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ORIGINAL ARTICLE

Targeting MCL-1 sensitizes FLT3-ITD-positive leukemias to cytotoxic therapies

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Patients suffering from acute myeloid leukemias (AML) bearing FMS-like tyrosine kinase-3-internal tandem duplications (FLT3-ITD) have poor outcomes following cytarabine- and anthracyclin-based induction therapy. To a major part this is attributed to drug resistance of FLT3-ITD-positive leukemic cells. Against this background, we have devised an antibody array approach to identify proteins, which are differentially expressed by hematopoietic cells in relation to activated FLT3 signaling. Selective upregulation of antiapoptotic myeloid cell leukemia-1 (MCL-1) was found in FLT3-ITD-positive cell lines and primary mononuclear cells from AML patients as compared with FLT3-wild-type controls. Upregulation of MCL-1 was dependent on FLT3 signaling as confirmed by its reversion upon pharmacological inhibition of FLT3 activity by the kinase inhibitor PKC412 as well as siRNA-mediated suppression of FLT3. Heterologously expressed MCL-1 substituted for FLT3 signaling by conferring resistance of hematopoietic cells to antileukemia drugs such as cytarabine and daunorubicin, and to the proapoptotic BH3 mimetic ABT-737. Conversely, suppression of endogenous MCL-1 by siRNA or by flavopiridol treatment sensitized FLT3-ITD-expressing hematopoietic cells to cytotoxic and targeted therapeutics. In conclusion, MCL-1 is an essential effector of FLT3-ITD-mediated drug resistance. Therapeutic targeting of MCL-1 is a promising strategy to overcome drug resistance in FLT3-ITD-positive AML.

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INTRODUCTION

Activating mutation of the FMS-like tyrosine kinase-3 receptor (FLT3) occurs in 30–40% of patients with acute myeloid leukemia (AML).[1,2] Two different types of mutations have been described: internal tandem duplications (ITD) within the juxtamembrane domain and tyrosine kinase domain 1, and point mutations within the tyrosine kinase domain 1.[3-6] Both types of mutations cause constitutive activation of the FLT3 receptor resulting in constitutive downstream signaling via the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and the phosphatidylinositol-3 kinase/AKT (PI3K/AKT) pathways. This may lead to enhanced cellular proliferation, reduced apoptosis and a block in the differentiation of hematopoietic blasts.[7-9] In addition to canonical FLT3 downstream signaling, activation of further pathways has been described in FLT3-ITD-positive cells. Activation of signal transducer and activator of transcription 5 (STAT5) by FLT3-ITD is associated with increased expression of the antiapoptotic protein BCL-X_L, as compared with FLT3-WT-expressing cells or cells harboring mutations of the FLT3 kinase domain.[10]

AML patients bearing FLT3-ITD mutations have a poor prognosis due to increased relapse rates following induction chemotherapy. This translates into reduced progression-free and overall survival rates as compared with the AML patients without ITD mutations.[4,11,12] Functionally, FLT3-ITD-expressing leukemic cells are more resistant to cytotoxic drugs and residual blasts or at least FLT3-ITD-positive leukemic stem cells are thought to contribute to the higher relapse rate and the dismal outcome of FLT3-ITD-positive AML. Hence, targeting the aberrant FLT3-ITD receptor using specific tyrosine kinase inhibitors (TKIs) is a promising therapeutic approach.[13,14] Several small molecule TKIs targeting FLT3 have been investigated in phase 1/2 clinical trials. Clinical responses have been observed with, midostaurin (PKC412), lestaurtinib (CEP-701), tandutinib (MLN-518) and SU11248, respectively, in chemotherapy refractory patients or patients not eligible for standard chemotherapy.[15-20] Furthermore, FLT3-TKIs seem to resensitize FLT3-ITD-positive leukemic blasts to cytotoxic antileukemic drugs. Currently, combination therapies are under clinical investigation to translate these findings into clinical practice.[20-24] However, primary and secondary resistance mechanisms such as gatekeeper mutations interfering with drug target interaction or rewired signaling of the FLT3 receptor were observed in patients treated with FLT3 TKIs, which might interfere with this direct therapeutic strategy.[25,26]

Inhibition of aberrantly activated antiapoptotic pathways is one mechanism of action of FLT3-TKIs. This can result in direct induction of apoptotic death of FLT3-ITD-dependent leukemic cells. Further, this may lead to resensitization of FLT3-ITD-positive blasts and leukemic stem cells to conventional antileukemic drugs. In particular, upregulation of antiapoptotic members of the BCL-2 family has been described in FLT3-ITD-expressing cells.[14,27,28] BCL-2 family proteins are the main regulators of the 'mitochondrial' apoptosis pathway. They consist of anti- and proapoptotic proteins that determine the balance between survival and programmed cells death.[29] The antiapoptotic family members BCL-2, BCL-X_L, myeloid cell leukemia-1 (MCL-1), BCL-W and A1 counteract the apoptotic effector proteins BAX, BAK, and

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possibly BOK, to prevent their activation and mitochondrial outer membrane permeabilization leading to release of cytochrome *c* and subsequent caspase activation.[30] Proapoptotic BH3 proteins, such as BAD, BIM, BIK, NOXA and PUMA, enable activation of BAX and BAK either by neutralizing antiapoptotic BCL-2 family proteins or by direct action.[29] In many cancer types, the balance between pro- and antiapoptotic BCL-2 family members is disturbed. Frequently, this occurs as a consequence of aberrant activation of MEK/ERK, PI3K/AKT and/or STAT5 pathways.[29,31]

Here, we have identified upregulation of the antiapoptotic BCL-2 family member MCL-1 in FLT3-ITD-expressing cell lines and primary AML blasts as compared to FLT3-WT cells. Elevated *MCL-1* RNA expression in FLT3-ITD-positive leukemic cells was suppressed by siRNA-mediated downregulation of the mutant receptor as well as by inhibitory concentrations of the protein kinase inhibitor PKC412. MCL-1 functionally contributed to the resistance phenotype of FLT3-ITD-positive leukemic cells. Suppression of endogenous MCL-1 by siRNA or by flavopiridol treatment sensitized FLT3-ITD cells to antileukemic therapies. These findings provide a rational basis for combination therapy strategies in FLT3-ITD-positive AML to eliminate residual leukemic blasts and stem cells after induction chemotherapy to overcome the poor prognosis of these patients.

MATERIALS AND METHODS

Cell models

Murine 32D cells were kindly provided by T Skorski (Philadelphia, PA, USA) and were maintained in RPMI 1640 with 10% fetal calf serum, 20 mM HEPES, pH 7.3, 50 mM β-mercaptoethanol, 2 mM L-glutamine and 10% WEHI-3B-conditioned medium as a source of interleukin-3. The human AML cell lines MV4;11 and Molm-13 harboring a FLT3-ITD mutation and the human acute lymphoblastic leukemia cell line RS4;11 harboring the FLT3-WT receptor (all obtained from the DSMZ, Braunschweig, Germany) were maintained in RPMI 1640 with 10% fetal calf serum, 20 mM HEPES, pH 7.3, 50 mM β-mercaptoethanol and 2 mM L-glutamine. All cells were grown at 37 °C, in a 5% CO_2-humidified incubator.[32]

Isolation of primary AML blasts and FLT3-ITD mutation screening

Heparin-treated peripheral blood samples (20 ml) were obtained from 12 AML patients at the time of diagnosis or relapse. Informed consent was obtained in accordance with the Declaration of Helsinki. Mononuclear cells enriched in AML leukemic blasts were isolated as described.[5] Genomic DNA from mononuclear cells was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). ITD mutation screening by PCR was performed as described.[5]

Plasmids, antibodies and reagents

The human FLT3-WT and a human FLT3-ITD construct, both subcloned into the pAL expression vector under control of the 5′ long terminal repeat of the Moloney murine sarcoma virus (MoMSV) and the plasmid pMAM/BSD, were used as described previously and were a kind gift from H Serve (University of Frankfurt, Germany).[5] This ITD allele (36 bp/12 amino acids (aa)) integrates between codons 598 and 599 in the JM domain of FLT3. The FLT3-ITD627E construct was subcloned into the pAL vector as described.[25] The ITD627E allele (93 bp/31 aa) integrates at codon 627 in the β2 sheet of the tyrosine kinase domain 1 of FLT3 and leads to an amino-acid exchange at codon 627 (alanine to glutamate).[6] The p3xFLAG-CMV10 vector containing the coding sequence of murine Mcl-1 under control of a CMV promotor was kindly provided by H Schulze-Bergkamen (National Center for Tumor Diseases, Heidelberg, Germany). All vector constructs were verified by nucleotide sequencing.

Flavopiridol was purchased from Sigma Aldrich (Munich, Germany), PKC412 was kindly provided by Novartis (Basel, Swizerland), ABT-737 was kindly provided by Abbott (Abott Park, IL, USA); cytarabine and daunorubicin were purchased from the Hospital Pharmacy of the University Hospital Essen, Germany.

The following primary antibodies were used: antiphospho-FLT3 (Y591), antiphospho-extracellular signal-related kinase 1/2 (ERK1/2; T202/Y204), anti-ERK1/2, antiphospho-S6 protein (S240/S244) and anti-S6 protein (all from Cell Signaling Technology, Danvers, MA, USA); anti-FLT3, anti-MCL-1, anti-BCL-X_L (H5), anti-BCL-2 (C2) (all from Santa Cruz, Heidelberg, Germany), anti-Actin (MP Biomedicals, Aurora, OH, USA), anti-FLAG (M2) (Sigma Aldrich). Recombinant human interleukin-3 (IL3) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), FLT3 ligand (FL) was purchased from R&D-Systems (Minneapolis, MN, USA).

Transfections

Transfection of 32D cells with different *FLT3*-DNA constructs was performed as described previously.[5] Functional receptor expression was confirmed by immunoblotting and FACS analyses as described previously.[5] 32D FLT3-ITD mMCL-1 cells and RS4;11 mMCL-1 cells, ectopically overexpressing MCL-1, were generated by transfection of 32D FLT3-ITD or RS4;11 cells with 20 μg p3xFLAG-CMV10 vector containing the coding sequence of murine *Mcl-1* by electroporation. Cells were selected with 0.5 mg/ml G418 and polyclonal cell lines were used for experiments.

Protein extract preparation and immunoblotting

Cells were serum starved for 5 h before treatment. 32D FLT3-WT and RS4;11 were treated with 100 ng/ml FL for 30 min. 32D FLT3-ITD, MV4;11 and Molm-13 cells were treated with flavopiridol or PKC412 in the indicated concentrations. Protein lysates were prepared for immunoblotting analyses as described previously.[22]

Apoptosis assays

The percentage of apoptotic cells was determined by measuring the sub-G1 fraction upon propidium iodide incorporation using flow cytometry as described.[33]

Drug combination index calculation

For calculation of combination indices (CI), cells were treated with flavopiridol, cytarabine, daunorubicin, ABT-737 and combinations of flavopiridol with the different drugs. Apoptotic cell death (fractional effect) was measured by flow cytometry. Pharmacological interaction of flavopiridol with cytarabine, daunorubicin and ABT-737 was analyzed using the CalcuSyn software (Biosoft, Cambridge, UK). CI ranging from 0.1 to 0.85 indicate strong to moderate synergism, indices from 0.85 to 0.9 slight synergism, indices from 0.9 to 1.1 nearly additive interaction and indices from 1.1 to 10 slight to strong antagonism, respectively.[34]

Gene suppression by siRNA

MV4;11 2×10^6 were transfected by electroporation with h-FLT3 (siGENOME SMARTpool, M-003137-01; Dharmacon, Lafayette, CO, USA), h-MCL-1 (sc-35 877, Santa Cruz) specific siRNAs. siRNAs were dissolved to a final concentration of 20 μM stock solution and for each transfection 10 μl siRNA stock solution was used. As a negative control, an equivalent concentration of AllStars Negative Control siRNA (QIAGEN) was used. For verification of effective gene suppression, protein lysates were prepared after 24 h and target protein expression was assayed by immunoblotting. For assessment of apoptosis, cells were left to recover from the transfection for 6 h and were then treated with or without cytarabine. Percentage of apoptotic cells was assessed following 24 h and 48 h incubation.

Proteomic analyses

32D FLT3-WT and FLT3-ITD were serum starved for 5 h. Cellular protein lysates of FL stimulated 32D FLT3-WT (100 ng/ml for 30 min) and non-stimulated 32D FLT3-ITD cells were prepared using lysis buffer, supplemented with protease and phosphatase inhibitors, supplied by Kinexus Bioinformatics (Vancouver, BC, Canada). Dye labeling, hybridization and analysis of the Kinex antibody array were done by Kinexus. Data obtained from the antibody array was filtered as follows: (1) Flag 0, this means spot quality is acceptable based on morphology and background; (2) change

from control (CFC) >20% or <−20% CFC, the percentage change from control is a measure of the change in normalized signal intensity averages between the experimental sample (32D FLT3-ITD) and the control sample (32D FLT3-WT + FL); (3) total error range: < 30%. The total error range is the sum of '% error range' from the experimental and control samples; and (4) signal to noise (s/n) ratio: >1.5. The s/n-ratio is the ratio between the measurement of the spot intensity and the spot's local background.

Gene expression analysis

Cells were serum starved for 5 h before treatment with growth factors and inhibitors for the indicated time points. Total RNA was isolated using the High Pure RNA Isolation Kit protocol (Roche Diagnostics, Mannheim, Germany) and cDNA synthesis was performed according to the protocol of Transcription High Fidelity cDNA Synthesis Kit (Roche Diagnostics). An amount of 1–5 μg was transcribed with anchored-oligo(dT)18 primer. qRT-PCR was performed according to the protocol of SYBRE Green 1 Master (Roche Diagnostics). The following primers were used for murine *Mcl-1*: 5′-TGTCAAACAAAGAGGCTGGGATGG-3′ and 5′-ATTTCTGATGCCGCCTTCTA GGTC-3′, human *MCL-1*: 5′-AGAAAGCTGCATCGAACCAT-3′ and 5′-CCAGC TCCTACTCCAGCAAC-3′, human actin: 5′-TCAGCTGTGGGGTCCTGT-3′ and 5′-GAAGGGACAGGCAGTGAG-3′, and *GAPDH*: 5′-CGTCCCACCACCATGGAGA-3′ and 5′-CGGCCATCACGCCACAGTTT-3′.

RESULTS

Drug resistance of FLT3-ITD-expressing cells correlates with differential expression of antiapoptotic proteins

Leukemic cells expressing a mutant FLT3-ITD receptor were described to be resistant to chemotherapeutic drugs.[13,14] In agreement, the FLT3-ITD-positive human leukemic cell line MV4;11 was more resistant to cytarabine-induced apoptosis as compared with the FLT3-WT cell line RS4;11 (data not shown). We hypothesized that activation of signal transduction pathways in FLT3-ITD-expressing cells accounted for this phenotype. To dissect differential protein expression and phosphorylation profiles in response to FLT3-ITD, we performed antibody array analyses (Kinex, Kinexus Bioinformatics) of the murine 32D cell line transfected with a wild-type human FLT3 (FLT3-WT 32D cells) or a mutant human FLT3-ITD receptor (FLT3-ITD 32D cells) construct.[5,22] Profiling was performed in FL-stimulated FLT3-WT 32D cells and in untreated FLT3-ITD 32D cells using the Kinex platform, which allowed parallel analysis of expression and phosphorylation status of 615 different proteins (Figure 1a). We identified 85 differentially expressed/phosphorylated proteins in protein lysates from FLT3-WT 32D cells as compared with FLT3-ITD 32D cells (Supplementary Tables 1A and B). A major proportion of these proteins was known to be involved in growth factor signal transduction. Based on the drug-resistant phenotype conferred by FLT3-ITD, we focused on proteins implied in the regulation of drug-induced apoptosis, such as the BCL-2 protein family.[35,36] Antibody array analysis of the major antiapoptotic BCL-2 family proteins revealed a slight downregulation of BCL-2 (approximately 20%) and a robust upregulation of BCL-XL (by 40%) in FLT3-ITD 32D cells (Supplementary Table 1). Antibody array analysis of differential expression of MCL-1 was not feasible due to a low signal-to-noise ratio (data not shown). To confirm these results and determine MCL-1 expression levels, we performed immunoblot analyses of protein lysates generated from FLT3-WT RS4;11 cells stimulated with FL, and from unstimulated FLT3-ITD MV4;11 cells, which supported the results concerning BCL-2 and BCL-XL obtained by the antibody array (Figures 1b and c). Interestingly, a significant upregulation of MCL-1 was observed in both FLT3-ITD models, MV4;11 cells and FLT3-ITD 32D cells, as compared to the respective FLT3-WT controls or to parental 32D cells (Figure 1d). Interleukin-3 stimulation of parental 32D cells strongly induced MCL-1 protein expression (Figure 1d). In keeping, qRT-PCR analysis revealed more than fivefold higher *MCL-1* transcript levels in FLT3-ITD-expressing MV4;11 and 32D cells as compared to FLT3-WT RS4;11 and 32D cells (Figure 1e and data not shown). In addition to leukemic cell lines, we investigated MCL-1 protein levels in primary mononuclear cells from 12 AML patients. A pronounced MCL-1 expression was detected in all six patients harboring a FLT3-ITD mutation, whereas MCL-1 was only expressed in two out of six samples from FLT3-WT patients (Figure 1f).

MCL-1 is a downstream target of FLT3 receptor signaling

To study whether FLT3-mediated signal transduction leads to activation of *MCL-1*, we stimulated FLT3-WT RS4;11 and 32D cells with FL and analyzed *MCL-1* RNA transcript levels and protein expression. *MCL-1* transcript and protein levels were induced within 5 h of FL stimulation (Figures 2a and b). Conversely, RNAi-mediated suppression of the endogenous, constitutively active FLT3-ITD receptor in MV4;11 cells resulted in significant downregulation of MCL-1 protein expression (Figure 2c). Taken together MCL-1 appears to be a direct downstream target of FL-stimulated FLT3 signaling as well as aberrantly activated FLT3-ITD in hematopoietic cells.

MCL-1 is an essential effector of FLT3-ITD-mediated drug resistance

To study the consequences of FLT3 inhibition at a functional level, we applied PKC412, a protein kinase inhibitor with activity against FLT3-ITD.[37] Incubation of FLT3-ITD-expressing MV4;11 and 32D cells with PKC412 at a concentration of 100 nM led to dephosphorylation of the FLT3-ITD receptor and of S6 protein, a downstream target of FLT3-activated signaling (Figure 2d). This was accompanied by significant downregulation of MCL-1 in a time-dependent manner and induction of apoptosis (Figure 2d and data not shown). As expected, expression of a FLAG-tagged murine *Mcl-1* cDNA (FLAG-*Mcl-1*) from a heterologous promotor in FLT3-ITD 32D cells was not affected by PKC412. PKC412 activity was evidenced by dephosphorylation of S6 protein (Figure 2e). Accordingly, FLT3-ITD 32D cells expressing FLAG-MCL-1 were significantly protected against PKC412-induced apoptosis (Figure 2f). Enforced FLAG-MCL-1 expression also conferred resistance to the cytotoxic agents cytarabine and daunorubicin, which are the mainstay of AML-induction therapy (Figures 2g and h).

Based on these findings MCL-1 constitutes a rational target for combination therapies overcoming drug resistance in FLT3-ITD-positive AML. To further explore this hypothesis, we devised siRNA specifically suppressing endogenous MCL-1 in FLT3-ITD-positive MV4;11 cells (Figure 3a). MCL-1 suppression was associated with enhanced spontaneous apoptosis and sensitization to cytarabine (Figure 3b). To confirm these findings in a clinically more applicable model, we used flavopiridol that has been described to inhibit *MCL-1* transcription by disturbing the STAT3/DNA interaction.[38] In phase I clinical trials in hematological diseases plasma levels up to 1.57 μM of flavopiridol could be achieved by a bolus-infusion schedule.[39] Therefore, concentrations ranging from 50 nM to 10 μM were used for further experiments. A dose-dependent downregulation of *MCL-1* transcript levels by flavopiridol was achieved in FLT3-ITD-expressing MV4;11 cells and in an additional FLT3-ITD-positive leukemia model, Molm-13 (Figure 4a). In both models, suppression of *MCL-1* transcript levels was associated with reduced MCL-1 protein expression (Figures 4b and c). To exclude non-specific downregulation of several BCL-2 family members by flavopiridol, we also analyzed the protein expression of BCL-2 and BCL-X_L, which both remained unchanged (Figure 4c and data not shown). MCL-1 expression levels were reported to be regulated by MAPK signaling.[40] Analyzing the basal phosphorylation levels of ERK1/2 by immunoblots using a phospho-specific antibody, we could not observe different MAPK pathway activation in

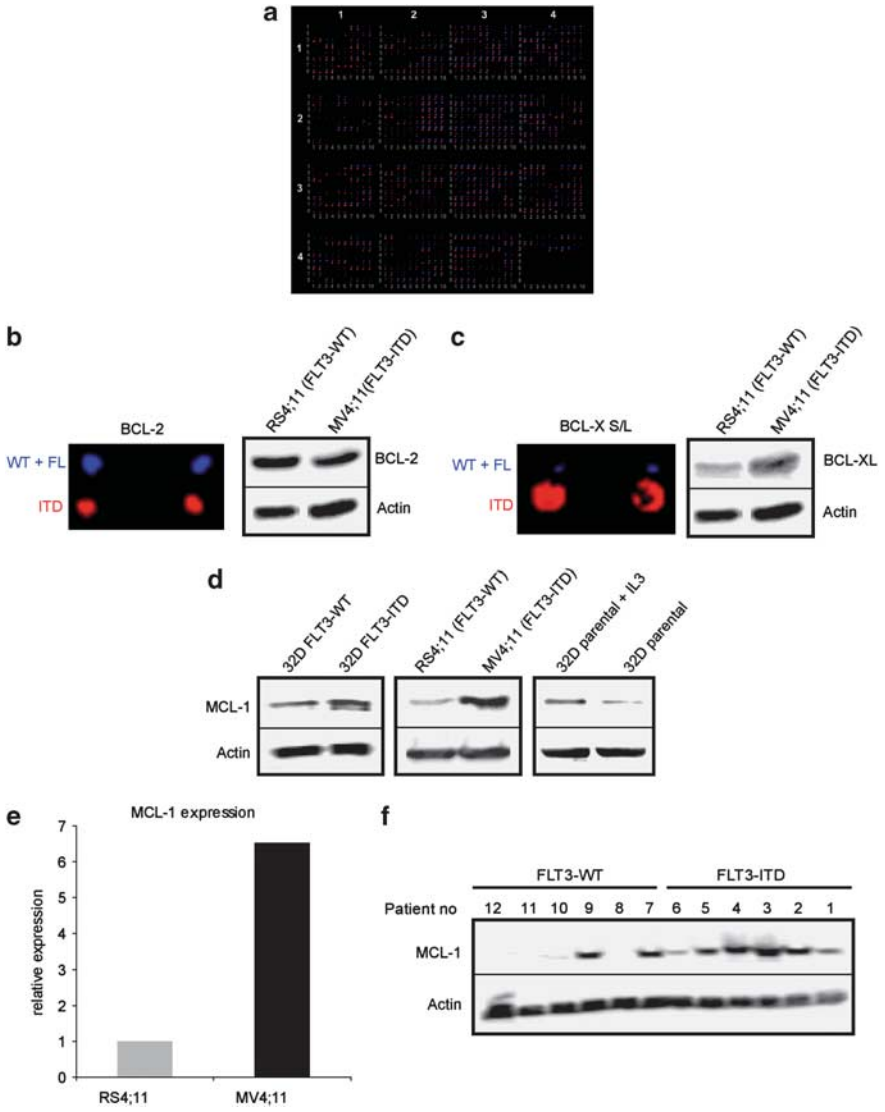

Figure 1. Differential expression of antiapoptotic BCL-2 family members in FLT3-ITD-positive cell lines and primary AML blasts. (**a**) Antibody array analyzing expression and/or phosphorylation of 615 proteins in duplicates using lysates from FL stimulated 32D FLT3-WT (100 ng/ml for 30 min) and non-stimulated 32D FLT3-ITD cells. Data obtained from the antibody array was filtered as described. (**b–d**) FLT3-ITD-expressing cells show upregulation of BCL-X_L and MCL-1, but not BCL-2, as compared with FLT3-WT-expressing cells or cells without FLT3 receptor expression. Whole-cell lysates from non-stimulated FLT3-ITD harboring MV4;11 and 32D cells, from FL stimulated (100 ng/ml for 30 min) FLT3-WT-harboring RS4;11 and 32D cells and IL3 stimulated parental 32D cells were analyzed by immunoblotting using specific antibodies for BCL-2 (**b**), BCL-X_L (**c**) and MCL-1 (**d**). To control equal loading, the blots were reprobed with an antibody recognizing Actin. (**e**) *MCL-1* RNA is upregulated in FLT3-ITD-positive cells. qRT-PCR analysis of FLT3-ITD-positive MV4;11 cells and FLT3-WT RS4;11 cells using specific primers for *MCL-1*. Expression levels of *MCL-1* were normalized to the housekeeping gene *Beta-Actin* and related to the basal transcription level in MV4;11 cells. (**f**) MCL-1 is upregulated in primary FLT3-ITD-positive AML blasts. Mononuclear cells from 12 AML patients were separated by Ficoll-Hypaque density-gradient centrifugation at the time of diagnosis or relapse. Whole-cell lysates were analyzed by immunoblotting using specific antibodies for MCL-1. To control equal loading, the blot was reprobed with an antibody recognizing Actin. Note that MCL-1 protein level was detected in all six patients harboring a FLT3-ITD mutation, whereas MCL-1 was only detected in two out of six patients harboring FLT3-WT.

FLT3-ITD-positive MV4;11 cells and FLT3-WT RS4;11. The phosphorylation status of ERK1/2 of MV4;11 and RS4;11 cells was not decreased by the presence or absence of flavopiridol (Figure 4d). This confirmed that flavopiridol had no impact on constitutive MAPK activation, an alternative mechanism of MCL-1 upregulation. To exclude interference of flavopiridol with FLT3 kinase activity, FLT3-ITD-positive MV4;11 cells were incubated with flavopiridol and PKC412 as positive control. No significant suppression of FLT3 receptor phosphorylation by flavopiridol was observed at concentrations as high as 10 μM, whereas MCL-1 was completely suppressed by flavopiridol at concentrations of 200 nM and above (Figure 4e). Dephosphorylation of FLT3 by PKC412 was also associated with a reduction of MCL-1 protein level (Figure 4e).

Pharmacological targeting of MCL-1 in FLT3-ITD-positive AML

We hypothesized that downregulation of MCL-1 by flavopiridol could lead to enhanced apoptosis in FLT3-ITD-positive cells. Cell cycle analyses were performed in MV4;11, FLT3-ITD 32D and Molm-13 cells following incubation with increasing concentrations of flavopiridol. A dose-dependent induction of apoptosis was

observed in all FLT3-ITD-positive cell lines (Figure 5a). Interestingly, flavopiridol also induced strong apoptosis in 32D cells expressing a non-JM FLT3-ITD receptor with an additional point mutation at position 627: These cells were shown to be resistant to PKC412 due to a differential signal transduction originating from an unusual FLT3-ITD receptor leading to upregulation of MCL-1 (Figure 5a).[25] ABT-737 is a pharmacological BH3 mimetic that can induce apoptosis by neutralizing BCL-2 and BCL-X_L.[35,41] To study whether flavopiridol-mediated suppression of MCL-1 sensitized FLT3-ITD-positive MV4;11 and Molm-13 cells to ABT-737, we treated cells with ABT-737, flavopiridol or combinations of both. Although ABT-737 or flavopiridol alone were only moderately toxic, combination therapy was effectively inducing apoptosis in FLT3-ITD-expressing cell lines (Figures 5b and c and data not shown). To elucidate possible synergism between the two compounds, we calculated CI for a broad range of flavopiridol

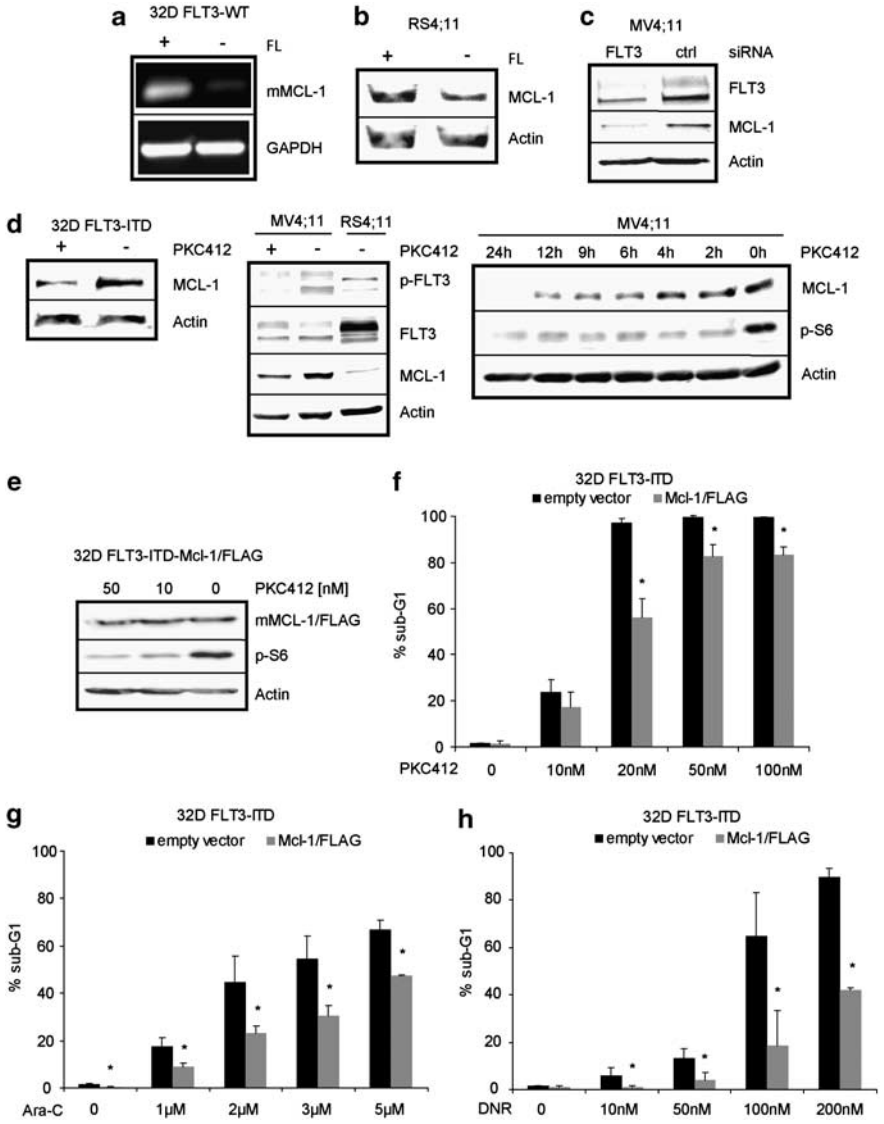

Figure 2. MCL-1 upregulation is dependent on FLT3 receptor signaling. (**a**) *MCL-1* RNA is upregulated in FLT3-WT cells following stimulation with FL. Semiquantitative RT-PCR analysis of FL (100 ng/ml for 5 h)-stimulated FLT3-WT expressing 32D cells using specific primers for murine *Mcl-1*. Expression was normalized to the housekeeping gene *GAPDH*. (**b**) MCL-1 is upregulated upon FL stimulation of FLT3-WT cells. Whole-cell lysates from FLT3-WT RS4;11 cells were analyzed by immunoblotting using a specific antibody for MCL-1 after stimulation with FL (100 ng/ml for 5 h). Reprobing with anti-Actin served as loading control. (**c**, **d**) Knockdown of the FLT3-ITD receptor by siRNA (**c**) or receptor tyrosine kinase inhibition by PKC412 (**d**) led to time dependent downregulation of MCL-1. FLT3 and MCL-1 expression was assessed in MV4;11 cells by immunoblotting 24 h after siRNA-mediated knockdown of FLT3 (**c**). 32D FLT3-ITD cells and MV4;11 cells were incubated with the FLT3-TKI PKC412 at a concentration of 100 nM for the indicated time periods. Whole-cell lysates were analyzed by immunoblotting using primary antibodies against phospho-FLT3, FLT3, phospho-S6 and MCL-1 (**d**). Blots were reprobed for Actin expression to ensure equal protein loading. (**e**) Stable expression of a FLAG-tagged murine *Mcl-1* cDNA (FLAG-MCL-1) led to sustained transgene expression in the presence of PKC412. Whole-cell lysates were obtained from FLAG-MCL-1-32D FLT3-ITD cells following incubation with PKC412 at different concentrations for 7 h, and were analyzed by immunoblotting using the indicated primary antibodies. (**f**) Expression of FLAG-MCL-1 confers resistance to PKC412. FLAG-MCL-1 32D FLT3-ITD cells were incubated with the indicated concentrations of PKC412 for 24 h. The percentage of apoptotic cells with subgenomic DNA was flow cytometrically assessed. Asterisks denote statistically significant ($P<0.05$, *t*-test). (**g**, **h**) Expression of FLAG-MCL-1 confers resistance to cytarabine and daunorubicin. FLAG-MCL-1 32D FLT3-ITD cells were incubated for 24 h at the indicated drug concentrations. The percentage of apoptotic cells with subgenomic DNA was flow cytometrically assessed. Asterisks denote statistically significant ($P<0.05$, *t*-test).

concentrations when combined with effective concentrations of ABT-737 (25 and 50 nM). A moderate to strong synergism was revealed for all combinations indicated by CI-values below 1 (Figure 5c and Table 1a). Next, we investigated whether flavopiridol-induced downregulation of MCL-1 sensitized FLT3-ITD-expressing cells to conventional cytotoxic drugs. FLT3-ITD 32D cells were incubated for 24 h with flavopiridol in combination with cytarabine or daunorubicin, respectively. Flavopiridol effectively sensitized FLT3-ITD-expressing cells to cytarabine- and daunorubicin-mediated apoptosis (Figures 6a-d). CI for flavopiridol and cytarabine (3 μM) or daunorubicin (100 nM) revealed moderate to strong synergism for all combinations (Figures 6b, d and Table 1b). To further establish downregulation of MCL-1 as the major mechanism of flavopiridol-induced cytotoxicity in FLT3-ITD-positive cells, we stably expressed FLAG-MCL-1 in FLT3-ITD 32D cells. At flavopiridol concentrations effectively suppressing endogenous MCL-1, heterologous FLAG-MCL-1 was still present in these double transgenic cell populations (Figure 7a). Interestingly, sustained expression of FLAG-MCL-1 mediated resistance to the proapoptotic effects of flavopiridol (Figure 7b). Additionally, FLAG-MCL-1-expressing cells were less susceptible to flavopiridol-mediated sensitization to cytarabine- or daunorubicin-induced apoptosis (Figures 7c to f). Only the highest concentration of flavopiridol synergized with cytarabine or daunorubicin ($CI < 1$), whereas all other combinations revealed only additive or even antagonistic effects (CI: 0.9–1.1 or $CI > 1.1$) (Figures 7d, f and Table 1b). Collectively these findings support the functional importance for MCL-1 in FLT3-ITD-mediated resistance of leukemia cells. Consequently, we proposed that MCL-1 could be targeted as an 'Achilles' heel' in FLT3-ITD-driven AML.

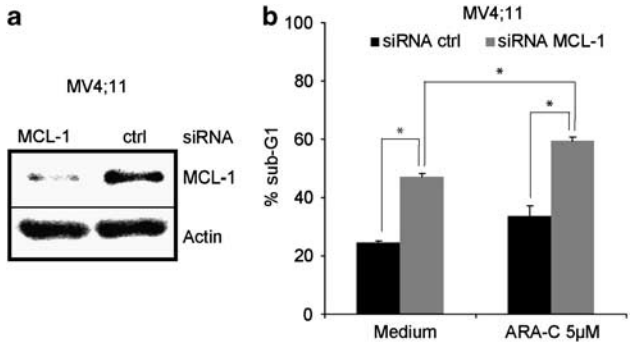

Figure 3. Suppression of MCL-1 by siRNA sensitizes FLT3-ITD-positive cells to cytotoxic drugs. (**a**) MV4,11 cells were transfected with MCL-1-specific siRNA or scrambled control. Whole-cell lysates were analyzed for protein expression of MCL-1 and Actin (as loading control). (**b**) MV4,11 cells treated as in (**a**) were incubated with or without cytarabine (5 μM) for 24 h, and the fraction of apoptotic cells with subgenomic DNA was quantified by flow cytometry. Asterisks denote statistically significant ($P < 0.05$, *t*-test).

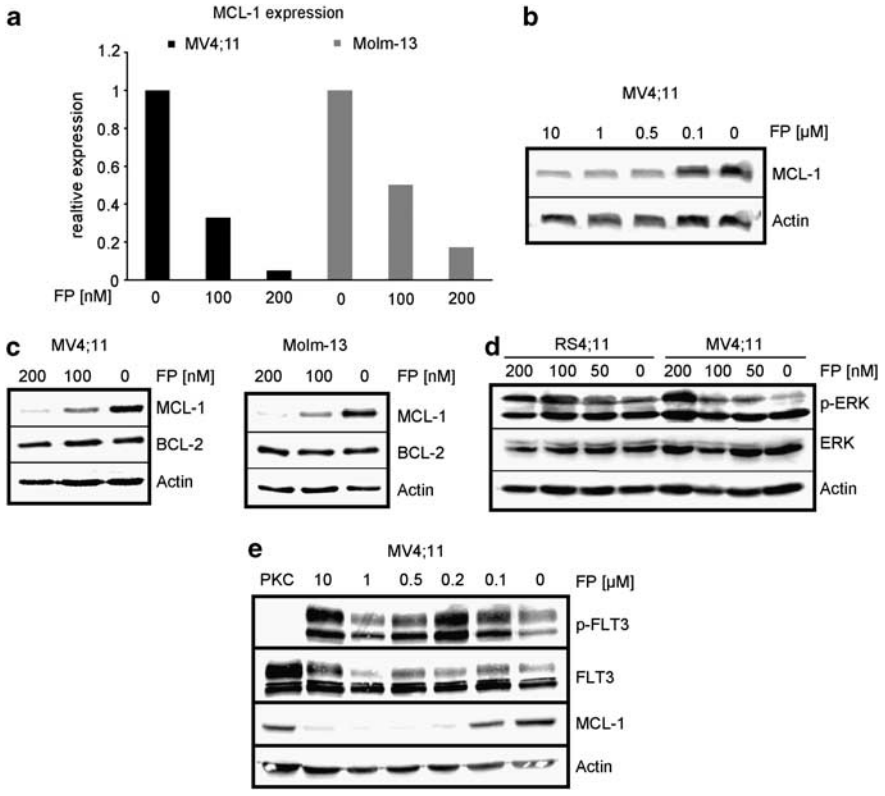

Figure 4. Flavopiridol suppresses MCL-1 in FLT3-ITD-positive cells. (**a**) Flavopiridol suppresses *MCL-1* RNA expression in FLT3-ITD-positive cells. MV4;11 and Molm13 cells were incubated for 5 h with flavopiridol at several concentrations, followed by RNA extraction and reverse transcription. Expression of *MCL-1* and *Beta-Actin* was measured by qRT-PCR; *MCL-1* levels were normalized to *Beta-Actin*. Values were related to the untreated medium control. (**b**) Dose-dependent suppression of MCL-1 protein expression in FLT3-ITD-positive cells by flavopiridol. MV4;11 cells were incubated for 5 h with flavopiridol at incremental concentrations. Whole-cell lysates were analyzed for MCL-1 and Actin expression (as loading control) by immunoblotting. (**c**) Flavopiridol suppresses MCL-1 but not BCL-2 in FLT3-ITD-positive cells. MV4;11 and Molm-13 cells were incubated for 5 h with flavopiridol at incremental concentrations as indicated. Whole-cell lysates were analyzed for expression of MCL-1, BCL-2 and Actin (as loading control) by immunoblotting. Flavopiridol used at MCL-1-suppressing concentrations does not act on MAPK activation (**d**) or FLT3 autophosphorylation (**e**). MV4;11 and RS4;11 cells were incubated for 5 h with flavopiridol at incremental concentrations or PKC412 100 nM, respectively. Whole-cell lysates were analyzed by immunoblotting for expression of p-ERK, ERK and Actin (**d**) or p-FLT3, FLT3, MCL-1 and Actin (**e**).

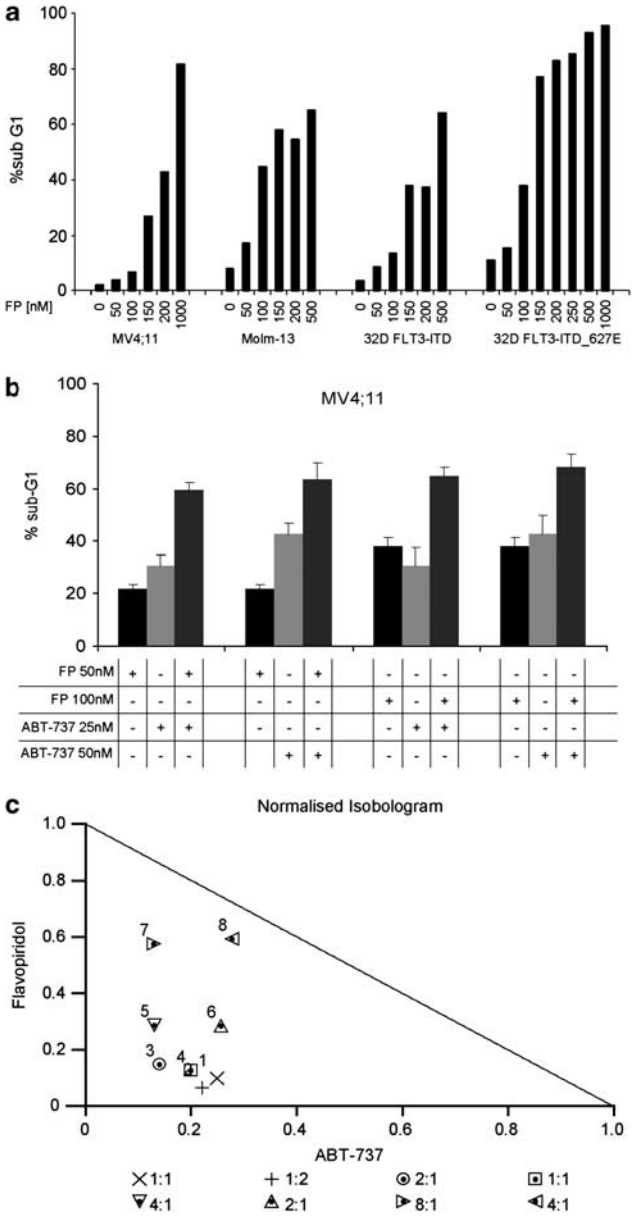

Figure 5. Activity of flavopiridol monotherapy and in combination with anticancer agents in FLT3-ITD-positive cells. (**a**) MV4;11, Molm-13, 32D FLT3-ITD and 32D FLT3-ITD_627E cells were incubated with flavopiridol at the indicated concentrations for 24 h (MV4;11 and 32D) or 48 h (Molm-13). The percentage of apoptotic cells with subgenomic DNA was assessed by flow cytometry. (**b**) MV4;11 cells were incubated for 48 h with flavopiridol (FP), ABT-737 or combinations thereof, and the percentage of apoptotic cells with subgenomic DNA was assessed by flow cytometry. (**c**) MV4;11 cells were incubated for 48 h with flavopiridol (25, 50, 100, 200 and 500 nM) or ABT-737 (10, 25, 50, 100, 200 and 500 nM). In addition, cells were incubated with flavopiridol (25, 50, 100 and 200 nM) in combination with ABT-737 (25 or 50 nM). Percentage of apoptotic cells with subgenomic DNA (fractional effect) was assessed by flow cytometry. Combination indices were calculated using the CalcuSyn software; normalized isobolograms are represented. The actual combination indices for all treatments are listed in Table 1a. Combination indices ranging from 0.1 to 0.85 indicate strong to moderate synergism, indices from 0.85 to 0.9 slight synergism, indices from 0.9 to 1.1 nearly additive interaction and indices from 1.1 to 10 slight to strong antagonism, respectively.

DISCUSSION

Here, we identify the antiapoptotic protein MCL-1 as promising target in FLT3-ITD-positive leukemia. MCL-1 expression was found to be increased in FLT3-ITD-positive leukemic cell lines and primary AML blasts compared with FLT3 wild-type controls and was strictly dependent on FLT3 signaling as confirmed by its reversion upon pharmacological inhibition of FLT3 activity as well as siRNA-mediated suppression of FLT3. As patients harboring FLT3-ITD mutations have a worse prognosis with higher relapse rates, reduced disease-free and overall survival rates compared to patients harboring wild-type FLT3, we assume that MCL-1 contributes to chemotherapy resistance of residual FLT3-ITD-positive blasts and/or leukemic stem cells.[4,11,12] In fact, we and others observed a reduced sensitivity to the cytotoxic drug cytarabine in myeloid leukemic cells transfected with a FLT3-ITD receptor.[13,42] Heterologously expressed MCL-1 substituted for FLT3 signaling by conferring resistance of hematopoietic cells to antileukemia drugs such as cytarabine and daunorubicin, and to the proapoptotic BH3 mimetic ABT-737. Conversely, suppression of endogenous MCL-1 by siRNA or by flavopiridol treatment sensitized FLT3-ITD-expressing hematopoietic cells to chemotherapy and targeted therapeutics.

Even though we observed increased MCL-1 RNA transcript and protein levels in our FLT3-ITD-positive cells, the exact mechanism how MCL-1 overexpression is linked to activation of FLT3 has to be clarified at this point. Recently, Yoshimoto *et al.*[14] observed a FLT3-ITD-dependent upregulation of MCL-1 in primary leukemic stem cells via activation of STAT5. In our cell models a STAT5-dependent upregulation of MCL-1 seems unlikely because FL stimulation of wild-type FLT3-expressing cells also enhanced *MCL-1* RNA transcription and protein levels, while STAT5 is not known to be activated by the wild-type FL-stimulated FLT3 receptor.[5,43] Using siRNA approaches or the MEK/ERK inhibitor U0126, we recently demonstrated that suppression of STAT3 or inhibition of the MAPK/ERK pathway had no impact on MCL-1 expression in FLT3-ITD_627E cells,[25] making this mode of action questionable in the present models of FLT3-ITD-positive hematopoietic cells. However, even though the exact mechanisms how deregulated MCL-1 expression is linked to activation of FLT3 needs to be determined, inhibition of overexpressed MCL-1 might be a promising therapeutic approach to sensitize residual blasts or leukemic stem cells to chemotherapy and to overcome the poor prognosis of FLT3-ITD-positive AML patients. This could be achieved by 'non-targeted' agents, like the semisynthetic flavonoid flavopiridol, which is derived from the indigenous Indian plant *Dysoxylum binectariferum*. Originally being described as an inhibitor of cyclin-dependent kinases, flavopiridol was shown here to be a potent inhibitor of RNA and protein expression of *MCL-1*, but not *BCL-2* or *BCL-X_L*, in FLT3-ITD-positive cells. Flavopiridol was described to disrupt the STAT3/DNA-binding, leading to reduced transcription of STAT3-induced genes.[38] As suppression of STAT3 had no impact on MCL-1 expression, though in FLT3-ITD_627E cells presumably showing a different signaling, this mode of action might not apply to our present models.[25] Additionally, we excluded inhibition of the MAPK/ERK-pathway, which reportedly can be involved in MCL-1 upregulation, as well as a direct inhibition of the FLT3 receptor kinase by flavopiridol.[40] However, flavopiridol sensitized FLT3-ITD-expressing cells to the cytotoxic drugs cytarabine and daunorubicin and to targeted therapies like the BH3-mimetic drug ABT-737, which was described to be ineffective in MCL-1-overexpressing cells.[35,41] Although at this point the exact mechanism for MCL-1 suppression by flavopiridol remains to be clarified, the compound could serve as a model for the development of more specific MCL-1-targeting agents to eliminate residual FLT3-ITD leukemic blasts and stem cells that are resistant to cytotoxic agents.

Table 1. Combination index (CI) for flavopiridol and ABT-737 flavopiridol and cytarabine or daunorubicin

FP [nM]	*ABT-737 [nM]*	*CI MV4;11*
25	25	0.346
50	25	0.290
100	25	0.418
200	25	0.705
25	50	0.288
50	50	0.327
100	50	0.543
200	50	0.872

FP [nM]	*Ara-C [μM]*	*CI 32D FLT3-ITD*	*CI 32D FLT3-ITD MCL-1/FLAG*
25	3	0.563	4.668
50	3	0.378	2.789
100	3	0.780	4.486
200	3	0.421	0.507

FP [nM]	*DNR [nM]*	*CI 32D FLT3-ITD*	*CI 32D FLT3-ITD MCL-1/FLAG*
25	100	0.583	1.046
50	100	0.415	0.936
100	100	0.409	1.011
200	100	0.904	0.778

Abbreviations: Ara-C, cytarabine; DNR, daunorubicin; FP, flavopiridol. Combination indices ranging from 0.1 to 0.3 indicate strong synergism, indices from 0.3 to 0.7 indicate synergism, indices from 0.7 to 0.85 indicate moderate synergism, indices from 0.85 to 0.9 slight synergism, indices from 0.9 to 1.1 nearly additive interaction, indices from 1.1 to 1.2 slight antagonism, indices from 1.2 to 1.45 moderate antagonism, indices from 1.45 to 3.3 antagonism and from 3.3 to 10 strong antagonism, respectively.

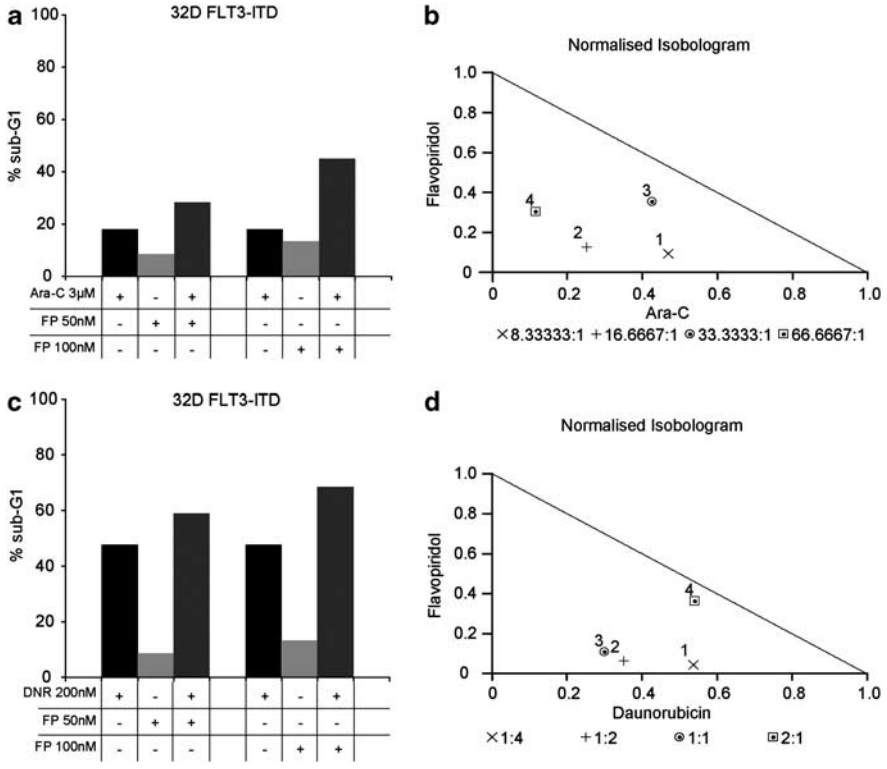

Figure 6. Activity of flavopiridol in combination with cytotoxic drugs in FLT3-ITD-positive cells. 32D FLT3-ITD-positive cells were incubated for 24 h with flavopiridol (FP), cytarabine (Ara-C) (**a**), daunorubicin (DNR) (**c**) or combinations thereof. The percentage of apoptotic cells with subgenomic DNA was assessed by flow cytometry. (**b**, **d**) 32D FLT3-ITD cells were incubated for 24 h with flavopiridol (25, 50, 100, 200 and 500 nM), cytarabine (1, 2, 3, 5, 10 and 20 μM) or daunorubicin (25, 50, 100, 150, 200 and 500 nM). In addition, cells were incubated with flavopiridol (25, 50, 100 and 200 nM) in combination with cytarabine (3 μM) (**b**) or daunorubicin (100 nM) (**d**). Percentage of apoptotic cells with subgenomic DNA (fractional effect) was assessed by flow cytometry. Combination indices were calculated using the CalcuSyn software; normalized isobolograms are represented. The actual combination indices for all treatments are listed in Table 1b. Combination indices ranging from 0.1 to 0.85 indicate strong to moderate synergism, indices from 0.85 to 0.9 slight synergism, indices from 0.9 to 1.1 nearly additive interaction and indices from 1.1 to 10 slight to strong antagonism, respectively.

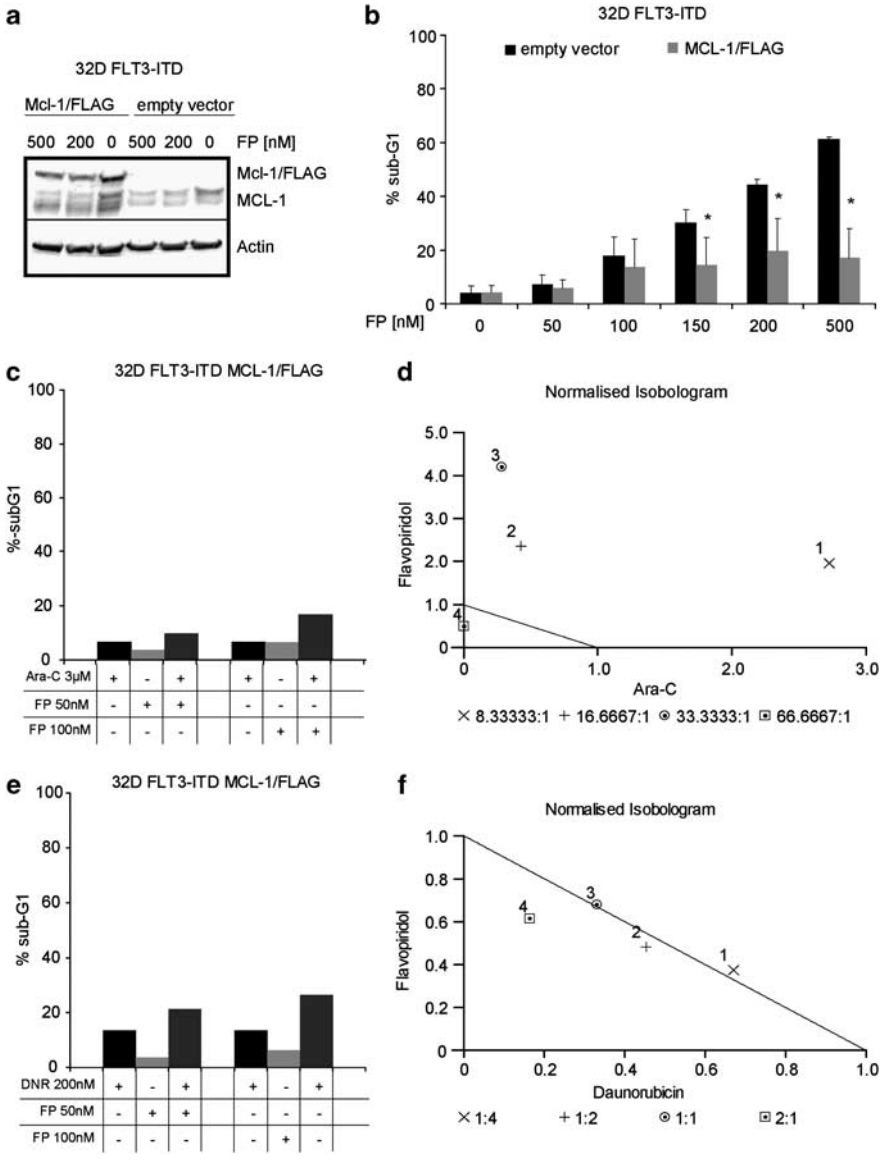

Figure 7. MCL-1 protects FLT3-ITD-positive cells against the proapoptotic activity of flavopiridol. (**a**) 32D FLT3-ITD cells were transfected to stably express a FLAG-MCL-1 construct or control vector. Expression of endogenous MCL-1 and transgenic FLAG-MCL-1 in relation to flavopiridol (FP, 5 h) treatment was assessed by immunoblotting. Actin was probed as loading control. (**b**) Expression of FLAG-MCL-1 rescues FLT3-ITD-positive cells from flavopiridol-induced apoptosis. 32D FLT3-ITD cells transfected with FLAG-MCL-1 or control vectors were incubated for 24 h with flavopiridol (FP) at incremental concentrations. The percentage of apoptotic cells with subgenomic DNA was quantified by flow cytometry. Asterisks denote statistically significant ($P<0.05$, *t*-test). (**c**, **e**) Expression of FLAG-MCL-1 protects FLT3-ITD-positive cells against apoptosis induced by cytarabine (Ara-C) (**c**), daunorubicin (DNR) (**e**) alone and in combination with flavopiridol (FP). 32D FLT3-ITD cells transfected with FLAG-MCL-1 or control vectors were incubated for 24 h with flavopiridol (FP), cytarabine (Ara-C), daunorubicin (DNR) or combinations thereof. The percentage of apoptotic cells with subgenomic DNA was quantified by flow cytometry. (**d**, **f**) 32D FLT3-ITD cells transfected with FLAG-MCL-1 were incubated for 24 h with flavopiridol (25, 50, 100, 200 and 500 nM), cytarabine (1, 2, 3, 5, 10 and 20 μM) or daunorubicin (25, 50, 100, 150, 200 and 500 nM). In addition, cells were incubated with flavopiridol (25, 50, 100 and 200 nM) in combination with cytarabine (3 μM) (**d**) or daunorubicin (100 nM) (**f**). Percentage of apoptotic cells with subgenomic DNA (fractional effect) was assessed by flow cytometry. Combination indices were calculated using the CalcuSyn software; normalized isobolograms are represented. The actual combination indices for all treatments are listed in Table 1b. Combination indices ranging from 0.1 to 0.85 indicate strong to moderate synergism, indices from 0.85 to 0.9 slight synergism, indices from 0.9 to 1.1 nearly additive interaction and indices from 1.1 to 10 slight to strong antagonism, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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