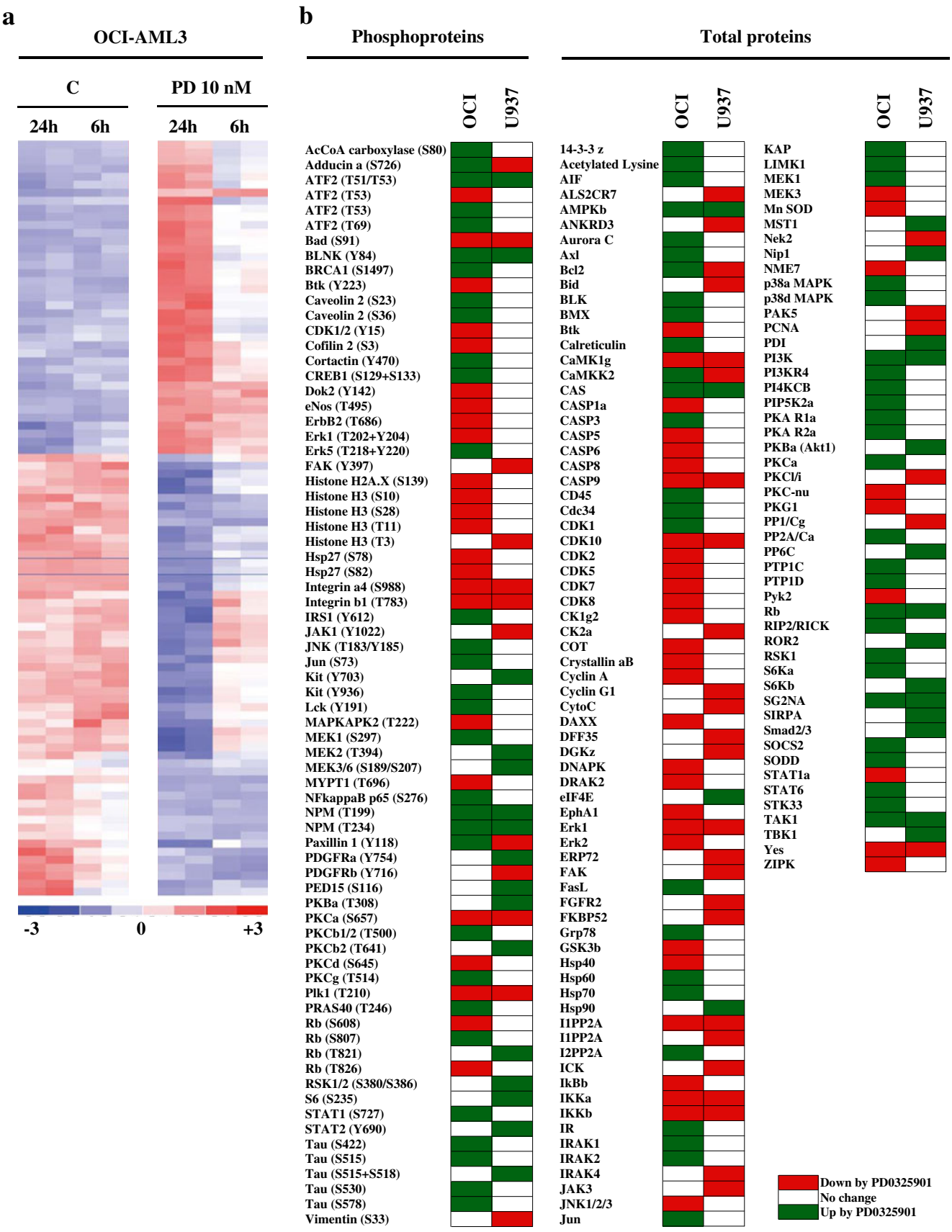
We next tested the hypothesis that activation of "parallel" signaling through the PI3K/AKT/mTOR pathway may play a role in functional resistance to MEK inhibition. In sensitive OCI-AML3 cells, high concentrations of PD0325901 ultimately led to concomitant inhibition of AKT (S473) phosphorylation (Fig. 5a), although AKT was transiently hyperphosphorylated at early time points (Fig. 3 and Fig. S6). Conversely, in a resistant OCI-AML3 sub-line (Fig. 5b and Fig. S7) and in U937 (Fig. 5c) cells, complete inhibition of ERK phosphorylation required at least 100-fold higher PD0325901 concentrations and AKT (S473) phosphorylation was induced, particularly at suboptimal PD0325901 concentrations. Similar results, in terms of incomplete inhibition of ERK phosphorylation, were obtained also in resistant lymphoid and breast cancer cell lines (data not shown). Consistent with the concept that

Fig. 2 Gene and protein expression profiling. **a** OCI-AML3 cells were exposed to either 10 nM PD0325901 or a matched concentration of vehicle. At the indicated time points, cells were collected, total RNA was extracted and purified, and gene expression profiles were performed using Affymetrix U133A 2.0 gene chips (see "Experimental procedures"). Supervised analysis shows 80 genes differentially expressed between PD0325901- and vehicle control-treated OCI-AML3 cells, after 6 and 24 h (see Table S3 for a list of PD0325901-modulated genes). Results relative to two independent experiments are shown. **b** OCI-AML3 and U937 cells were exposed to PD0325901 (10 and 1,000 nM for OCI-AML3 and 1,000 nM for U937) or matched vehicle control for 6 h, then lysed, and subjected to proteomic analysis of 500 unmodified and 300 phosphorylated epitopes, using a high throughput Kinex™ Antibody Microarray (Kinexus). Raw data were then analyzed using specific filtering criteria, which included the $lLog_2$-ratio of treated and control averages, used to calculate the differential signal intensities of each protein. Results are expressed using a color code indicating epitopes upregulated (*green*), downregulated (*red*), or without significant changes (*white*) in response to PD0325901 treatment in each cell line. For OCI-AML3 cells, both the 10 nM and the 1,000 concentrations were considered in the analysis (significant changes with either dose and concordant changes with both doses were retained, while discordant changes in response to the two concentrations of PD0325901 were discarded)

MEK blockade may inhibit or activate the PI3K/AKT/mTOR pathway in functionally sensitive and resistant cells, respectively, transcriptomic analysis revealed that PD0325901 upregulated PTEN expression in OCI-AML3 cells and mTOR expression in U937 cells (Fig. 5d). In addition, PD0325901 also induced AKT expression and phosphorylation (T308) and S6 phosphorylation in U937 cells, as evidenced by proteomic analysis (Fig. 2b). Notably, comparative transcriptomic and proteomic analysis of PD0325901-sensitive (OCI-AML3) and PD0325901-resistant (U937) cell lines revealed marked downregulation of PTEN and upregulation of mTOR messenger RNA, upregulation of PI3K expression, increased AKT1 expression and phosphorylation (T308 and S473), inhibitory PTEN phosphorylation (S380+S382+S385), increased S6Ka and b expression, and increased S6 phosphorylation (S235) in resistant U937 cells (Fig. S8 and S9). From a functional standpoint, combined MEK inhibition (by PD0325901) and mTOR blockade downstream of AKT (by temsirolimus) resulted in growth inhibitory synergism, up to a fraction affected ≤0.8, in both OCI-AML3 (CI at the ED_{50}, ED_{75}, and ED_{90}: 0.33, 0.55, and 0.95, respectively; Fig. 5e) and MOLM-13 (CI at the ED_{50}, ED_{75}, and ED_{90}: 0.12, 0.50, and 2.07, respectively; Fig. 5f).

Discussion

The MEK/ERK kinase module is an attractive therapeutic target in hematological malignancies [5, 13]. Here, we demonstrate that the selective MEK inhibitor PD0325901 exerts potent antileukemic effects, particularly in AML cell lines


a
OCI-AML3
C
PD 10 nM
24h
6h
24h
6h
-3
0
+3
b
Phosphoproteins
Total proteins
OCI
U937
AcCoA carboxylase (S80)
Adducin a (S726)
ATF2 (T51/T53)
ATF2 (T53)
ATF2 (T53)
ATF2 (T69)
Bad (S91)
BLNK (Y84)
BRCA1 (S1497)
Btk (Y223)
Caveolin 2 (S23)
Caveolin 2 (S36)
CDK1/2 (Y15)
Cofilin 2 (S3)
Cortactin (Y470)
CREB1 (S129+S133)
Dok2 (Y142)
eNos (T495)
ErbB2 (T686)
Erk1 (T202+Y204)
Erk5 (T218+Y220)
FAK (Y397)
Histone H2A.X (S139)
Histone H3 (S10)
Histone H3 (S28)
Histone H3 (T11)
Histone H3 (T3)
Hsp27 (S78)
Hsp27 (S82)
Integrin a4 (S988)
Integrin b1 (T783)
IRS1 (Y612)
JAK1 (Y1022)
JNK (T183/Y185)
Jun (S73)
Kit (Y703)
Kit (Y936)
Lck (Y191)
MAPKAPK2 (T222)
MEK1 (S297)
MEK2 (T394)
MEK3/6 (S189/S207)
MYPT1 (T696)
NFkappaB p65 (S276)
NPM (T199)
NPM (T234)
Paxillin 1 (Y118)
PDGFRa (Y754)
PDGFRb (Y716)
PED15 (S116)
PKBa (T308)
PKCa (S657)
PKCb1/2 (T500)
PKCb2 (T641)
PKCd (S645)
PKCg (T514)
Plk1 (T210)
PRAS40 (T246)
Rb (S608)
Rb (S807)
Rb (T821)
Rb (T826)
RSK1/2 (S380/S386)
S6 (S235)
STAT1 (S727)
STAT2 (Y690)
Tau (S422)
Tau (S515)
Tau (S515+S518)
Tau (S530)
Tau (S578)
Vimentin (S33)
14-3-3 z
Acetylated Lysine
AIF
ALS2CR7
AMPKb
ANKRD3
Aurora C
Axl
Bcl2
Bid
BLK
BMX
Btk
Calreticulin
CaMK1g
CaMKK2
CAS
CASP1a
CASP3
CASP5
CASP6
CASP8
CASP9
CD45
Cdc34
CDK1
CDK10
CDK2
CDK5
CDK7
CDK8
CK1g2
CK2a
COT
Crystallin aB
Cyclin A
Cyclin G1
CytoC
DAXX
DFF35
DGKz
DNAPK
DRAK2
eIF4E
EphA1
Erk1
Erk2
ERP72
FAK
FasL
FGFR2
FKBP52
Grp78
GSK3b
Hsp40
Hsp60
Hsp70
Hsp90
I1PP2A
I1PP2A
I2PP2A
ICK
IkBb
IKKa
IKKb
IR
IRAK1
IRAK2
IRAK4
JAK3
JNK1/2/3
Jun
KAP
LIMK1
MEK1
MEK3
Mn SOD
MST1
Nek2
Nip1
NME7
p38a MAPK
p38d MAPK
PAK5
PCNA
PDI
PI3K
PI3KR4
PI4KCB
PIP5K2a
PKA R1a
PKA R2a
PKBa (Akt1)
PKCa
PKCl/i
PKC-nu
PKG1
PP1/Cg
PP2A/Ca
PP6C
PTP1C
PTP1D
Pyk2
Rb
RIP2/RICK
ROR2
RSK1
S6Ka
S6Kb
SG2NA
SIRPA
Smad2/3
SOCS2
SODD
STAT1a
STAT6
STK33
TAK1
TBK1
Yes
ZIPK
Down by PD0325901
No change
Up by PD0325901

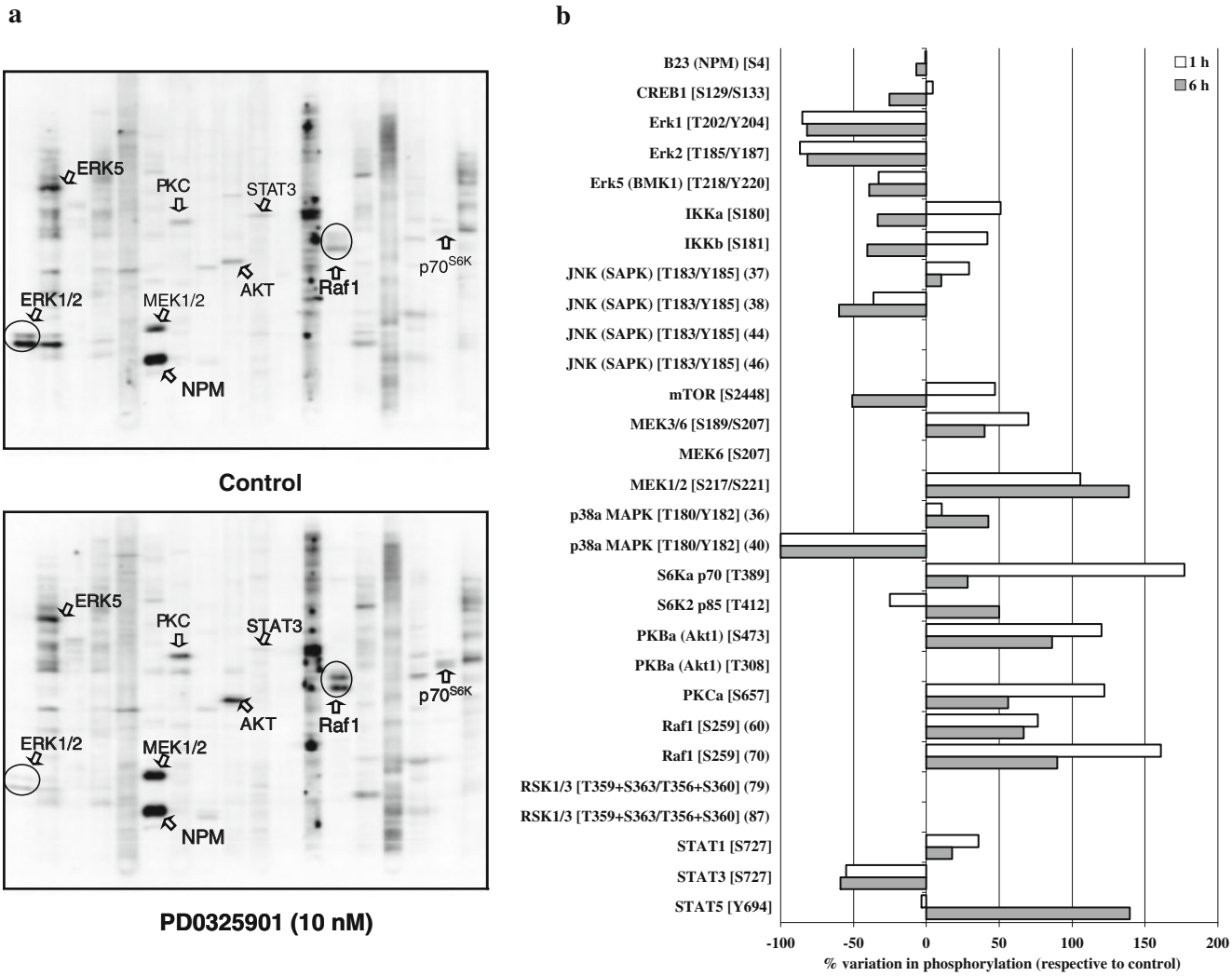


Fig. 3 PD0325901-induced changes in protein phosphorylation patterns. OCI-AML3 cells were exposed to 10 nM PD0325901 or a matched concentration of vehicle for 1 and 6 h. Protein were then extracted and subjected to multiplex Western blotting with antibodies specific for the phosphorylated form of 18 different proteins using a custom Kinexus Phospho-Protein Screen assay (Kinetworks™ KCPS 1.1), as described in "Experimental procedures." **a** The migration positions of selected target phosphoproteins are indicated on a representative Western blotting map obtained after 1 h of exposure to either vehicle control (DMSO, *top panel*) or 10 nM PD0325901 (*bottom panel*). **b** Densitometric quantification of the intensity of individual bands corresponding to the indicated phosphoproteins. Results represent the mean of two individual experiments and are expressed as percent change in band intensity in PD0325901-treated cells relative to vehicle control, after 1 (*white bars*) and 6 h (*gray bars*) of exposure

and primary samples, through a combination of inhibition of proliferation and induction of apoptosis. Using the FDC-P1 model, we also show that hematopoietic cell transformation by activated oncogenes that lie immediately upstream of MEK makes myeloid cells exquisitely sensitive to the proapoptotic action of PD0325901. Finally, phospho-proteomic profiling shows that MEK inhibition by PD0325901 triggers the activation of putative "escape" pathways, both within the same signaling cascade (Raf-1, MEK) and through parallel pathways (PI3K/AKT/mTOR), which could be exploited therapeutically to build rational, MEK inhibition-based combinations with synergistic antileukemic effects.

In agreement with previous data from our group [7], several AML cell lines were sensitive to PD0325901-induced growth inhibition and apoptosis induction, which were also evident in a substantial proportion of primary AML samples. Of note, cell line models of MM also displayed an intermediate level of sensitivity to MEK inhibition by PD0325901, in accordance to the recently proposed role for constitutive MEK/ERK activation as a promising therapeutic target in this disease [18, 19]. PD0325901 exerts its growth inhibitory effects primarily through inhibition of cell cycle progression, consistent with the reported role of ERK at the G1/S transition [20]. Several other genes/proteins potentially relevant to the functional effects induced by

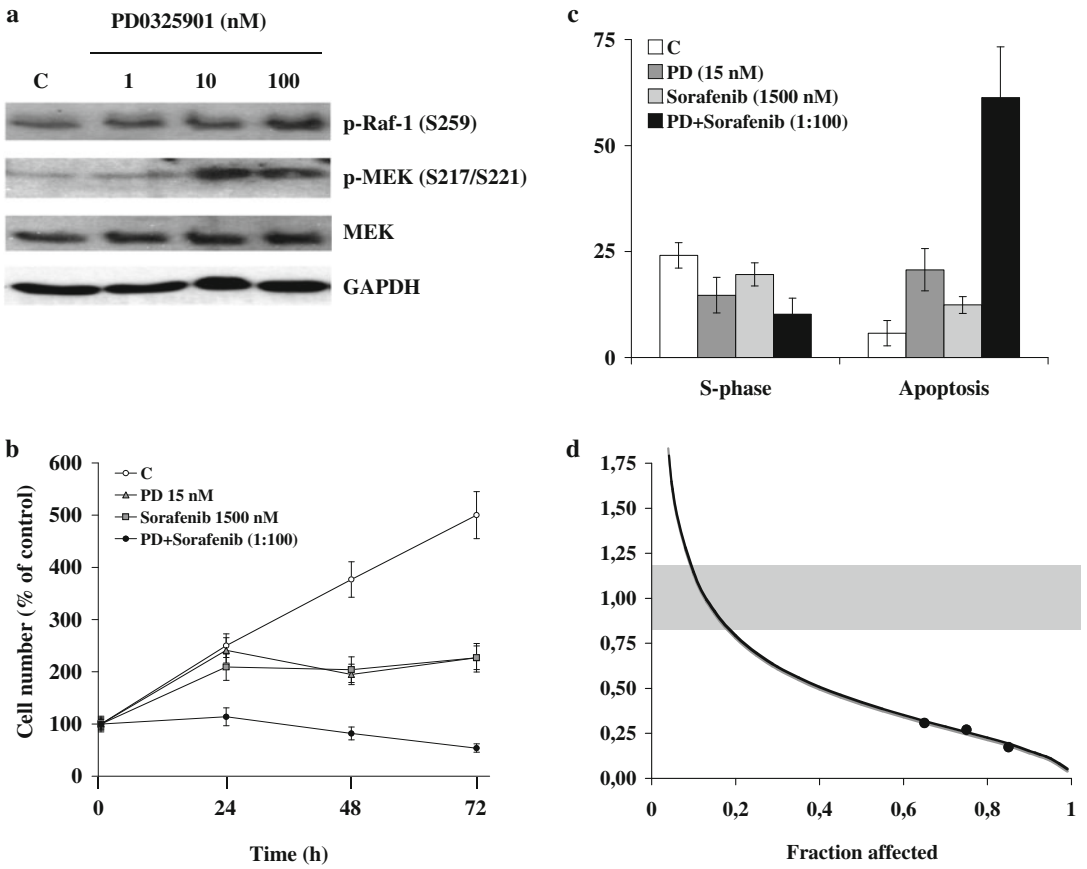


Fig. 4 Effects of combined MEK and RAF blockade. **a** OCI-AML3 cells were exposed to PD0325901 for 1 h, lysed, and subjected to Western blot analysis with the indicated antibodies. Western blot with antibodies specific for GAPDH is shown as protein loading and blotting control. Results from one experiment representative of at least three independent experiments performed with superimposable results are shown. **b** OCI-AML3 cells were exposed to PD0325901 (15 nM), sorafenib (1,500 nM), or both for the indicated periods of time. Cell counts and viability were then assessed by trypan blue exclusion counting. Results are expressed as percentage of cells plated at the beginning of the experiment (time 0) and represent the average±SD of four independent experiments. **c** OCI-AML3 cells were treated as in **b** for 48 h. Distribution of cells in the different phases of cell cycle was assessed by acridine orange DNA/RNA staining; apoptosis induction was assessed by annexin V/PI staining. Results are expressed as percentage of cells in the S-phase of the cell cycle or as percentage of apoptotic cells and represent the average±SD of four independent experiments. **d** OCI-AML3 cells were exposed to a range of PD0325901 (5–15 nM) and sorafenib (500–1,500 nM) concentrations, either alone or combined at a fixed ratio (1:100). Growth inhibition, as compared with vehicle control-treated cells, was then assessed after 72 h and CI were calculated by conservative isobologram analysis, using the Chou–Talalay method and the Calcusyn software. CIs are plotted against the fraction affected; *continuous line* represents calculated simulations, while *solid dots* represent actual experimental data points for the combination. Using this method, CI values below 0.8 indicate strong synergism, between 0.8 and 1.2 additivity, above 1.2 antagonism

PD0325901 were modulated in OCI-AML3, including genes involved in DNA or ribosomal RNA synthesis, chromatin remodeling, and protein translation, genes directly related to leukemia development (FLT3, WT1, and MAFB), genes involved in MAPK signaling (DUSP6), and genes implicated in microenvironmental interactions (SEMA6A) (Table S3). Interestingly, a number of genes are concordantly regulated by PD0325901 in both AML and malignant melanoma models, suggesting that they may be part of a "shared" signature of functional sensitivity to MEK inhibitors [21, 22], as recently reported in other model systems [23].

Unfortunately, the molecular basis for sensitivity to MEK inhibitors remains elusive [23]. Although a general correlation between baseline levels of ERK phosphorylation and sensitivity to PD0325901 was evident in both cell lines and primary samples (Fig. S1), only a weak correlation between ERK phosphorylation and clinical outcome has been observed in clinical trials of MEK inhibitors reported so far [24]. Data obtained in the oncogene-transformed FDC-P1 model clearly indicate that constitutive activation of pathway elements that lie immediately upstream of ERK and/or overexpression of selected upstream tyrosine kinase receptors powerfully sensitize leukemic cells to the proapoptotic action of PD0325901; however, no mutations in the genes driving functional sensitivity/resistance to MEK inhibition in other tumor models [25] were found in the panel of hematological cell lines tested (Table S1). One possible

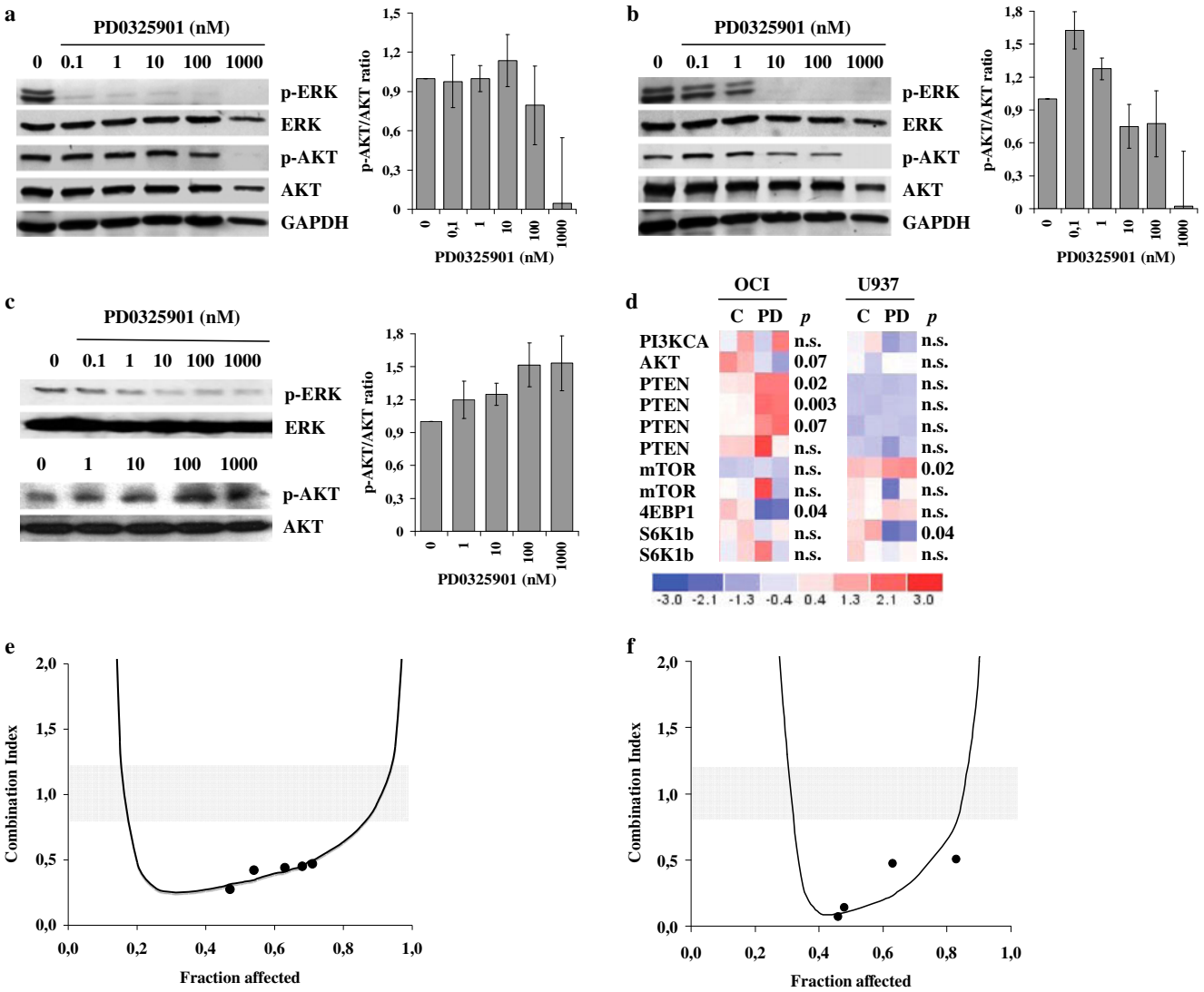


Fig. 5 PD0325901-induced signaling perturbations in sensitive and resistant AML model cell lines and growth-inhibitory effects of combined MEK and mTOR blockade. **a**, **b** Parental OCI-AML3 (**a**) or OCI-AML3 grown in the presence of 3 μM CI-1040 for prolonged periods of time (**b**, see also Fig. S7) were exposed to PD0325901 at the indicated concentrations for 1 h, lysed and subjected to Western blot analysis using the indicated antibodies. Results from one experiment representative of at least three independent experiments performed with superimposable results are shown. **c** U937 cells were exposed to PD0325901 at the indicated concentrations for 1 h, lysed, and subjected to Western blot analysis using the indicated antibodies. Results from one experiment representative of at least three independent experiments performed with superimposable results are shown. For Western blot experiments (**a–c**), densitometric analysis is shown in side panels; results represent the average±SEM of three independent experiments and are expressed as the OD ratio of p-AKT/AKT for each individual sample normalized to untreated control. **d** OCI-AML3 and U937 cells were exposed to 10 or 1,000 nM PD0325901, respectively, or a matched concentration of vehicle for 24 h. Cells were then collected, total RNA was extracted and purified, and gene expression profiles were performed using Affymetrix U133A 2.0 gene chips (see "Experimental procedures"). Supervised analysis shows genes belonging to the PI3K/AKT/mTOR pathway that are differentially expressed between PD0325901- and vehicle control-treated cells, with their relative *p* value (see also Fig. 2, Figure S4, and Tables S3 and S4 for a complete list of PD0325901-modulated genes). Results relative to two independent experiments are shown. **e** OCI-AML3 cells were exposed to a wide range of PD0325901 (1–10 nM) and CCI-779 (temsirolimus, 100–1,000 nM) concentrations, either alone or combined at a fixed ratio (1:100). Growth inhibition, as compared with vehicle control-treated cells, was then assessed after 72 h and CI were calculated by conservative isobologram analysis, as described in Fig. 4d. **f** MOLM-13 cells were exposed to a wide range of PD0325901 (5–100 nM) and CCI-779 (temsirolimus, 50–1,000 nM) concentrations, either alone or combined at a fixed ratio (1:10). Growth inhibition, as compared with vehicle control-treated cells, was then assessed after 72 h, and CI were calculated by conservative isobologram analysis, as described in Fig. 4d

alternative is to pursue rational, mechanism-based combinations with agents interfering with putative "escape" pathways. In agreement with data obtained in different tumor models [26], proteomic analysis and gene expression profiling indicate that MEK blockade interrupts a negative feedback loop, by which ERK activation inhibits signaling

through upstream components of the Ras/Raf/MEK/ERK cascade. This is consistent with previous observations of prolonged growth-factor-mediated RAF activation in response to MEK inhibition [27] and appears to be mediated, at least in part, by downregulation of dual-specificity phosphatases (DUSP-4 and DUSP-6) and adaptor proteins that either positively or negatively regulate MAPK signaling (SPRY-2 and SPRY-4, KSR1) (Table S3 and [21, 28]). In addition, our data on combined MEK and RAF inhibition provide unequivocal evidence of a striking pro-apoptotic synergism of such a "vertical" combination strategy in AML, extending findings recently reported in solid tumors and lymphomas [29, 30] and providing the rationale for ongoing clinical trials (www.clinicaltrials.gov/ct2/show/NCT00785226).

Compensatory activation of parallel signaling through the PI3K/AKT/mTOR pathway in response to MEK inhibition is an emerging theme in cancer cell signal transduction; indeed, several recent reports highlight the importance of a functional cross-talk between the MEK/ERK and PI3K/AKT/mTOR in different models of cancer progression and response to individual and combined pathway inhibitors [31]. In AML models, under certain conditions, MEK blockade may indeed cross-modulate signaling through the PI3K/AKT/mTOR pathway: in PD0325901-sensitive cell lines (OCI-AML3), MEK blockade transiently increases AKT phosphorylation but ultimately results in inhibition of AKT activity, as recently described in melanoma models by different groups including ours [32, 33]; conversely, in MEK inhibition-resistant cells, MEK blockade actually induces AKT phosphorylation [32], particularly in inherently resistant cells, such as U937, in which the PI3K/AKT/mTOR pathway is constitutively upregulated and further activated by PD0325901, and in OCI-AML3 cells with acquired resistance to MEK inhibition, in which AKT activation is induced at suboptimal PD0325901 concentrations but can be overcome by increased concentrations of the MEK inhibitor. As a consequence, functional synergism with combined MEK and mTOR inhibition is observed in MEK inhibition-sensitive OCI-AML3 and MOLM-13 cells at suboptimal concentrations of each agent but is lost at a fraction affected >0.8. The same situation is observed in melanoma models, where PD0325901, at concentrations that completely inhibit MEK-to-ERK signaling, is able to cross-inhibit the AKT/mTOR module, resulting in the lack of synergistic effects in terms of inhibition of VEGF production and tumor cell growth by combined MEK/mTOR blockade at fractions affected >0.8. This observation led us to the identification of novel cross-talk mechanisms, whereby constitutive ERK activity suppresses PTEN expression, while its inhibition restores PTEN levels and results in the concomitant inhibition of the PI3K/AKT/mTOR pathway [33]. At concentrations of PD0325901 and temsirolimus that are currently used in the clinic as single agents, pharmacologic interactions between these two agents would potentially be in the synergistic range in AML patients; however, the role of rebound AKT activation upon MEK inhibition will need to be comprehensively assessed in both cell line models and primary samples and the conditions under which combined MEK and PI3K/AKT/mTOR inhibition may result in synergistic antileukemic activity will have to be precisely defined before these results can be effectively translated into the clinical setting. In that respect, tumor genotype will need to be taken into account, as our own data suggest that PTEN status may play an important role in determining the functional outcome of single and combined MEK and mTOR inhibition [33] and recent clinical data highlight the potential for increased antileukemic activity of single-agent MEK inhibitors in RAS-mutant AML [34].

Overall, the findings reported herein support the continued development of MEK inhibitors as promising therapeutic agents in hematological malignancies, particularly AML, and support the investigation of both "vertical" and "lateral" combination strategies that may result in synergistic antitumor effects. Deeper insights into the molecular mechanisms of action of PD0325901 and other MEK-targeted agents will likely increase our chances to successfully translate such exciting preclinical findings into effective new therapeutic approaches for AML and, possibly, other cancers.

Acknowledgments The authors wish to thank Dr. Lara Felicioni (Clinical Research Center, Center of Excellence on Aging, University-Foundation, Chieti, Italy) for her contribution to the characterization of the mutational status of the cell lines employed. This work was supported in part by grants from the Italian Association for Cancer Research (AIRC, to M.M. and D.D.B.), the Italian Ministry of Health (to M.M.), and the Cariplo Foundation (to M.M.). L.C. is a fellow of the Italian Foundation for Cancer Research (FIRC). A.T. and M.M. equally contributed to this manuscript.

Disclosure statements The authors have nothing to disclose.

References

1. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A et al (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773:1263–1284
2. Sebolt-Leopold JS, Herrera R (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 4:937–947
3. Gregorj C, Ricciardi MR, Petrucci MT, Scerpa MC, De Cave F, Fazi P, Vignetti M, Vitale A, Mancini M, Cimino G et al (2007) ERK1/2 phosphorylation is an independent predictor of complete remission in newly diagnosed adult acute lymphoblastic leukemia. Blood 109:5473–5476
4. Kornblau SM, Womble M, Qiu YH, Jackson CE, Chen W, Konopleva M, Estey EH, Andreeff M (2006) Simultaneous activation of multiple signal transduction pathways confers

poor prognosis in acute myelogenous leukemia. Blood 108:2358–2365
5. Milella M, Precupanu CM, Gregorj C, Ricciardi MR, Petrucci MT, Kornblau SM, Tafuri A, Andreeff M (2005) Beyond single pathway inhibition: MEK inhibitors as a platform for the development of pharmacological combinations with synergistic anti-leukemic effects. Curr Pharm Des 11:2779–2795
6. Lauchle JO, Kim D, Le DT, Akagi K, Crone M, Krisman K, Warner K, Bonifas JM, Li Q, Coakley KM et al (2009) Response and resistance to MEK inhibition in leukaemias initiated by hyperactive Ras. Nature 461:411–414
7. Milella M, Kornblau SM, Estrov Z, Carter BZ, Lapillonne H, Harris D, Konopleva M, Zhao S, Estey E, Andreeff M (2001) Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. J Clin Invest 108:851–859
8. Milella M, Estrov Z, Kornblau SM, Carter BZ, Konopleva M, Tari A, Schober WD, Harris D, Leysath CE, Lopez-Berestein G et al (2002) Synergistic induction of apoptosis by simultaneous disruption of the Bcl-2 and MEK/MAPK pathways in acute myelogenous leukemia. Blood 99:3461–3464
9. Milella M, Konopleva M, Precupanu CM, Tabe Y, Ricciardi MR, Gregorj C, Collins SJ, Carter BZ, D'Angelo C, Petrucci MT et al (2007) MEK blockade converts AML differentiating response to retinoids into extensive apoptosis. Blood 109:2121–2129
10. Lunghi P, Costanzo A, Salvatore L, Noguera N, Mazzera L, Tabilio A, Lo-Coco F, Levrero M, Bonati A (2006) MEK1 inhibition sensitizes primary acute myelogenous leukemia to arsenic trioxide-induced apoptosis. Blood 107:4549–4553
11. Lunghi P, Giuliani N, Mazzera L, Lombardi G, Ricca M, Corradi A, Cantoni AM, Salvatore L, Riccioni R, Costanzo A et al (2008) Targeting MEK/MAPK signal transduction module potentiates ATO-induced apoptosis in multiple myeloma cells through multiple signaling pathways. Blood 112:2450–2462
12. Tortora G, Bianco R, Daniele G, Ciardiello F, McCubrey JA, Ricciardi MR, Ciuffreda L, Cognetti F, Tafuri A, Milella M (2007) Overcoming resistance to molecularly targeted anticancer therapies: rational drug combinations based on EGFR and MAPK inhibition for solid tumours and haematologic malignancies. Drug Resist Updat 10:81–100
13. McCubrey JA, Steelman LS, Abrams SL, Bertrand FE, Ludwig DE, Basecke J, Libra M, Stivala F, Milella M, Tafuri A et al (2008) Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy. Leukemia 22:708–722
14. McCubrey JA, Milella M, Tafuri A, Martelli AM, Lunghi P, Bonati A, Cervello M, Lee JT, Steelman LS (2008) Targeting the Raf/MEK/ERK pathway with small-molecule inhibitors. Curr Opin Investig Drugs 9:614–630
15. Lyubynska N, Gorman MF, Lauchle JO, Hong WX, Akutagawa JK, Shannon K, Braun BS (2011) A MEK inhibitor abrogates myeloproliferative disease in Kras mutant mice. Sci Transl Med 3:76ra27
16. Chou TC, Talalay P (1984) Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
17. Blalock WL, Pearce M, Steelman LS, Franklin RA, McCarthy SA, Cherwinski H, McMahon M, McCubrey JA (2000) A conditionally-active form of MEK1 results in autocrine tranformation of human and mouse hematopoietic cells. Oncogene 19:526–536
18. Tai YT, Fulciniti M, Hideshima T, Song W, Leiba M, Li XF, Rumizen M, Burger P, Morrison A, Podar K et al (2007) Targeting MEK induces myeloma-cell cytotoxicity and inhibits osteoclastogenesis. Blood 110:1656–1663
19. Annunziata CM, Hernandez L, Davis RE, Zingone A, Lamy L, Lam LT, Hurt EM, Shaffer AL, Kuehl WM, Staudt LM (2011) A mechanistic rationale for MEK inhibitor therapy in myeloma based on blockade of MAF oncogene expression. Blood 117:2396–2404
20. Chambard JC, Lefloch R, Pouyssegur J, Lenormand P (2007) ERK implication in cell cycle regulation. Biochim Biophys Acta 1773:1299–1310
21. Ciuffreda L, Del Bufalo D, Desideri M, Di Sanza C, Stoppacciaro A, Ricciardi MR, Chiaretti S, Tavolaro S, Benassi B, Bellacosa A et al (2009) Growth-inhibitory and antiangiogenic activity of the MEK inhibitor PD0325901 in malignant melanoma with or without BRAF mutations. Neoplasia 11:720–731
22. Milella M, Chiaretti S, Ricciardi MR et al (2007) Comparative gene profiling of acute myeloid leukemia (AML) and malignant melanoma (MEL) cell lines exposed to the MEK inhibitor PD0325901 reveals common effectors of the MEK/ERK kinase module. Blood 110:3470, ASH Annual Meeting Abstracts
23. Dry JR, Pavey S, Pratilas CA, Harbron C, Runswick S, Hodgson D, Chresta C, McCormack R, Byrne N, Cockerill M et al (2010) Transcriptional pathway signatures predict MEK addiction and response to selumetinib (AZD6244). Cancer Res 70:2264–2273
24. Wang D, Boerner SA, Winkler JD, LoRusso PM (2007) Clinical experience of MEK inhibitors in cancer therapy. Biochim Biophys Acta 1773:1248–1255
25. Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, Ye Q, Lobo JM, She Y, Osman I et al (2006) BRAF mutation predicts sensitivity to MEK inhibition. Nature 439:358–362
26. Friday BB, Yu C, Dy GK, Smith PD, Wang L, Thibodeau SN, Adjei AA (2008) BRAF V600E disrupts AZD6244-induced abrogation of negative feedback pathways between extracellular signal-regulated kinase and Raf proteins. Cancer Res 68:6145–6153
27. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489–27494
28. McKay MM, Ritt DA, Morrison DK (2009) Signaling dynamics of the KSR1 scaffold complex. Proc Natl Acad Sci USA 106:11022–11027
29. Nguyen TK, Jordan N, Friedberg J, Fisher RI, Dent P, Grant S (2010) Inhibition of MEK/ERK1/2 sensitizes lymphoma cells to sorafenib-induced apoptosis. Leuk Res 34:379–386
30. Ou DL, Shen YC, Liang JD, Liou JY, Yu SL, Fan HH, Wang DS, Lu YS, Hsu C, Cheng AL (2009) Induction of Bim expression contributes to the antitumor synergy between sorafenib and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor CI-1040 in hepatocellular carcinoma. Clin Cancer Res 15:5820–5828
31. Ciuffreda L, McCubrey JA, Milella M (2009) Signaling intermediates (PI3K/PTEN/AKT/mTOR and RAF/MEK/ERK pathways) as therapeutic targets for anti-cancer and anti-angiogenesis treatments. Curr Signal Transduct Ther 4:130–143
32. Gopal YN, Deng W, Woodman SE, Komurov K, Ram P, Smith PD, Davies MA (2010) Basal and treatment-induced activation of AKT mediates resistance to cell death by AZD6244 (ARRY-142886) in Braf-mutant human cutaneous melanoma cells. Cancer Res 70:8736–8747
33. Ciuffreda L, Di Sanza C, Cesta Incani U, Eramo A, Desideri M, Biagioni F, Passeri D, Falcone I, Sette G, Bergamo P et al. (2012) The mitogen-activated protein kinase (MAPK) cascade controls phosphatase and tensin homolog (PTEN) expression through multiple mechanisms. J Mol Med (Berl) doi:10.1007/s00109-011-0844-1
34. Ravandi F, Pigneux A, DeAngelo DJ, Dombret H, Delaunay J, Thomas X, Kadia T, Luepfert C, Asatiani E, Donica M et al (2011) Preliminary clinical, pharmacokinetic (PK) and pharmacodynamic (PD) results of the safety run-in part of a phase II trial of orally available MEK-inhibitor MSC1936369 in patients (Pts) with advanced hematological malignancies (HM). Blood 118:1554 (ASH Annual Meeting Abstracts)