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ORIGINAL ARTICLE

PI-103, a dual inhibitor of Class IA phosphatidylinositide 3-kinase and mTOR, has antileukemic activity in AML

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The phosphatidylinositol 3-kinase (PI3K)/Akt and mammalian target of rapamycin complex 1 (mTORC1) signaling pathways are frequently activated in acute myelogenous leukemia (AML). mTORC1 inhibition with RAD001 induces PI3K/Akt activation and both pathways are activated independently, providing a rationale for dual inhibition of both pathways. PI-103 is a new potent PI3K/Akt and mTOR inhibitor. In human leukemic cell lines and in primary blast cells from AML patients, PI-103 inhibited constitutive and growth factor-induced PI3K/Akt and mTORC1 activation. PI-103 was essentially cytostatic for cell lines and induced cell cycle arrest in the G1 phase. In blast cells, PI-103 inhibited leukemic proliferation, the clonogenicity of leukemic progenitors and induced mitochondrial apoptosis, especially in the compartment containing leukemic stem cells. In contrast, apoptosis was not induced with RAD001 and IC87114 association, which specifically inhibits mTORC1 and p110δ activity, respectively. PI-103 had additive proapoptotic effects with etoposide in blast cells and in immature leukemic cells. Interestingly, PI-103 did not induce apoptosis in normal CD34$^+$ cells and had moderate effects on their clonogenic and proliferative properties. Here, we demonstrate that multi-targeted therapy against PI3K/Akt and mTOR with PI-103 may be of therapeutic value in AML.

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Keywords: AML; PI-103; class I PI3K; mTOR; leukemic stem cells; neoplasia

Introduction

Acute myelogenous leukemia (AML) is a clonal hematopoietic disorder caused by differentiation arrest and proliferation of immature progenitors.[1] The malignant clone arises from multipotent cells with intrinsic or acquired self-renewal properties, namely leukemic stem cells (LSCs), by analogy with normal hematopoiesis.[2,3] As LSCs are generally quiescent, they are insensitive to cycle-dependent chemotherapy and widely implicated in AML relapse.[2] The minimal cell population containing LSCs has been defined in the CD34$^+$ CD38$^{low/neg}$ CD123$^+$ population.[4]

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The phosphatidylinositol 3-kinase (PI3K) signaling pathway is frequently activated in blast cells from patients with AML as well as in LSCs.[4–8] Class IA PI3K enzymes are heterodimers, required to generate phosphatidylinositol 3,4,5-trisphosphate, which is critical for Akt activation. We and others recently demonstrated that blast cells constitutively express the p110δ isoform of class IA PI3K.[8,9] IC87114, a highly specific p110δ inhibitor, totally suppresses Akt activation and reduces blast cell proliferation, demonstrating that PI3K activity in AML blast cells is mainly dependent on p110δ activity. The mammalian target of rapamycin complex 1 (mTORC1), activated downstream of PI3K/Akt in several models, controls cell growth through p70S6K and 4E-BP1.[10] mTORC1 has been reported to be activated in 50 to 70% of AML samples and the mTORC1 inhibitor rapamycin decreases the clonogenicity of leukemic progenitors.[11,12] We have recently shown, in fresh AML samples, that mTORC1 inhibition by RAD001 (a rapamycin analog) induced Akt overactivation as a result of an IGF-1/IGF-1R autocrine loop.[13] Furthermore, we also observed that mTORC1 activation is independent of PI3K activation.[13] These results led us to assess the rationale for a combined inhibition of both mTORC1 and PI3K/Akt pathways in AML.[13]

PI-103 is a recently synthesized molecule of the pyridofuropyrimidine class that targets both class I PI3K and mTOR.[14,15] PI-103 has been primarily tested in glioma cell lines, where it induced a strong cell growth inhibition.[16,17] Here, we report data demonstrating the antileukemic activity of PI-103 in primary blast cells from AML patients, with, in contrast, moderate effects on normal CD34$^+$ cells. Interestingly, PI-103 also induced significant apoptosis in the compartment of cells containing LSCs, suggesting a potential clinical interest.

Materials and methods

Patients

Bone marrow samples were obtained at diagnosis from 16 patients with *de novo* AML, included in the AML2001 trial of chemotherapy initiated by the GOELAMS, as previously reported.[8] Their main characteristics are listed in Table 1. All biological studies were approved by the GOELAMS Institutional Review Board, and signed informed consent provided, according to the Declaration of Helsinki Principles. CD34$^+$ cells were

Table 1 Characteristics of AML patients

	FAB	*BM (%)*	*Cytogenetics*	*PI3K*	*mTORC1*	*Apoptosis in LSC-FC*	*Apoptosis in the bulk*	*CFU-L*
1	AML4	70	45, XY, del(5)(q12q33), del(7)(q22q35),del(q22q35), +der(11)t(11;15)(p1?4,q?21), −15, −16, add(17)(p1?3), variation [11]	+	+	D	D	D
2	AML1	85	46, XX [20]	+	+	D	D	D
3	AML4	80	46, XY, t(8;21) [20]	+	+	ND	ND	ND
4	AML2	70	46, XX, del(5)(q15q33) [11]/46, XX [9]	−	+	ND	ND	ND
5	AML4	92	46, XY [20]	+	+	ND	D	ND
6	AML5	85	88–95, XXXX, ?inv(3)(p21q11), del(5)(q13q33), X2, −17, −17, −21, −21, +4, −6, idic(21)(q22), +mar, +mar2, +variation [25]	+	+	ND	ND	D
7	AML4	90	46, XY [20]	−	+	ND	ND	D
8	AML2	92	46, XX, t(8;21), del9q [20]	−	+	D	D	D
9	AML0	100	46, XY [20]	−	+	D	D	D
10	AML1	100	46, XX [20]	+	+	D	D	D
11	AML4	70	46, XX [20]	−	+	ND	D	D
12	AML1	87	46, XY, trisomy 14 [5/20]/46, XY [15/20]	−	+	D	D	D
13	AML2	50	45, XY, t(3;17), del(7), del(3q)	−	ND	D	D	ND
14	AML1	63	45, XY, inv(3), del(7)	−	ND	D	D	ND
15	AML4	71	46, XX, inv(16) [20]	+	+	ND	D	ND
16	AML4	70	46, XX [20]	−	+	ND	D	ND

Abbreviations: AML, acute myelogenous leukemia; Apoptosis in LSC-FC, apoptosis analysis in leukemic stem cells (LSCs) by flow cytometry; BM (%), bone marrow blast percentage; CFU-L, colony-forming-unit-leukemia; D, done; mTORC1, status of constitutive mTORC1 activation; ND, not done; PI3K, status of constitutive PI3K activation.

obtained from five allogenic donors, after informed consent, and were purified from the peripheral blood, after cytapheresis.

Cells

Bone marrow cells were obtained before induction chemotherapy. Bone marrow samples were subjected to Ficoll-Hypaque density gradient separation to isolate mononuclear cells. Only samples with over 70% of blast cells were used for experiments except for samples 13 and 14, which were analyzed by flow cytometry. Cell viability was assessed by trypan blue assay, and only samples with less than 5% trypan blue-positive cells were further processed.

The MOLM14 and OCI-AML3 leukemic cell lines were obtained from ATCC. MV4-11 cell line was kindly provided by Dr JF Peyron (INSERM U895/C3M, Nice, France).

Cell culture

Patient cells were starved in cytokine and serum-free medium for 4 h to evaluate constitutive activation of the signaling pathways, as described previously.[6] For long-term (48 h) experiments, cells were cultured in MEM containing 10% fetal calf serum (FCS) to improve cell viability. Leukemic cell lines were cultured in 10% FCS MEM. Cells were incubated without or with the following inhibitors: 10 nM RAD001 (Everolimus, Novartis, Basel, Switzerland), 10 μM IC87114 (Icos Corp., Seattle, WA, USA), 25 μM LY294002 (Sigma, St Louis, MO, USA) or different concentrations of PI-103 (kindly provided by Z Knight, University of California, San Francisco, CA, USA). In some experiments, etoposide (Pierre Fabre, Toulouse, France) was used at concentrations ranging from 0.1 to 10 μM. Anti-Fas agonist antibody (CH11) was purchased from Immunotech (Becton Dickinson, Palo Alto, CA, USA).

Western blot analyses

After incubation, cells were solubilized in boiling Laemmli sample buffer and protein extracts from 10^6 cells were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with specific antibodies. Proteins were visualized using a secondary antibody, conjugated to HRP, and a chemiluminescence detection system (Enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or SuperSignal West Femto (Pierce, Rockford, IL, USA)). The images were captured using Multigauge software version 3.0 from Fujifilm. The antibodies directed against p-Akt (Ser473), Akt, p-p70S6 kinase (Thr389), p70S6 kinase, p-FOXO3a (Thr32), p-p44/42 MAPK (Thr202/Tyr204), caspase-9 and caspase-8 were purchased from Cell Signaling (Beverly, MA, USA). The anti-p110δ antibody was from Abcam (Paris, France). The anti-p110α and p110β antibodies were kindly provided by Dr B Vanhaesebroeck (Ludwig Institute for Cancer Research, London, UK) and the anti-p85 antibody by Dr Verdier (Institut Cochin, INSERM U567, Paris, France). The anti-β-actin antibody was from Sigma. The antibodies directed against cyclin D1, SCF^{Skp2}, Bcl-XL (S18), Mcl-1 (S19) and PARP (N20) were from Santa Cruz (Santa Cruz, CA, USA). The antibodies against caspase-3 and $p27^{kip1}$ were from BD Pharmingen (San Diego, CA, USA). Goat anti-rabbit IgG and horse anti-mouse IgG secondary antibodies conjugated to HRP were purchased from Cell Signaling.

Flow cytometry

Apoptosis was quantified by staining with annexin V-PE (Becton Dickinson, Le Pont-De-Claix, France), according to the manufacturer's instructions. Mitochondrial membrane potential was assessed with HIDC iodide staining (Sigma-Aldrich). Positive controls were set up with 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCmClP; Sigma-Aldrich). To assess the cell cycle, DNA staining was performed with Draq5 (Alexis Biochemicals, Lausen, Switzerland). DNA content analysis included determination of the mean channel fluorescence and the coefficient of variation of the G1 peaks, number of cell doublets. All data were generated using the autoanalysis function of the Modfit LT 3.1 program (Verity Software House, Topsham, ME, USA), with active aggregates and debris

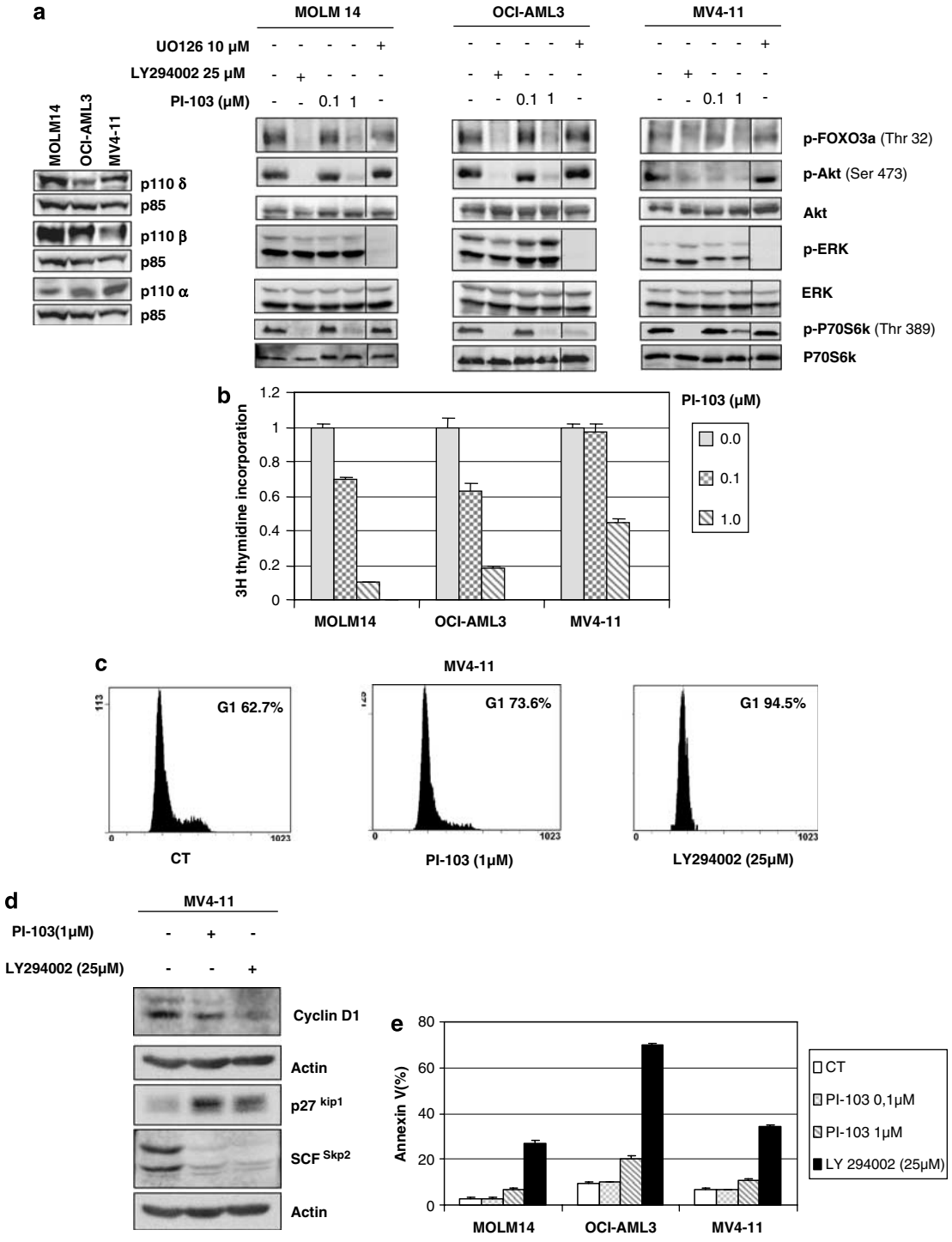

Figure 1 PI-103 inhibits PI3K/Akt and mTORC1 activity and blocks proliferation in human leukemic cell lines. (**a**) Protein extracts from 10^6 MOLM14, OCI-AML3 and MV4-11 cells were analyzed by western blot using antibodies directed against p110α, p110β, p110 δ and p85. MOLM14, OCI-AML3 and MV4-11 cells were incubated at 10^7 cells/ml in 10% fetal calf serum (FCS), without or with the following inhibitors for 1 h: 0.1 μM PI-103, 1 μM PI-103, 25 μM LY294002 or 10 μM UO126. Protein extracts from 10^6 cells were analyzed by western blot using antibodies directed against p-Akt, p-FOXO3a, p-P70S6K, p-p44/42 ERK/MAPK, Akt, p-70S6k and p44/42 ERK/MAPK antibodies. (**b**) For proliferation assays, MOLM14, OCI-AML3 and MV4-11 cells were cultured during 48 h at 10^5 cells/ml, in triplicate, in 10% FCS, without or with 0.1 or 1 μM PI-103, and then pulsed 6 h with 1 μCi (37 kBq) [^{3}H]-thymidine, as reported.[8] The amounts of radioactivity were determined after trichloracetic acid precipitation. Results are expressed as a ratio to the control, for each condition. Vertical bars indicate s.d. (**c**) MV4-11 cells were incubated at 5×10^5 cells/ml in 10% FCS without or with 1 μM PI-103 or 25 μM LY294002 during 18 h. A total of 2×10^5 cells were stained with 0.25 μl of Draq5 in 100 μl of PBS, for 15 min. DNA content was analyzed in the FL4 channel after eliminating the doublets. (**d**) MV4-11 cells were incubated at 5×10^5 cells/ml in 10% FCS, without or with the following inhibitors during 18 h: 1 μM PI-103, 25 μM LY294002. Protein extracts from 10^6 cells were analyzed by western blotting using antibodies directed against cyclin D1, p27^{kip1}, SCF^{Skp2} and actin. (**e**) MOLM14, OCI-AML3 and MV4-11 cells were incubated in duplicate at 2.5×10^5 cells/ml for 48 h in 10% FCS MEM, under the following conditions: vehicle alone, increasing doses of PI-103 or 25 μM LY294002, and then stained with annexin V-PE.

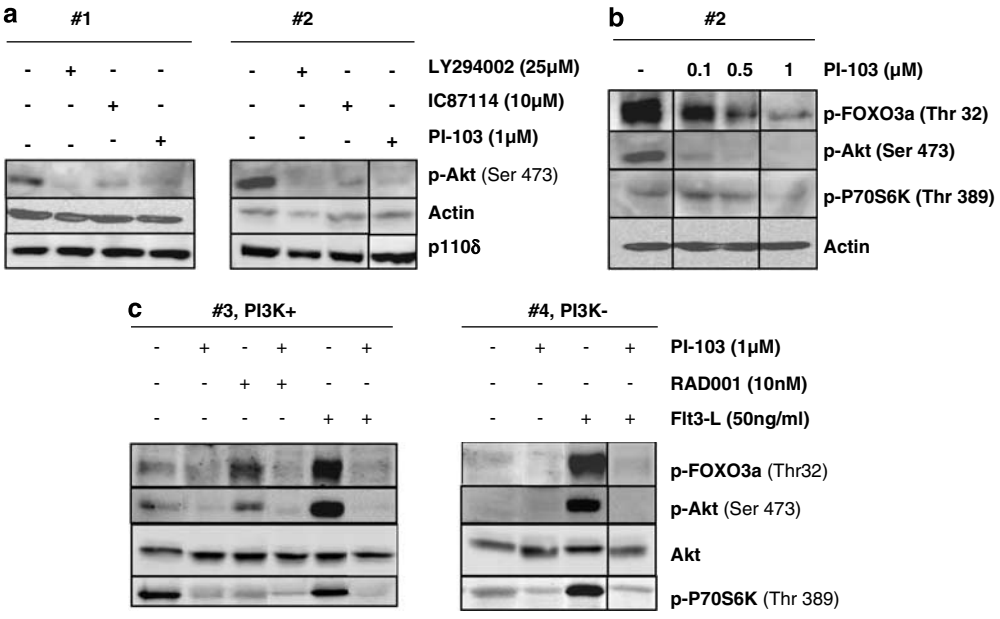

Figure 2 PI-103 inhibits p110 δ activity in acute myelogenous leukemia (AML) samples with constitutive PI3K activation and spontaneous and Flt3L-induced Akt and P70S6K phosphorylation. (**a**) Blast cells were cultured for 4 h at 10^7 cells/ml in cytokine and serum-free medium, without or with the following inhibitors, added during the last hour: 1 μM PI-103, 25 μM LY294002 or 10 μM IC87114. Protein extracts from 10^6 cells were analyzed by western blotting using antibodies directed against p-Akt, p110 δ and actin. (**b**) AML blast cells were cultured for 4 h at 10^7 cells/ml in cytokine and serum-free medium, without or with PI-103, added during the last hour at 0.1, 0.5, or 1 μM concentrations. Protein extracts from 10^6 cells were analyzed by western blot using antibodies directed against p-Akt, p-P70S6K, p-FOXO3a and Actin. (**c**) Blast cells from two AML samples (3 and 4) were starved for 4 h in cytokine and serum-free medium. During the last hour, 10 nM RAD001 or 1 μM PI-103 was added. Growth factor stimulation with Flt3L (50 ng/ml) was performed for 15 min. Western blot analyses were performed with anti-p-Akt, anti-p-FOXO3a, anti-p-P70S6K and anti-Akt antibodies.

modeling, according to the software manufacturer's recommendations. To characterize the $CD34^+$ $CD38^{low/neg}$ $CD123^+$ compartment, the following antibodies were used: CD38-FITC, CD123-PC5 and CD34-PC7 (Beckman Coulter, Miami, FL, USA).[4]

Colony assays

Clonogenic assays of normal $CD34^+$ hematopoietic progenitors. Normal $CD34^+$ cells were purified (>95% $CD34^+$ cells) on MIDI-MACS immunoaffinity columns (Miltenyi Biotech, Bergish Badgach, Germany), then plated at a concentration of 2.5×10^4 cells/ml on 35 mm plastic culture dishes in duplicate, in 1 ml semisolid culture medium (0.8% methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada, H4100), 10% FCS, 0.8% bovine serum albumin, 1.7 mM glutamine, 10^{-4} mol/l β-mercaptoethanol), and 1 UI/ml EPO, 2.5 ng/ml GM-CSF, 1000 UI/ml IL-3, 10 ng/ml SCF, 20 ng/ml IL-6 and 2.5 ng/ml G-CSF. The erythroid (BFU-E), granulo-macrophagic (CFU-GM) and granulo-erythroid-megakaryocytic-monocytic (CFU-GEMM) colony-forming units were counted under an inverted microscope.[18]

Colony-forming-unit-leukemia assays. Colony-forming-unit-leukemia (CFU-L) assays were performed as previously reported.[12] Briefly, blast cells were cultured at 10^5 cells/ml in H4230 methyl cellulose medium (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with 10% 5637 conditioned medium (5637 is a cell line derived from bladder carcinoma).[12] Cells were then plated in 35 mm Petri dishes in duplicate, and incubated for 7 days in a humidified CO_2 incubator (5% CO_2, 37 °C). At day 7, CFU-L colonies were scored under an inverted microscope.

[^{3}H]-Thymidine incorporation assay

Blast cells were incubated for 48 h in triplicate at 10^5 cells/ml in 10% FCS MEM with or without the different inhibitors, as previously reported.[8] Normal $C34^+$ cells were incubated for 48 h in triplicate at 10^5 cells/ml in RTM with 25 ng/l SCF, 10 ng/l Flt3L and 10 ng/l TPO. They were then pulsed for 6 h with [^{3}H]-thymidine (1 μCi, 37 kBq). The amount of radioactivity incorporated was determined by trichloracetic acid precipitation.

Statistical analyses

All assays were performed in triplicate, and data expressed as mean values and s.d. Statistical significance of differences observed between experimental groups were determined using Student's *t*-test.

Results

PI-103 inhibits PI3K/Akt and mTORC1 activity and blocks proliferation in human leukemic cell lines

MOLM14, OCI-AML3 and MV4-11 leukemic cell lines have constitutive activation of PI3K/Akt (p-Akt Ser 473 and p-FOXO3a Thr32), mTORC1 (p-P70S6K Thr389) and ERK/MAPK (p-p42/44 ERK/MAPK Thr202/Tyr204) signaling pathways (Figure 1a). These cell lines express the p110α, p110β and p110δ isoforms of class IA PI3K (Figure 1a). A 1 μM concentration of PI-103 inhibited constitutive PI3K/Akt and mTORC1 activity, as assessed by the inhibition of Akt, FOXO3a and p70S6-kinase phosphorylation, respectively (Figure 1a). At this concentration, PI-103 presents high specificity and does not significantly inhibit other kinases in a panel of 317 kinases recently tested.[19] PI-103 did not inhibit ERK/MAPK activation,

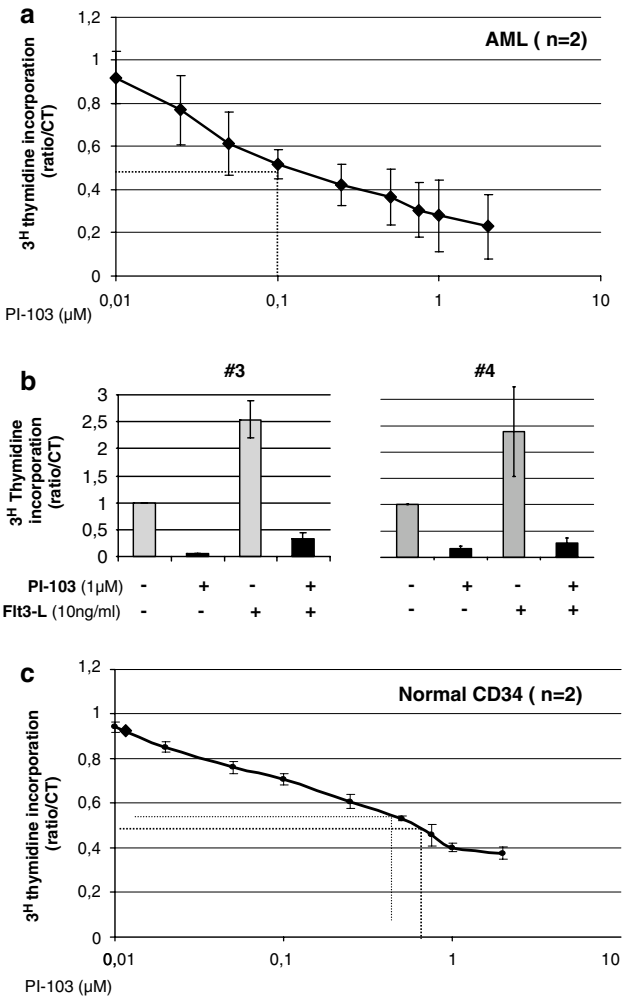

Figure 3 PI-103 inhibits spontaneous and growth factor-induced proliferation of acute myelogenous leukemia (AML) blast cells and has moderate effects on normal $CD34^+$ cell proliferation. (**a**) For proliferation assays, AML blasts cells from two PI3K+ samples (1 and 2) were cultured for 48 h at 10^5 cells/ml, in triplicate, in 10% fetal calf serum (FCS), with increasing doses of PI-103, and then pulsed 6 h with 1 μCi (37 kBq) [^{3}H]-thymidine, as reported.[8] The amounts of radioactivity were determined after trichloracetic acid precipitation. Results are expressed as a ratio to the control, for each condition. Vertical bars indicate s.d. (**b**) Blast cells from patients 3 and 4 were incubated for 48 h at 10^5 cells/ml in triplicate in MEM containing 10% FCS, under the following conditions: vehicle alone (control), 1 μM PI-103, without (−) or with (+) 10 ng/ml Flt3L, and then pulsed 6 h with 1 μCi (37 kBq) [^{3}H]-thymidine. The amounts of radioactivity were determined after trichloracetic acid precipitation. Results are expressed as a ratio to the control condition, for each condition. Vertical bars indicate s.d. (**c**) Normal $CD34^+$ cells purified from two allogenic donors were cultured for 48 h at 10^5 cells/ml, in triplicate, in RTM medium with 25 ng/ml SCF, 10 ng/ml Flt3L and 10 ng/ml IL-3, and increasing doses of PI-103, and then pulsed 6 h with 1 μCi (37 kBq) [^{3}H]-thymidine, as described above. Results are expressed as a ratio to the control condition, for each condition. Vertical bars indicate s.d.

whereas UO126, a MEK inhibitor, fully suppressed ERK1/2 phosphorylation. UO126 also induced a decrease in p70S6K phosphorylation, but only in OCI-AML3 cells (Figure 1a).

We then determined the functional activity of PI-103 in these cell lines. PI-103 inhibited cell proliferation at 1 μM (Figure 1b). In the MV4-11 cell line, PI-103 induced cell cycle arrest in the G1 phase (73.6% with PI-103 versus 62.7% in control;

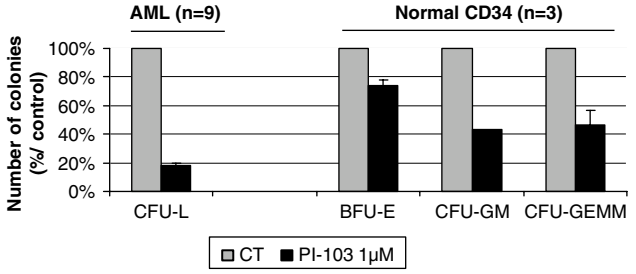

Figure 4 PI-103 profoundly inhibits leukemic progenitor clonogenicity, with moderate effects on normal hematopoiesis. Blast cells from nine acute myelogenous leukemia samples were adjusted to 10^5 cells/ml. These were grown in methylcellulose medium supplemented with 10% supernatant of the 5637-CM cell line, without or with 1 μM PI-103, in duplicate. At day 7, the CFU-L were counted.[11] Normal $CD34^+$ cells, purified from three donors, were incubated in duplicate in RTM medium, with 25 ng/ml SCF, 10 ng/ml Flt3L and 10 ng/ml IL-3, without or with 1 μM PI-103. The erythroid (BFU-E), granulo-macrophagic (CFU-GM) and granulo-erythroid-megakaryocytic-monocytic (CFU-GEMM) colony-forming units were counted after 14 days. Results are expressed as a percentage of the control, for each condition. Vertical bars indicate s.d. CFU-L, colony-forming-unit-leukemia.

Figure 1c). This antiproliferative effect of PI-103 correlated with a decrease in cyclin D1 and SCF^{Skp2} protein expression, and an accumulation of $p27^{Kip1}$ protein (Figure 1d). However, PI-103 was essentially cytostatic in these leukemic cell lines and the rate of apoptosis was not significantly increased at 1 μM, in contrast to that induced by LY294002 (Figure 1e).

Here, we demonstrate that PI-103 strongly inhibits the proliferation of leukemic cell lines, related to the suppression of the PI3K/Akt and mTORC1 pathways.

PI-103, as well as IC87114, inhibits constitutive p110δ activity in AML blast cells

We and others previously reported that IC87114, which specifically inhibits the activity of the p110δ isoform of class I PI3K, fully suppresses Akt phosphorylation in AML blasts, indicating that PI3K activity is mainly due to p110δ.[8,9] We determined whether PI-103 could inhibit the activity of the p110δ isoform in 12 AML patients. Figure 2a presents the results for two representative AML samples with constitutive PI3K activity (PI3K+). In agreement with our previous results,[8] blast cells from these patients strongly expressed p110δ and IC87114 inhibited Akt phosphorylation on Ser473 as efficiently as LY294002 (Figure 2a), confirming that in AML cells, most PI3K activity was due to p110δ. PI-103 inhibited Akt phosphorylation as efficiently as IC87114 or LY294002 in these patients, demonstrating that PI-103 exerts a potent anti-p110δ activity and is suitable for PI3K/Akt inhibition in AML.

PI-103 inhibits constitutive PI3K/Akt and mTORC1 activities and growth factor-induced activation of both pathways

PI-103 is a dual inhibitor of both PI3K, including p110δ, and mTORC1.[15] We determined the concentration of PI-103 required to fully inhibit the activity of both pathways, as it was reported that the concentration of PI-103 sufficient to suppress mTORC1 activity is higher than that necessary to inhibit PI3K activity.[15] Blast cells from patient 2 were incubated with increasing concentrations of PI-103 (Figure 2b). This patient was PI3K+ and had constitutive mTORC1 activation,

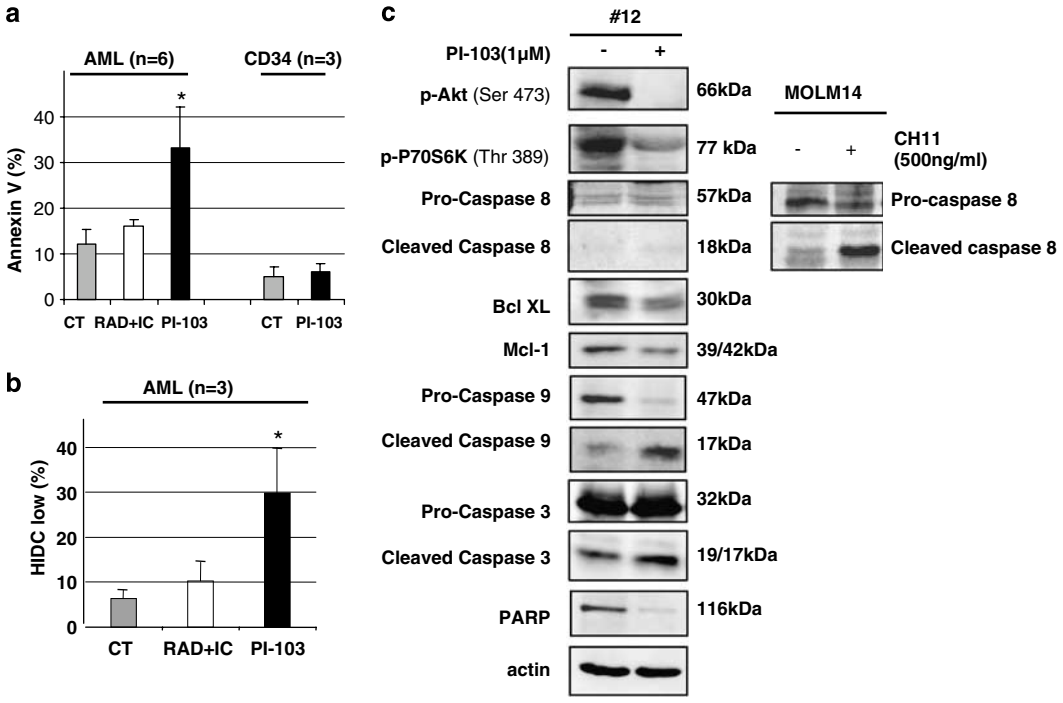

Figure 5 PI-103 induces significant apoptosis in primary acute myelogenous leukemia (AML) blast cells. (**a**) Blast cells from six AML samples were incubated in duplicate at 2.5×10^5 cells/ml during 48 h in 10% fetal calf serum (FCS) MEM, under the following conditions: medium alone, 10 μM IC87114 (IC) and 10 nM RAD001 (RAD), or 1 μM PI-103, then stained with annexin V-PE. Normal $CD34^+$ cells purified from three donors were incubated in duplicate in RTM medium with 25 ng/ml SCF, 10 ng/ml Flt3L and 10 ng/ml IL-3, for 48 h, without or with 1 μM PI-103. Cells were stained for annexin V-PE. Results from six experiments are pooled and are expressed as a percentage of annexin V-stained cells. Vertical bars indicate s.d. (**b**) Blast cells from three AML samples were incubated in duplicate at 2.5×10^5 cells/ml for 48 h in 10% FCS MEM, under the following conditions: vehicle alone (CT), 10 μM IC87114 (IC) and 10 nM RAD001 (RAD), or 1 μM PI-103, and stained with HIDC. Results of three independent experiments are pooled and expressed as a percentage of low HIDC-staining-positive cells, reflecting cells that present a mitochondrial depolarization. (**c**) Blast cells from a PI3K+ patient (no. 12) were cultured during 48 h at 5×10^5 cells/ml in 10% FCS, without or with 1 μM PI-103. Protein extracts from 10^6 cells were analyzed by western blotting using antibodies directed against caspase-8, Mcl-1, Bcl-XL, caspase-9, caspase-3, PARP, p-Akt and p-p70S6K. MOLM14 cells treated for 24 h with the Fas agonist antibody CH11 (500 ng/ml) were used as the positive control for caspase-8 cleavage.

as revealed by phosphorylation of P70S6 kinase (Figure 2b). The effective concentration of PI-103 to fully inhibit PI3K/Akt and mTORC1 pathways was 1 μM (Figure 2b).

We previously reported that a 4 h serum and cytokine deprivation of AML blast cells distinguished PI3K+ from PI3K− samples.[6] After a 4 h deprivation, Flt3L induced (PI3K− blasts) or increased (PI3K+ blasts) PI3K/Akt and mTORC1 activation in all AML samples. We determined whether PI-103 could counteract the cytokine-activated PI3K/Akt and mTORC1 signaling pathways (Figure 2c). In patient 3 with constitutive PI3K/Akt and mTORC1 activation, 1 μM PI-103 totally inhibited both constitutive and Flt3L-stimulated Akt, FOXO3a and P70S6K phosphorylation (Figure 2c, left panel). In the same patient, RAD001-mediated mTORC1 inhibition upregulated Akt and FOXO3a phosphorylation, as previously reported.[13] A 1 μM concentration of PI-103 fully reversed this RAD001-induced PI3K/Akt overactivation. Sample 4 was PI3K− but had constitutive mTORC1 activation as revealed by p-P70S6K (Thr389), which was inhibited by 1 μM PI-103 (Figure 2c, right panel). In this patient, PI-103 also fully inhibited Flt3L-induced activation of PI3K/Akt and mTORC1. Similar results were observed when blast cells were stimulated with SCF or IGF1 (data not shown). Overall, these results show that, in AML primary blast cells, PI-103 inhibits both constitutive and growth factor-induced PI3K/Akt and mTORC1 activation, and reverses the positive feedback of mTORC1 inhibition on PI3K/Akt.

PI-103 inhibits spontaneous and Flt3L-induced blast cell proliferation and has moderate effects on normal $CD34^+$ proliferation

We determined the effect of increasing concentrations of PI-103 on AML blast cell proliferation, using [^{3}H]-thymidine incorporation assay, in two PI3K+ AML samples (patients 1 and 2). We observed a dose-dependent decrease in cell growth with increasing concentrations of PI-103, with an IC50 of 0.1 μM (mean of both samples) (Figure 3a). Growth inhibition was maximal for 1 μM PI-103, which is also the concentration required for full Akt and mTORC1 inhibition (Figures 2a and b). We then studied the effect of 1 μM PI-103 on blast cell proliferation of samples 3 and 4 after stimulation by Flt3L (Figure 3b). We observed that PI-103 strongly inhibited both basal and growth factor-induced blast cell proliferation in the two samples, independently of their PI3K status (Figure 3b). As shown in Figure 3c, PI-103 also decreased the proliferation of normal $CD34^+$ cells, but these cells appeared less sensitive to PI-103 than AML cells, as the IC50 of PI-103 for normal $CD34^+$ cells was 0.75 μM.

PI-103 inhibits the clonogenic properties of AML blast cells

We then determined whether PI-103 could inhibit the clonogenicity of AML progenitors. Nine AML samples were cultured

in methylcellulose assay, without or with 1 μM PI-103. We observed a highly significant decrease in the CFU-L number with 1 μM PI-103 (decrease of 82 ± 2%, $P = 0.010$; Figure 4). The clonogenic potential of normal $CD34^+$ cells (three different samples) was less impaired by PI-103, with a 26% (± 2%), 57% (± 1%) and 53% (± 4%) decrease in the BFU-E, CFU-GM and CFU-GEMM numbers, respectively (Figure 4). These results suggest that normal hematopoietic progenitors may be less sensitive to the inhibitory effect of PI-103, when compared with AML blast cells.

PI-103 induces apoptosis in AML blast cells but not in normal $CD34^+$ cells

We previously reported that the concomitant and specific inhibition of mTORC1 and PI3K/Akt by RAD001 and IC87114, respectively (RAD + IC), induced additive antiproliferative effects in blast cells.[13] We determined if RAD + IC could induce apoptosis in primary blast cells from six AML patients. As shown in Figure 5a, apoptosis of AML blast cells was not increased significantly by treatment with RAD + IC for 48 h. In contrast, 1 μM PI-103, under identical experimental conditions, significantly induced apoptosis, as shown by increased annexin V staining from 12% (± 3%) in controls to 33% (± 9%) with PI-103 ($P = 0.001$). Interestingly, we noted that PI-103 did not induce apoptosis in normal $CD34^+$ cells from three different donors, suggesting a favorable therapeutic index of PI-103 (Figure 5a).

We also observed that PI-103 significantly increased the percentage of HIDC low cells in the three patients tested, indicative of mitochondrial depolarization (6.3% ± 1.9% in CT versus 29.9% ± 10% with PI-103, $P = 0.01$; Figure 5b). Again, the RAD + IC association did not significantly modify the mitochondrial integrity (Figure 5b). In a representative PI3K+ patient (no. 12) (Figure 5c), PI-103 induced the proteolytic cleavage of caspase-9, caspase-3 and of its substrate PARP, but caspase-8 was not cleaved in PI-103-treated cells. As a positive control for caspase-8 cleavage, MOLM14 cells were treated for 24 h with a CH11 antibody that activates the death domain of Fas receptor and induces extrinsic apoptosis. Furthermore, the expression level of the antiapoptotic proteins Bcl-XL and Mcl-1 decreased in PI-103-treated AML cells. Overall, these results suggest that PI-103 may activate the intrinsic mitochondrial apoptotic pathway and not the extrinsic, death-receptor-mediated pathway.

PI-103 induces apoptosis in the $CD34^+$ $CD38^{low/neg}$ $CD123^+$ population and potentiates etoposide-induced apoptosis in AML blasts

A key issue in AML therapy is to target the compartment of LSCs involved in AML resistance and relapse. The $CD34^+$ $CD38^{low/neg}$ $CD123^+$ population has been previously reported as a compartment containing the LSCs.[4] Six AML samples were treated with 1 μM PI-103, and apoptosis was analyzed by annexin V staining in this specific population.[4] PI-103 induced significant apoptosis in all the samples tested (7% in controls (± 1%) versus 25% with PI-103 (± 2%); $P = 0.002$; Figure 6). These results indicate that PI-103 may target this immature leukemic compartment.

We investigated whether PI-103 could enhance the proapoptotic effect of etoposide. In four samples, a 24 h exposition to etoposide induced dose-dependent apoptosis (Figure 7a). In the concentration range of 0.1–5 μM etoposide, addition of 1 μMPI-103 increased the rate of etoposide-induced apoptosis by 15%

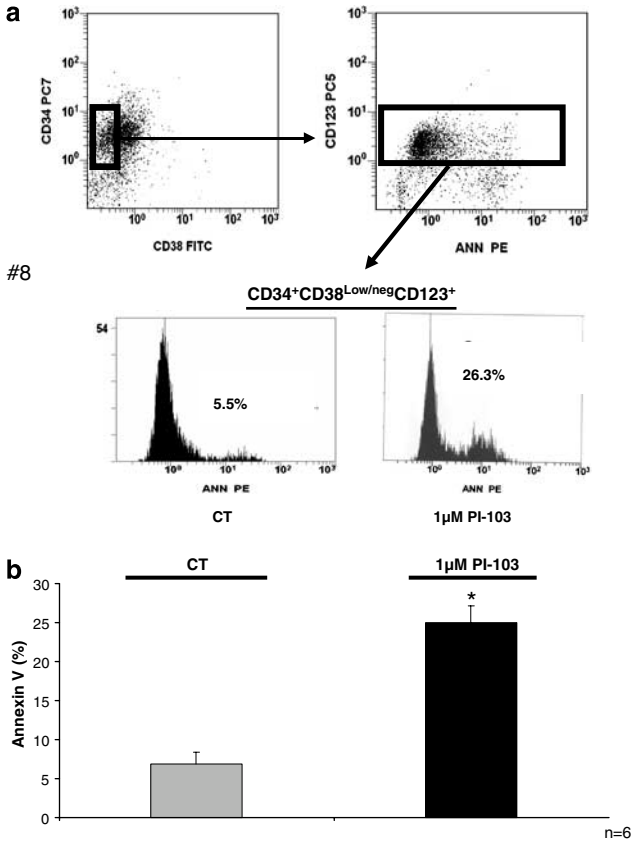

Figure 6 PI-103 induces apoptosis in the $CD34^+$ $CD38^{low/neg}$ $CD123^+$ compartment. Blast cells from six acute myelogenous leukemia patients were incubated in duplicate under the conditions reported in Figure 5, and then subjected to FC analysis with CD38-FITC, CD123-PC5, CD34-PC7 and annexin V-PE antibodies. The $CD34^+$ $CD38^{low/neg}$ $CD123^+$ subpopulation was analyzed for annexin-V PE staining. (**a**) Annexin V-positive cells in the selected $CD34^+$ $CD38^{low/neg}$ $CD123^+$ subpopulation are shown for patient 8. (**b**) Results obtained from the six experiments are pooled and expressed as a percentage of annexin V-stained cells. Vertical bars indicate s.d.

in all samples, suggesting an additive effect. Above 5 μM etoposide, PI-103 induced synergistic effects (Figure 7a). Moreover, a similar pattern of effect was detected in the $CD34^+$ $CD38^{low/neg}$ $CD123^+$ population when PI-103 was associated to etoposide (Figure 7b).

Discussion

The PI3K/Akt and mTORC1 signaling pathways have been recently identified as potential targets for therapeutic intervention in AML.[5,11,20–23] In contrast to several cellular systems where mTORC1 is a downstream target of Akt, we have shown that constitutive mTORC1 activation is independent of PI3K/Akt activity in AML.[13] These results have been confirmed by Recher and co-workers,[24] who demonstrated that mTORC1 could be activated by the Src kinase Lyn, independent of the PI3K/Akt pathway. In agreement with these previous results, all patients in our series, including the PI3K− patients, had constitutive mTORC1 activation. Moreover, mTORC1 negatively regulates the PI3K/Akt pathway in AML.[13] Indeed, P70S6K, a major relay of mTORC1, downregulates an IGF-1/IGF1-R autocrine loop in AML blasts by phosphorylating the molecular adapter IRS2 and

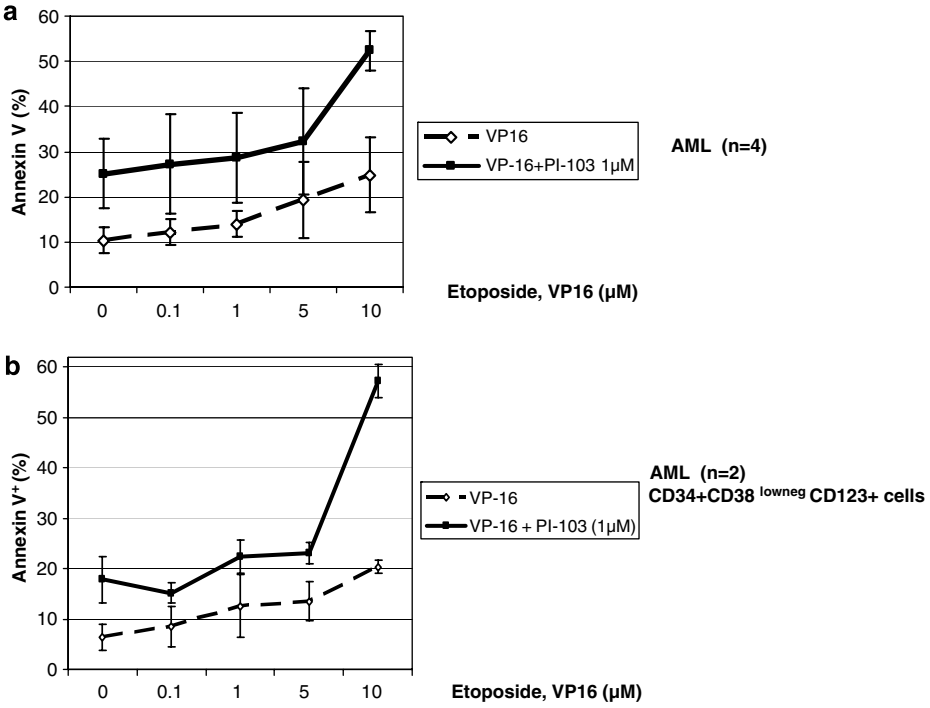

Figure 7 PI-103 is additive with etoposide to induce apoptosis in acute myelogenous leukemia (AML) blast cells and in the $CD34^+$ $CD38^{low/neg}$ $CD123^+$ compartment. (**a**) Blast cells from four AML samples were incubated in duplicate at 2.5×10^5 cells/ml during 48 h in 10% fetal calf serum MEM, under the following conditions: vehicle alone, increasing doses of etoposide alone or with 1 μM PI-103, and then stained with annexin V-PE. Results obtained from four experiments are pooled and expressed as a percentage of annexin V-stained cells. Vertical bars indicate s.d. (**b**) Blast cells from two of these AML samples were subjected to FC analysis with CD38-FITC, CD123-PC5, CD34-PC7 and annexin V-PE antibodies. The $CD34^+$ $CD38^{low/neg}$ $CD123^+$ subpopulation was analyzed for annexin-V PE staining. Results obtained from the two experiments are pooled and expressed as a percentage of annexin V-stained cells. Vertical bars indicate s.d.

inducing its degradation.[13] As IRS2 is required for intracellular signal transduction of the IGF-1 receptor, blocking mTORC1 by RAD001 relieves the inhibitory effect of P70S6K on the IGF-1/IGF1-R autocrine loop and increases PI3K/Akt activation.[13] These observations provide a rationale for the concomitant inhibition of the PI3K/Akt and mTORC1 pathways in AML. Accordingly, we observed that the specific inhibition of mTORC1 and PI3K/Akt by RAD001 and IC87114 induced additive antiproliferative effects.[13]

PI-103 is a recently synthesized molecule of the pyridofuropyrimidine class that targets both class I PI3K and mTOR.[14,15] *In vitro*, PI-103 also inhibits DNA-PK belonging to the PIKK family, and some class II PI3K, at similar concentrations. Much higher concentrations appear to be required to inhibit the class III PI3K VPS34 and other members of the PIKK family, such as ATM or ATR.[15]

We first studied the effects of PI-103 in the MV4-11, MOLM14 and OCI-AML3 human leukemic cell lines. We observed that PI-103 inhibited the proliferation of these cells and induced a cell cycle arrest in the G1 phase. This was explained by a decreased expression of the cell cycle inducer cyclin D1 and of the SCF^{Skp2} E3 ubiquitin ligase, leading to $p27^{Kip1}$ accumulation.

As reported in glioma cell lines, PI-103 was essentially cytostatic and did not induce significant apoptosis in leukemic cell lines.[16,17] These results are surprising, given that PI3K/Akt is generally believed to control cell survival. However, as pointed out by Raynaud *et al.*,[25] this conclusion has been frequently drawn from experiments using the low specificity PI3K inhibitor LY294002. This compound also inhibits many other kinases involved in cell survival and stimulates the production of reactive oxygen species.[25] Thus, the requirement of PI3K activity for cell survival should be carefully verified for each cellular model.

Although PI-103 has been reported to inhibit p110δ *in vitro*,[25] it has been essentially described as an inhibitor of the p110α subunit of class IA PI3K *in vivo*.[15,26] As most PI3K activity detected in AML blasts is due to the activity of the p110δ isoform,[8,9] we first established that PI-103 suppressed Akt phosphorylation as efficiently as IC87114, a highly specific p110δ inhibitor, thereby demonstrating that PI-103 is active against the p110δ isoform of PI3K. Experiments with increasing concentrations of PI-103 from 0.1 to 1 μM led us to determine that 1 μM was the concentration required to inhibit Akt, FOXO3a phosphorylations and mTORC1 activation. This concentration fully suppressed both constitutive and Flt3L-stimulated activation of PI3K/Akt and mTORC1. We observed a strong antiproliferative effect of PI-103 on blast cells cultured in basal conditions or induced to intensively proliferate in response to Flt3L stimulation. This confirms our previous results showing that the PI3K and mTORC1 pathways are necessary for blast cell proliferation. Moreover, PI-103 induced 82% reduction of AML progenitor clonogenicity in nine samples tested. Its anti-clonogenic effect on normal $CD34^+$ cells was less pronounced.

In contrast to the results obtained in AML cell lines, we observed that 1 μM PI-103 induced significant apoptosis in blast cells. Our study is the first to report proapoptotic effects of PI-103 in primary AML cells. We found that mitochondrial apoptosis was induced and possibly triggered by the down-regulation of Bcl-XL and Mcl-1 antiapoptotic proteins. In contrast, we did not find evidence for an activation of the extrinsic apoptosis pathway by PI-103. However, whether this proapoptotic effect is due to the simultaneous inhibition of PI3K and mTORC1 is questionable, as treatment of AML blast cells with RAD + IC did not induce apoptosis. Efficiency of PI-103 to inhibit PI3K/Akt and mTORC1 in AML was not higher than that

of RAD + IC. This suggests that inhibition of another target(s) of PI-103, such as mTORC2, could be required to induce apoptosis in AML cells. However, in four AML samples, the specific inhibition of the mTORC2 complex with a small interfering RNA, anti-Rictor, did not induce significant apoptosis (data not shown).

It is now well demonstrated that only a small subset of cells, called LSCs, are able to maintain the leukemic cell pool in the long term. These cells are $CD34^+$, $CD38^{low/neg}$ $CD123^+$, although all cells harboring this phenotype are probably not true LSCs. Interestingly, PI-103 increased apoptosis in this population, whereas it did not impair the survival of normal $CD34^+$ hematopoietic progenitor cells. Thus, PI-103 could target not only the bulk but also a compartment of immature leukemic cells.

The antiproliferative, anticlonogenic and proapoptotic effects of PI-103 were not different in PI3K+ versus PI3K− samples. The presence of FCS and growth factors in the culture medium used for *in vitro* functional tests, which stimulates PI3K/Akt activity, probably explains this result. This suggests that all AML patients could be responsive to PI-103 treatment, irrespective of their constitutive PI3K status.

In conclusion, our results show that targeting both PI3K and mTOR pathways, using multifunctional inhibitors such as PI-103, is a promising therapy in AML. Indeed, this compound has been previously used in mouse models and, despite its rapid metabolism, has demonstrated valuable antitumor activity.[25] Moreover, the therapeutic index of this compound is favorable and is therefore promising for future developments in clinical trials.

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