Published in final edited form as:

Cancer Lett. 2013 February 1; 329(1): 45–58. doi:10.1016/j.canlet.2012.09.020.

Mechanisms of apoptosis induction by simultaneous inhibition of PI3K and FLT3-ITD in AML cells in the hypoxic bone marrow microenvironment

Linhua Jin^{1,2,*}, Yoko Tabe^{1,3,*}, Hongbo Lu³, Gautam Borthakur³, Takashi Miida¹, Hagop Kantarjian³, Michael Andreeff³, and Marina Konopleva³

¹Department of Clinical Laboratory Medicine, Juntendo University School of Medicine, Tokyo, Japan

²Sportology Center, Juntendo University School of Medicine, Tokyo, Japan

³Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Abstract

We investigated the antileukemia effects and molecular mechanisms of apoptosis induction by simultaneous blockade of PI3K and mutant FLT3 in AML cells grown under hypoxia in co-cultures with bone marrow stromal cells. Combined treatment with selective class I PI3K inhibitor GDC-0941 and sorafenib reversed the protective effects of bone marrow stromal cells on FLT3-mutant AML cells in hypoxia, which was associated with downregulation of Pim-1 and Mcl-1 expression levels. These findings suggest that combined inhibition of PI3K and FLT3-ITD may constitute a targeted approach to eradicating chemoresistant AML cells sequestered in hypoxic bone marrow niches.

Keywords

acute myeloid leukemia; bone marrow microenvironment; hypoxia; GDC-0941; sorafenib

1. Introduction

Phosphatidylinositol 3-kinase (PI3K) is the major activator of the serine/threonine protein kinase Akt, which modulates a variety of downstream substrates involved in regulation of cell-cycle progression, cell survival, and transcription. Activation of the PI3K/Akt/mTOR

© 2012 Elsevier Ireland Ltd. All rights reserved.

Corresponding author contact information Marina Konopleva, M.D., Ph.D. Department of Leukemia The University of Texas MD Anderson Cancer Center 1515 Holcombe Blvd. Houston, Texas 77030 USA Telephone: 713-794-1628 mkonople@mdanderson.or.

*LJ and YT contributed equally to this work

AUTHORSHIP

+ L.J performed research, analyzed data and wrote the paper. Y.T designed and performed research, analyzed data and wrote the paper. H.L performed research. G.B. performed research. T.M analyzed and interpreted data. H.K analyzed and interpreted data. M.A. designed research and wrote the paper. M.K. designed research, analyzed data and wrote the paper.

CONFLICT OF INTEREST STATEMENTS

The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

pathway is one of the most frequent molecular events in acute myeloid leukemia (AML), documented in approximately 70% of patients [1]. PI3Ks are divided into three classes, I, II, and III. Only the class I PI3Ks, comprising the p110 α , β , δ , and γ isoforms, are able to generate the second messenger phosphatidylinositol 3,4,5-trisphosphate through activation of phosphoinositide-dependent kinase 1, which subsequently phosphorylates and activates Akt [2]. As one of the important downstream targets of Akt, mTOR influences many aspects of cellular physiology, including protein translation, by directly phosphorylating the key translation regulators p70 ribosomal S6 kinase (p70-S6K), which phosphorylates the 40S ribosomal protein S6, and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) [3,4].

mTOR exists as two complexes, mTOR complex 1 (mTORC1) and mTORC2. Akt and mTORC1/2 are linked to each other via positive and negative regulatory feedback circuits, which restrain their simultaneous hyperactivation. Once mTORC1 is activated through Akt, it antagonizes the formation of mTORC2 and reduces Akt activity. Thus, both mTORC1 and mTORC2 control Akt activation. We have recently reported that mTOR kinase inhibitor PP242 that inhibits activities of both, TORC1 and TORC2 complexes, effectively caused cell death of primary AML blasts co-cultured with bone marrow-derived stromal cells [5]. These findings provide rationale for targeting PI3K/Akt/mTOR pathway in AML [6,7]. The pan-PI3K inhibitors that inhibit all PI3K classes, such as LY294002 [8], have not been successful in clinical development mainly because of their toxic effects and unfavorable pharmacokinetic properties [9]. Recently, novel PI3K isoform-specific inhibitors have been developed as potential chemotherapeutic drugs, and have entered clinical trials [9]. GDC-0941 (Figure 1A) is a selective inhibitor of class I PI3Ks [10], which has been shown to inhibit p110 α , β , δ , and γ with median inhibitory concentrations (IC₅₀) of 3, 33, 3, and 75nM, respectively, and to weakly inhibit mTOR, with an IC₅₀ of 580nM [10].

In AML, activating mutations of the Fms-like tyrosine kinase-3 gene (*FLT3*) occur in approximately 30% of patients and are associated with poor prognosis [11,12]. Activation of downstream targets involved in the PI3K/AKT, RAS/mitogen-activated protein kinase (MAPK) pathways, and activator of transcription 5 (STAT5) [13,14] confers oncogenic properties such as dysregulated proliferation, resistance to apoptosis, and blocked differentiation [15]. The prosurvival serine/threonine-protein kinase Pim-1, a known downstream target of PI3K/Akt and FLT3/STAT5 signaling, has been shown to be overexpressed in FLT3-mutated leukemias [16]. Increased Pim-1 expression is implicated in transcription regulation, cell survival, tumorigenesis/leukemogenesis, and resistance to cytotoxic agents [17]. Sorafenib (Figure 1B) is a multikinase inhibitor that targets FLT3-ITD [18,19]. In a Phase I study of single agent sorafenib in AML, it was found to be particularly active in patients with FLT3-ITD-positive AML [20,21]. However, similar to other FLT3 inhibitors this agent produces near-complete clearing of the circulating peripheral blood blasts but only transient reduction of bone marrow-resident blasts, suggesting contribution of the microenvironmental non-pharmacological resistance. Indeed, our published data indicate stroma-mediated resistance to sorafenib and other FLT3 inhibitors [22]. We have previously shown that BM-derived stromal cells activate Akt in co-cultured leukemic cells, conferring survival advantage and chemoresistance [4]. Recent data indicate that the bone marrow microenvironment in physiological conditions is hypoxic [23], a condition even more pronounced in leukemias [24,25].

Culture of leukemic cells under hypoxia induces HIF-1 α expression, increases the release of pro-angiogenic cytokines and promotes metabolic switch to glycolysis, which may influence leukemogenesis and chemosensitivity of tumor cells [26]. Hypoxia durably activates the PI3K/Akt/mTOR signaling pathway, which may be one mechanism for leukemic cells to adapt and survive under conditions of hypoxia [27,28]. Therefore, effective targeting of the

PI3K/Akt signaling pathway could suppress AML cell survival in the hypoxic BM microenvironment.

In this study, we investigated the antileukemia effects and molecular mechanisms of apoptosis induction by simultaneous treatment with the selective class I PI3K inhibitor GDC-0941 and FLT3-ITD inhibitor sorafenib in AML cells bearing FLT3-ITD mutations in the context of the BM microenvironment. Hypoxia and interactions with BM stromal cells stimulated prosurvival Akt activation and Pim-1 expression, and decreased sensitivity to GDC-0941 or sorafenib as single agents in FLT3-ITD-positive AML cells. In turn, simultaneous blockade of PI3K and FLT3 pathways inhibited these signaling modules and promoted apoptosis. These data suggest that combined inhibition of PI3K/Akt and FLT3-ITD may constitute a targeted approach to eradicating chemoresistant AML cells sequestered in hypoxic BM niches.

2. Materials and methods

2.1 Cell lines, primary samples, and culture conditions

FLT3-ITD mutation-bearing AML cell line MV4;11 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), MOLM13 cells were provided by Hayashibara Biochemical Laboratories (Okayama, Japan) and MOLM14 cells were provided by Dr. Mark Levis, Johns Hopkins University School of Medicine, Baltimore, MD, respectively. Wild-type FLT3-bearing AML cell lines HL-60 and THP-1 were purchased from ATCC and OCI-AML3 was purchased from DSMZ (Braunschweig, Germany). Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO₂. Samples from AML patients with high (> 48%) blast count harboring FLT3-ITD mutation were obtained after informed consent in accordance with institutional guidelines set forth by The University of Texas MD Anderson Cancer Center per Declaration of Helsinki principles. Mononuclear cells were purified by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO) density-gradient centrifugation, and nonadherent cell were resuspended in RPMI 1640 medium supplemented with 10% FBS at a density of 6×10^6 cells/mL. Clinical characteristics of patients are summarized in Table I.

Mesenchymal stem cells (MSCs) obtained from healthy BM donors were cultured at a density of 5,000 to 6,000 cells/cm² in minimum essential medium alpha supplemented with 20% FBS, 1% L-glutamine, and 1% penicillin-streptomycin as described elsewhere [29]. The isolated, cultured MSCs at passage 3 comprised a single phenotypic population, as determined by flow cytometric analysis, positive for SH2 and SH3 and negative for markers of hematopoietic lineage, as described elsewhere [30]. Passage 3 or 4 MSCs were used for the co-culture experiments. To study the effect of BM stroma on AML cells, AML cell lines or primary AML cells were cultured, at a density of 1×10^5 or 1×10^6 , respectively, with or without a layer of MSCs plated at a density of 0.2×10^5 cells/cm². Co-cultured AML cells were separated from the MSC monolayer by careful pipetting with ice-cold phosphate-buffered saline solution (PBS), repeated twice. After the AML cells were collected, to rule out the possibility of contamination with MSCs, MSC monolayers were examined by microscopy ($\times 100$) to confirm that the monolayer was not damaged and that fewer than 10 leukemic cells/visual field remained attached. To verify lack of significant contamination in collected leukemic cells, the expression of CD45 was measured by flow cytometry as a discriminator between leukemic cells and MSCs [4]. Data acquisition and analysis were done using the CellQuest software. In indicated experiments, co-cultures were performed in the presence of GDC-0941 (Genentech Inc, South San Francisco, CA) and/or sorafenib (LC Laboratories, Woburn, MA). Chemical structures are shown in Figure 1.

For hypoxia experiments, leukemic cells from AML cell lines were cultured under 1% O₂ for at least 14 days to assure their continuous proliferation and survival. Primary AML cells were pre-incubated overnight.

2.2 Cell viability, apoptosis, and cell-cycle analysis

Cell proliferation was determined using the trypan blue dye exclusion method or the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI) following the company's protocol. Apoptotic cell death was analyzed by annexin V staining [29]. Briefly, fresh cells were washed twice with binding buffer (10 mM HEPES, 140 mM NaCl, and 5 mM CaCl₂, pH 7.4) and then stained with fluorescein isothiocyanate-conjugated annexin V (Roche Diagnostics, Indianapolis, IN) and propidium iodide (PI). Annexin V fluorescence was determined by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The flow cytometric data were analyzed by Cell Quest software (Becton Dickinson). The extent of drug-specific apoptosis was assessed by the formula: % specific apoptosis = (test – control) × 100 / (100 – control). Cell cycle distribution was analyzed by flow cytometric analysis of PI-stained nuclei. Cells were fixed in ice-cold ethanol (70% vol/vol) and stained with PI solution (25 µg/ml PI, 180 U/ml RNase, 0.1% Triton X-100, and 30 mg/ml polyethylene glycol in 4 mM citrate buffer, pH 7.8; Sigma-Aldrich). DNA content was determined by FACScan flow cytometer systems and CellQuest acquisition and analysis programs. Gating was set to exclude cell debris, cell doublets, and cell clumps.

2.3 Western blot analysis

Western blot analysis was performed as previously described [29]. Cells were solubilized in lysis buffer (PBS, 1× cell lysis buffer [Cell Signaling Technology, Beverly, MA], 1× protease inhibitor [Roche], 1× phosphatase inhibitor cocktails I and II [Calbiochem, San Diego, CA]) and incubated for 30 minutes on ice. Subsequently, the lysates were subjected to centrifugation for 10 minutes at 13,000 rpm at 4°C, and the supernatants were further analyzed. Protein concentration was determined by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Total protein (30 µg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad Laboratories) and transferred to polyvinylidene-fluoride membranes (0.45 µm, GE Healthcare, Buckinghamshire, UK), then probed with first and second antibodies according to the manufacturers' protocols. For immunoblotting, the following antibodies were used: α-tubulin (Sigma-Aldrich), Pim-1 (abcam, Cambridge, MA), HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA), p27^{KIP1} and Mcl-1 (BD-Pharmingen, San Diego, CA), Akt, phospho-(p-)Akt^{Ser473}, S6 ribosomal protein (S6K), p-S6K^{Ser235/Ser236}, p-4E-BP1^{Thr37/Thr46}, p-STAT5^{Tyr694}, Bim, Bcl2, Bcl-xL, cleaved caspase-3, and horseradish peroxidase-linked anti-mouse and anti-rabbit IgG (all from Cell Signaling Technology).

2.4 Statistical analysis

Results are shown as the mean plus or minus the SEM of the results of at least three experiments. The Student paired *t*-test was used for statistical comparison between groups. *P*-values less than 0.05 were considered statistically significant. Cytotoxicity of the combined treatments was assessed by the Chou-Talalay method [31] using Calcsyn software (Biosoft, Cambridge, UK). The combination index (CI) values indicate degree of synergism: strong synergism (0.3-0.7), moderate synergism (0.7-0.85), slight synergism (0.85 to 0.9), or nearly additive (0.90-1.10). The average combination index (CI) value for the drug combinations was calculated using ED₅₀, ED₇₅, and ED₉₀ (50%, 75% and 90% effective doses).

3. Results

3.1 Anti-leukemic efficacy of GDC-0941 in AML cells under normoxic and hypoxic conditions

To investigate the impact of hypoxia on AML cell responsiveness to GDC-0941, we first monitored viability of FLT3-mutated MV4;11, MOLM13 and MOLM14 AML cell lines under hypoxic or normoxic conditions treated with GDC-0941. GDC-0941 resulted in a dose-dependent reduction in cell viability accompanied by an increase of trypan blue-positive dead cells (Figure 2A,B). While hypoxia itself moderately inhibited cell growth with cytotoxicity, cells in hypoxia were less sensitive to GDC-0941 than those treated under the normoxic conditions as shown by the IC₅₀ for cell growth at 48 hours (MV4;11: 4.3μM in normoxia, 5.7μM in hypoxia; MOLM13: 5.4μM in normoxia, 9.2μM in hypoxia; MOLM14: 0.1μM in normoxia, 0.3μM in hypoxia). We next examined the potency of GDC-0941 against phosphorylated protein biomarkers of the PI3K pathway, p-Akt and p-S6K, under normoxic and hypoxic conditions (Figure 2C). Treatment with GDC-0941 (1μM) in normoxia nearly completely inhibited p-Akt Ser473 and p-S6K in both, MV4;11 and MOLM13 cells. In turn, hypoxia induced the phosphorylation of Akt and S6K in AML cells, reducing the potential of GDC-0941 to inhibit the pathway. Similar findings with respect to pAkt were observed in MOLM14 cells although we failed to detect pS6 in this cell line (Supplemental Figure 4).

We next investigated combined effects of GDC-0941 and FLT3 inhibitor sorafenib in FLT3-mutant AML cells cultured in medium without stromal support. As shown in Figure 3, combination of GDC-0941 and sorafenib was synergistic under normoxic condition (mean CI values 0.38 for MV4;11, 0.80 for MOLM13, 0.26 for MOLM14). In hypoxia, the GDC-0941/sorafenib combination produced synergistic effects in MOLM14 (mean CI =0.31), additive effects in MOLM13 (mean CI =0.97) but was antagonistic in MV4;11 cells (mean CI= 450.95). These results indicate that in certain cell types hypoxia may attenuate the anti-proliferative effects of GDC-0941 and to a lesser extent of GDC-0941/sorafenib combination.

The wild-type FLT3-bearing AML cells HL-60, THP-1, and OCI-AML3 cells were less sensitive to sorafenib compared to FLT3-mutated AML cells (IC₅₀ values: 0.01μM for MV4;11, 0.02μM for MOLM13, 0.04μM for MOLM14, 3.0μM for HL-60, 3.6μM for THP-1, and 0.7μM for OCI-AML3), concordant with the previous study [18], and no synergy upon combination with GDC-0941 was observed (Supplemental Figure 1).

3.2 Combined effects of GDC0941 with sorafenib on AML cells with FLT3-ITD co-cultured with MSC in hypoxia

We previously reported that the multikinase inhibitor sorafenib, which directly targets mutant FLT3 kinase [18], is effective in inducing growth arrest and apoptosis in AML cells bearing FLT3-ITD mutations[18] but its potency is diminished in stromal co-culture conditions [22]. We have further reported the activation of PI3K/AKT signaling in leukemic cells co-cultured with stroma[4]. We therefore evaluated the combined efficacy of GDC-0941 and sorafenib in FLT3-ITD-bearing AML cells co-cultured with primary human marrow-derived stromal cells (MSC) under conditions mimicking hypoxic BM microenvironment. In these studies, we utilized GDC-0941 at a concentration of 0.5μM, which is near the reported plasma concentration level required for tumor stasis with inhibition of Akt [32]. Sorafenib is well tolerated clinically, and the plasma concentration of sorafenib achieved in clinical trials exceeds the concentrations utilized in our experiments [20].

In all three FLT3-ITD cell lines, treatment with GDC-0941 (0.5 μ M) resulted in G₀/G₁ cell-cycle arrest without significant apoptosis induction, and this was not affected by co-culture with MSC in either the normoxic or hypoxic condition (Figure 4A,B, Supplemental Figure 2). The combined treatment with a low dose of sorafenib (0.01 μ M) resulted in increased annexin V-positive fractions under both, normoxic and hypoxic conditions. While co-culture with MSC significantly reduced apoptosis induced by combined GDC-0941/sorafenib treatment under normoxic conditions, it failed to do so under hypoxic stress (Figure 4B and Supplemental Table I). Concordant with these results, MSC partially reversed inhibition of cell growth by GDC-0941/sorafenib in normoxic condition, whereas the combination of GDC-0941/sorafenib was equally potent in the hypoxic condition under MSC co-culture conditions (Figure 4C). In primary FLT3-ITD(+) AML samples, we observed the protective effects of MSC on sorafenib-induced apoptosis in seven of the eight cases (#1-3,5-8) under normoxia and in four of the eight cases under hypoxia (#3,5,7,8). In turn, hypoxia diminished sorafenib-induced cell death in 5/8 cases compared to normoxia (#1,2,3,5,6). Combined treatment with GDC-0941 and sorafenib induced more apoptosis than each of the agents alone in all samples tested. This effect was evident in both, normoxic and hypoxic conditions and with or without MSCs. Co-culture with MSC, however, decreased the GDC-0941/sorafenib induced apoptosis in the majority (7/8) cases in normoxia (#1, #3-8) and in five of the eight cases in hypoxia (#3-7). (Figure 4D). Whereas the heterogeneity of the sensitivity to GDC-0941 or sorafenib and the extent of protective effects of MSC have been observed among FLT3-ITD patients, these findings suggest that the combined blockade of PI3K and mutant FLT3 promotes apoptosis both under normoxic and hypoxic conditions.

3.3 GDC-0941/sorafenib combination downregulates hypoxia-induced Pim-1 and MCL-1 in FLT3-ITD-expressing AML cells under hypoxic and MSC co-culture conditions

We next examined the molecular mechanisms of apoptosis induction by simultaneous treatment with GDC-0941 and sorafenib in AML cells co-cultured with MSC under normoxic and hypoxic conditions. In normoxic conditions, phosphorylation levels of Akt were increased by co-culture with MSCs in MV4;11 and MOLM14, and less so in MOLM13 cells with high constitutive levels of pAkt. Inhibition of PI3K by GDC-0941 resulted in complete downregulation of constitutive and stroma-induced pAkt in all three cell types. Hypoxia itself induced activation of Akt without further influence by MSC, and this effect was only partially inhabitable by GDC-0941 (Figure 5 and Supplemental Figure 3,4). In contrast, sorafenib-induced downregulation of p-Akt was partially inhibited by MSC but was independent of oxygen level. The GDC-0941/sorafenib combination almost completely abolished Akt phosphorylation although residual pAkt was detectable in MV4;11 and MOLM14 cells under hypoxia. Compared to its efficacy on p-Akt, GDC-0941 was less effective at downregulating the phosphorylation levels of S6K, which were increased by the hypoxic or MSC co-culture conditions. Sorafenib-induced downregulation of p-S6K levels was partially reversed by MSC in both, normoxic and hypoxic conditions. In turn, the GDC-0941/sorafenib combination was effective in inhibiting S6K phosphorylation irrespective of MSC co-culture or oxygen level. Phosphorylation of another mTOR downstream target, 4E-BP1, was also decreased by GDC-0941/sorafenib. Overall, the GDC-0941/sorafenib combination effectively blocked the PI3K/Akt/mTOR pathway that was activated in response to hypoxia or interactions with MSC.

We next examined regulation of other FLT3-ITD targets under conditions mimicking the BM microenvironment. Mutated FLT3 is known to induce active STAT-5 [13,14]. STAT-5 phosphorylation was unaffected by GDC0941 and completely blocked by sorafenib in all culture conditions.

The prosurvival serine/threonine-protein kinase Pim-1, a downstream target of Akt and FLT3 signaling, was upregulated by hypoxia in MV4;11, MOLM13 and MOLM14 cells. Under hypoxia, sorafenib decreased Pim-1 expression and this decrease was abrogated by MSC. While GDC-0941 alone did not affect hypoxia-induced Pim-1 levels, the GDC-0941/sorafenib combination downregulated Pim-1 expression in the hypoxic condition irrespective of MSC co-culture (Figure 5A, B and Supplemental Figure 4). Modulation of the mTOR, Akt and Pim under hypoxic MSC co-culture conditions is summarized in the Table II.

We next examined treatment-induced changes in the expression levels of proapoptotic and antiapoptotic Bcl-2 family proteins. Whereas treatment with sorafenib induced downregulation of Mcl-1 expression, MSC co-culture partially or fully reversed it. The GDC-0941/sorafenib combination decreased Mcl-1 levels, and this decrease was reversed by MSC co-culture in the normoxic but not the hypoxic condition. Expression of the proapoptotic Bim and cleaved caspase-3 were moderately increased by the GDC-0941/sorafenib combination in all tested conditions. These changes were associated with observed apoptotic responses. Expression levels of Bcl-2 were not significantly changed by any treatment or cell culture condition. Since we observed G₁ cell-cycle arrest by GDC-0941 or sorafenib in both MV4;11 and MOLM13 cells, we examined the effects of these agents on key regulators of the G₁-S cell-cycle transition. Expression of cell-cycle regulatory protein p27^{KIP1} was increased by GDC-0941 and/or sorafenib irrespective of MSC co-culture or oxygen condition (Figure 5A,B), with no change in p21 and cyclin D1 (Supplemental Figure 5). Hypoxia-induced HIF-1 α expression was affected by neither GDC-0941 nor sorafenib treatment (Figure 5A,B).

4. Discussion

The present study demonstrated that simultaneous inhibition of class I PI3Ks by GDC-0941 and of FLT3-ITD mutant protein by sorafenib effectively decreased growth of

AML cells bearing FLT3-ITD in conditions mimicking the hypoxic BM microenvironment. While constitutive activation of PI3K signaling has been previously implicated in resistance to targeted FLT3 inhibition in FLT3-ITD driven AML [33,34,35], our data suggest that co-culture with BM stromal cells further promotes PI3K/AKT/mTOR signaling insensitive to the blockade of mutant FLT3 by sorafenib. While sorafenib inhibited the mTOR signaling in cells cultured in suspension, it failed to fully inhibit MSC-induced S6K phosphorylation, indicating a potential role of the BM microenvironment to stimulate PI3K/mTOR signaling even in cells with high baseline PI3K/mTOR activity due to activated receptor tyrosine kinase. In turn, stroma-induced S6K phosphorylation was suppressed by GDC-0941, supporting our prior observations of the principal role of PI3K/AKT/mTOR pathway in stroma-mediated pro-survival signaling [4]. This stroma-triggered activation of PI3K may constitute one of the mechanisms of resistance to FLT3 kinase inhibitors, shown here in the *in vitro* co-cultures of primary AML cells with MSC, and consistent with reduced efficacy of sorafenib in bone marrow-resident blasts reported by us previously [20]. While multiple mechanisms of microenvironmental resistance to FLT3 inhibition have been described, including production of FLT3 ligand or other cytokines [36,37], secretion of chemokines (SDF-1 α) [22], integrin-mediated signals and others [4,38], all of these ultimately converge on downstream PI3K/AKT signaling, providing rationale for combination strategies.

We have further studied the contribution of hypoxia, recently shown by us and others as essential component of diseased leukemic microenvironment [26,39,40], in the sensitivity to FLT3 inhibitors. Hypoxia is known to confer pro-survival signals to tumor cells via multiple mechanisms including activation of PI3K/Akt/mTOR and HIF-1 α pathways [2,41,42].

Hypoxia-induced mTOR activation is modulated in a PI3K/Akt-dependent [43] and – independent manner [44], while mTOR itself mediates the downstream signaling of PI3K/Akt through increasing phosphorylation of Akt [45]. In this study, we demonstrated that in AML cells hypoxia induced phosphorylation levels of Akt and of mTOR target S6K, which may be one mechanism for leukemic cells to adapt and survive under conditions of hypoxic stress [27,28]. However, GDC-0941, which has high potency against class I PI3Ks but less against mTOR [46], showed less inhibitory effect on Akt and S6K phosphorylation levels under hypoxic conditions, and was not effective in downregulating hypoxia-induced HIF-1 α . These findings suggest that in AML cells hypoxia activates mTOR and HIF-1 α through alternative, PI3K-independent pathway(s), the nature of which remains to be elucidated. Curiously, hypoxia-induced S6K phosphorylation was partially abrogated by sorafenib. The molecular mechanism of sorafenib action under hypoxia requires further study; one possibility is the ability of this multikinase inhibitor to block Raf/MAPK signaling which is known to be activated by hypoxia and can be responsible for increased S6K phosphorylation [20,27]. To this end, simultaneous administration of GDC-0941 and sorafenib resulted in parallel inhibition of signaling pathways converging at the mTOR/S6K checkpoint and promote growth inhibition of FLT3-mutated AML cells under conditions mimicking the hypoxic BM microenvironment (Table II). It is notable, however, that in primary AML cells, unlike in cell lines, co-culture with stromal cells under hypoxic conditions resulted in decreased induction of apoptosis by GDC/sorafenib combination. Concomitant intra-pathway blockade or inhibition of parallel signaling pathways in addition to GDC-0941 treatment might be required to suppress the survival of AML cells adapted to hypoxic environmental stress in the BM microenvironment. As such, the extrinsic components including chemokine receptors (CXCR4), adhesion molecules (VLA-4 and CD44), and hypoxia-related proteins are known to influence the survival of AML cells in hypoxic BM microenvironment [38]. We have demonstrated that small-molecule CXCR4 inhibitor enhanced sorafenib-induced apoptosis in samples from primary AML patients with FLT3 mutation [22]. Likewise, ligation of CD44 with the H90 monoclonal antibody resulted in marked reduction of the leukemic burden in NOD-SCID mice transplanted with primary AML cells [51]. Affecting homing and adhesion through interference with chemokines and adhesion molecules may cause egress of leukemic cells out of protective microenvironmental niches and enhance antileukemic effects of FLT3 inhibitors.

To elucidate molecular mechanisms of the inhibitors under stromal co-cultures at different oxygen levels, we studied effects of MSC and hypoxia on major downstream intracellular signaling pathways activated in FLT3-ITD cells. Pim-1 kinase is known to promote hypoxia-induced chemoresistance, and is a target of PI3K/Akt signaling and of FLT3-ITD downstream STAT5 activation [20,47]. Recent studies have shown that Pim-1 inhibitor AR00459339 is preferentially cytotoxic to FLT3-ITD AML cells, promoting the de-phosphorylation of FLT3 target STAT5 [48]. We demonstrated that sorafenib repressed hypoxia-induced Pim-1, but this inhibition was partially reversed by MSC co-culture. The GDC-0941/sorafenib combination decreased Pim-1 expression irrespective of MSC co-culture condition or oxygen concentration. The finding that GDC-0941 was effective in inhibiting induction of Pim-1 expression by BM stromal cells indicates that PI3K/Akt signaling might play a dominant role in Pim-1 induction. In turn, STAT5 phosphorylation, moderately induced by MSC in MOLM13 cells and constitutively activated in MV4;11 cells, was completely diminished by sorafenib, indicating that STAT5 is an unlikely mediator of Pim-1 expression in the system used. Notably, phosphorylation of 4E-BP1 is well known as a target of Pim-1 [49] and of mTOR. Although MSC partially reversed sorafenib-induced repression of p-4E-BP1 in the hypoxic condition, co-treatment with GDC-0941 was effective in p-4E-BP1 inhibition. The downregulation of Pim-1 was associated with downregulation of its downstream target Mcl-1 [50] and with induction of cleaved caspase-3, which might be associated with the observed apoptotic responses. We

have further observed that the GDC-0941/sorafenib combination arrested the cell cycle in G1phase and induced p27^{Kip1}, known to be suppressed by activated Akt, irrespective of oxygen concentration or MSC co-culture. Thus, GDC-0941 and sorafenib induced inhibition of FLT3-ITD-bearing AML growth by blocking the separate signaling pathways that coordinately mediate mTOR, Pim-1, Mcl-1, and p27^{Kip1} expression (Figure 6). The contribution of individual signaling molecules downstream of FLT3 and PI3K to the effects of sorafenib and GDC-0941 under different conditions remains to be formally investigated in future studies.

In summary, this study demonstrates that the combination of GDC-0941 with sorafenib inhibits hypoxia- and BM stromal cell-induced signaling pathways in a coordinate manner in AML cells with FLT3-ITD mutations. These data suggest that simultaneous blockade of PI3K and mutant FLT3 is required to overcome the protective influence of the hypoxic BM microenvironment and may constitute a targeted approach to eradicating chemoresistant AML cells sequestered in hypoxic BM niches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Yasuhito Hatanaka, Tomomi Ikeda, Takako Ikegami and Akemi Koyanagi for technical assistance. We thank Laboratories of Molecular and Biochemical Research and Cell Biology, Research Support Center, Juntendo University Graduate School of Medicine for use of facilities. We thank Kathryn Hale for manuscript review, Melodie England for help in the preparation of the manuscript, and Genentech Inc. for the gift of GDC-0941.

This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan and Juntendo Institutional Research Grant of Environmental and Gender Specific Medicine(to Y.T.); Juntendo Institutional Project Research Program (to L.J.); 1R01CA155056-01, CDP-01, Leukemia and Lymphoma Society and DRP, Leukemia Spore 5 P50 CA100632-08 (to M.K.); and 5P01 CA55164-17 (to M.A.).

REFERENCES

- [1]. Kornblau SM, Womble M, Qiu YH, Jackson CE, Chen W, Konopleva M, Estey EH, Andreeff M. Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood*. 2006; 108:2358–2365. [PubMed: 16763210]
- [2]. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer*. 2002; 2:489–501. [PubMed: 12094235]
- [3]. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev*. 2004; 18:1926–1945. [PubMed: 15314020]
- [4]. Tabe Y, Jin L, Tsutsumi-Ishii Y, Xu Y, McQueen T, Priebe W, Mills GB, Ohsaka A, Nagaoka I, Andreeff M, Konopleva M. Activation of integrin-linked kinase is a critical prosurvival pathway induced in leukemic cells by bone marrow-derived stromal cells. *Cancer Res*. 2007; 67:684–694. [PubMed: 17234779]
- [5]. Zeng Z, Shi YX, Tsao T, Qiu Y, Kornblau SM, Baggerly KA, Liu W, Jessen K, Liu Y, Kantarjian H, Rommel C, Fruman DA, Andreeff M, Konopleva M. Targeting of mTORC1/2 by the mTOR kinase inhibitor PP242 induces apoptosis in AML under conditions mimicking the bone marrow microenvironment. *Blood*. 2012 e-pub.
- [6]. Martelli AM, Evangelisti C, Chiarini F, McCubrey JA. The phosphatidylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients. *Oncotarget*. 2010; 1:89–103. [PubMed: 20671809]
- [7]. Dunlop EA, Tee AR. Mammalian target of rapamycin complex 1: signaling inputs, substrates and feedback mechanisms. *Cell Signal*. 2009; 21:827–835. [PubMed: 19166929]

- [8]. Hu L, Zaloudek C, Mills GB, Gray J, Jaffe RB. In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). *Clin. Cancer Res.* 2000; 6:880–886. [PubMed: 10741711]
- [9]. Kong D, Yamori T. Advances in development of phosphatidylinositol 3-kinase inhibitors. *Curr. Med. Chem.* 2009; 16:2839–2854. [PubMed: 19689267]
- [10]. Folkes AJ, Ahmadi K, Alderton WK, Alix S, Baker SJ, Box G, Chuckowree IS, Clarke PA, Depledge P, Eccles SA, Friedman LS, Hayes A, Hancox TC, Kugendradas A, Lensun L, Moore P, Olivero AG, Pang J, Patel S, Pergl-Wilson GH, Raynaud FI, Robson A, Saghir N, Salphati L, Sohal S, Ultsch MH, Valenti M, Wallweber HJ, Wan NC, Wiesmann C, Workman P, Zhyvoloup A, Zvelebil MJ, Shuttleworth SJ. The identification of 2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thien o[3,2-d]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. *J. Med. Chem.* 2008; 51:5522–5532. [PubMed: 18754654]
- [11]. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood.* 2002; 100:1532–1542. [PubMed: 12176867]
- [12]. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, Sonoda Y, Fujimoto T, Misawa S. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia.* 1996; 10:1911–1918. [PubMed: 8946930]
- [13]. Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Müller C, Grüning W, Kratz-Albers K, Serve S, Steur C, Büchner T, Kienast J, Kanakura Y, Berdel WE, Serve H. Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. *Blood.* 2000; 96:3907–3914. [PubMed: 11090077]
- [14]. Choudhary C, Brandts C, Schwable J, Tickenbrock L, Sargin B, Ueker A, Böhmer FD, Berdel WE, Müller-Tidow C, Serve H. Activation mechanisms of STAT5 by oncogenic Flt3-ITD. *Blood.* 2007; 110:370–374. [PubMed: 17356133]
- [15]. Radomska HS, Bassères DS, Zheng R, Zhang P, Dayaram T, Yamamoto Y, Sternberg DW, Lokker N, Giese NA, Bohlander SK, Schnittger S, Delmotte MH, Davis RJ, Small D, Hiddemann W, Gilliland DG, Tenen DG. Block of C/EBP alpha function by phosphorylation in acute myeloid leukemia with FLT3 activating mutations. *J. Exp. Med.* 2006; 203:371–381. [PubMed: 16446383]
- [16]. Shah N, Pang B, Yeoh KG, Thorn S, Chen CS, Lilly MB, Salto-Tellez M. Potential roles for the PIM1 kinase in human cancer—a molecular and therapeutic appraisal. *Eur. J. Cancer.* 2008; 44:2144–2151. [PubMed: 18715779]
- [17]. Amaravadi R, Thompson CB. The survival kinases Akt and Pim as potential pharmacological targets. *J. Clin. Invest.* 2005; 115:2618–2624. [PubMed: 16200194]
- [18]. Lierman E, Lahortiga I, Van Mieghroet H, Mentens N, Marynen P, Cools J. The ability of sorafenib to inhibit oncogenic PDGFRbeta and FLT3 mutants and overcome resistance to other small molecule inhibitors. *Haematologica.* 2007; 92:27–34. [PubMed: 17229632]
- [19]. Metzelder S, Wang Y, Wollmer E, Wanzel M, Teichler S, Chaturvedi A, Eilers M, Enghofer E, Neubauer A, Burchert A. Compassionate use of sorafenib in FLT3-ITD-positive acute myeloid leukemia: sustained regression before and after allogeneic stem cell transplantation. *Blood.* 2009; 113:6567–6571. [PubMed: 19389879]
- [20]. Zhang W, Konopleva M, Shi YX, McQueen T, Harris D, Ling X, Estrov Z, Quintás-Cardama A, Small D, Cortes J, Andreeff M. Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia. *J. Natl. Cancer Inst.* 2008; 100:184–198. [PubMed: 18230792]
- [21]. Borthakur G, Kantarjian H, Ravandi F, Zhang W, Konopleva M, Wright JJ, Faderl S, Verstovsek S, Mathews S, Andreeff M, Cortes JE. Phase I study of sorafenib in patients with refractory or relapsed acute leukemias. *Haematologica.* 2011; 96:62–68. [PubMed: 20952518]
- [22]. Zeng Z, Shi YX, Samudio IJ, Wang RY, Ling X, Frolova O, Levis M, Rubin JB, Negrin RR, Estey EH, Konoplev S, Andreeff M, Konopleva M. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood.* 2009; 113:6215–6224. [PubMed: 18955566]
- [23]. Eliasson P, Jönsson JI. The hematopoietic stem cell niche: low in oxygen but a nice place to be. *J. Cell Physiol.* 2010; 222:17–22. [PubMed: 19725055]

- [24]. Mortensen BT, Jensen PO, Helledie N, Iversen PO, Ralfkiaer E, Larsen JK, Madsen MT. Changing bone marrow micro-environment during development of acute myeloid leukaemia in rats. *Br. J. Haematol.* 1998; 102:458–464. [PubMed: 9695960]
- [25]. Jensen PO, Mortensen BT, Hodgkiss RJ, Iversen PO, Christensen IJ, Helledie N, Larsen JK. Increased cellular hypoxia and reduced proliferation of both normal and leukaemic cells during progression of acute myeloid leukaemia in rats. *Cell Prolif.* 2000; 33:381–395. [PubMed: 11101010]
- [26]. Frolova O, Samudio I, Benito JM, Jacamo R, Kornblau SM, Markovic A, Schober W, Lu H, Qiu YH, Buglio D, McQueen T, Pierce S, Shpall E, Konoplev S, Thomas D, Kantarjian H, Lock R, Andreeff M, Konopleva M. Regulation of HIF-1 α signaling and chemoresistance in acute lymphocytic leukemia under hypoxic conditions of the bone marrow microenvironment. *Cancer Biol Ther.* 2012; 13:858–870. [PubMed: 22785211]
- [27]. Fiegl M, Samudio I, Mnjoyan Z, Korchin B, Fritsche H, Andreeff M. Physiological hypoxia promotes lipid raft and PI3K-dependent activation of MAPK 42/44 in leukemia cells. *Leukemia.* 2010; 24:1364–1367. [PubMed: 20508615]
- [28]. Alvarez-Tejado M, Naranjo-Suarez S, Jiménez C, Carrera AC, Landázuri MO, del Peso L. Hypoxia induces the activation of the phosphatidylinositol 3-kinase/Akt cell survival pathway in PC12 cells: protective role in apoptosis. *J. Biol Chem.* 2001; 276:22368–22374. [PubMed: 11294857]
- [29]. Tabe Y, Konopleva M, Munsell MF, Marini FC, Zompetta C, McQueen T, Tsao T, Zhao S, Pierce S, Igari J, Estey EH, Andreeff M. PML-RAR α is associated with leptin-receptor induction: the role of mesenchymal stem cell-derived adipocytes in APL cell survival. *Blood.* 2004; 103:1815–1822. [PubMed: 14630813]
- [30]. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Ma rshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999; 284:143–147. [PubMed: 10102814]
- [31]. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 1984; 22:27–55. [PubMed: 6382953]
- [32]. Salphati L, Wong H, Belvin M, Bradford D, Edgar KA, Prior WW, Sampath D, Wallin JJ. Pharmacokinetic-pharmacodynamic modeling of tumor growth inhibition and biomarker modulation by the novel phosphatidylinositol 3-kinase inhibitor GDC-0941. *Drug. Metab. Dispos.* 2010; 38:1436–1442. [PubMed: 20538720]
- [33]. Piloto O, Wright M, Brown P, Kim KT, Levis M, Small D. Prolonged exposure to FLT3 inhibitors leads to resistance via activation of parallel signaling pathways. *Blood.* 2007; 109:1643–1652. [PubMed: 17047150]
- [34]. Zeng Z, Samudio IJ, Zhang W, Estrov Z, Pelicano H, Harris D, Frolova O, Hail N Jr, Chen W, Kornblau SM, Huang P, Lu Y, Mills GB, Andreeff M, Konopleva M. Simultaneous inhibition of PDK1/AKT and Fms-like tyrosine kinase 3 signaling by a small-molecule KP372-1 induces mitochondrial dysfunction and apoptosis in acute myelogenous leukemia. *Cancer Res.* 2006; 7:3737–3746. [PubMed: 16585200]
- [35]. Weisberg E, Banerji L, Wright RD, Barrett R, Ray A, Moreno D, Catley L, Jiang J, Hall-Meyers E, Sauveur-Michel M, Stone R, Galinsky I, Fox E, Kung AL, Griffin JD. Potentiation of antileukemic therapies by the dual PI3K/PDK-1 inhibitor, BAG956: effects on BCR-ABL– and mutant FLT3-expressing cells. *Blood.* 2008; 111:3723–3734. [PubMed: 18184863]
- [36]. Levis M, Ravandi F, Wang ES, Baer MR, Perl A, Coutre S, Erba H, Stuart RK, Baccarani M, Cripe LD, Tallman MS, Meloni G, Godley LA, Langston AA, Amadori S, Lewis ID, Nagler A, Stone R, Yee K, Advani A, Douer D, Wiktor-Jedrzejczak W, Juliusson G, Litzow MR, Petersdorf S, Sanz M, Kantarjian HM, Sato T, Tremmel L, Bensen-Kennedy DM, Small D, Smith BD. Results from a randomized trial of salvage chemotherapy followed by lestaurtinib for patients with FLT3 mutant AML in first relapse. *Blood.* 2011; 117:3294–3301. [PubMed: 21270442]
- [37]. Zheng R, Bailey E, Nguyen B, Yang X, Piloto O, Levis M, Small D. Further activation of FLT3 mutants by FLT3 ligand. *Oncogene.* 2011; 30:4004–4014. [PubMed: 21516120]
- [38]. Konopleva MY, Jordan CT. Leukemia stem cells and microenvironment: biology and therapeutic targeting. *J. Clin. Oncol.* 2011; 29:591–599. [PubMed: 21220598]

- [39]. Benito J, Shi Y, Szymanska B, Carol H, Boehm I, Lu H, Konoplev S, Fang W, Zweidler-McKay PA, Campana D, Borthakur G, Bueso-Ramos C, Shpall E, Thomas DA, Jordan CT, Kantarjian H, Wilson WR, Lock R, Andreeff M, Konopleva M. Pronounced hypoxia in models of murine and human leukemia: high efficacy of hypoxia-activated prodrug PR-104. *PLoS One*. 2011; 6:e23108. [PubMed: 21853076]
- [40]. Azab AK, Hu J, Quang P, Azab F, Pitsillides C, Awwad R, Thompson B, Maiso P, Sun JD, Hart CP, Roccaro AM, Sacco A, Ngo HT, Lin CP, Kung AL, Carrasco RD, Vanderkerken K, Ghobrial IM. Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood*. 2012; 119:5782–94. [PubMed: 22394600]
- [41]. Lee SM, Lee CT, Kim YW, Han SK, Shim YS, Yoo CG. Hypoxia confers protection against apoptosis via PI3K/Akt and ERK pathways in lung cancer cells. *Cancer Lett*. 2006; 242:231–238. [PubMed: 16427189]
- [42]. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature*. 2006; 441:437–443. [PubMed: 16724055]
- [43]. Chen EY, Mazure NM, Cooper JA, Giaccia AJ. Hypoxia activates a platelet-derived growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway that results in glycogen synthase kinase-3 inactivation. *Cancer Res*. 2001; 61:2429–2433. [PubMed: 11289110]
- [44]. Vasseur S, Afzal S, Tardivel-Lacombe J, Park DS, Iovanna JL, Mak TW. DJ-1/PARK7 is an important mediator of hypoxia-induced cellular responses. *Proc. Natl. Acad. Sci. U S A*. 2009; 106:1111–1116. [PubMed: 19144925]
- [45]. Sun HL, Liu YN, Huang YT, Pan SL, Huang DY, Guh JH, Lee FY, Kuo SC, Teng CM. YC-1 inhibits HIF-1 expression in prostate cancer cells: contribution of Akt/NF-kappaB signaling to HIF-1alpha accumulation during hypoxia. *Oncogene*. 2007; 26:3941–3951. [PubMed: 17213816]
- [46]. Raynaud FI, Eccles SA, Patel S, Alix S, Box G, Chuckowree I, Folkes A, Gowan S, De Haven Brandon A, Di Stefano F, Hayes A, Henley AT, Lensun L, Pergl-Wilson G, Robson A, Saghir N, Zhyvoloup A, McDonald E, Sheldrake P, Shuttleworth S, Valenti M, Wan NC, Clarke PA, Workman P. Biological properties of potent inhibitors of class I phosphatidylinositide 3-kinases: from PI-103 through PI-540, PI-620 to the oral agent GDC-0941. *Mol. Cancer Ther*. 2009; 8:1725–1738. [PubMed: 19584227]
- [47]. Kojima K, Konopleva M, Tsao T, Andreeff M, Ishida H, Shiotsu Y, Jin L, Tabe Y, Nakakuma H. Selective FLT3 inhibitor FI-700 neutralizes Mcl-1 and enhances p53-mediated apoptosis in AML cells with activating mutations of FLT3 through Mcl-1/Noxa axis. *Leukemia*. 2010; 24:33–43. [PubMed: 19946262]
- [48]. Fathi AT, Arowojolu O, Swinnen I, Sato T, Rajkhowa T, Small D, Marmsater F, Robinson JE, Gross SD, Martinson M, Allen S, Kallan NC, Levis M. A potential therapeutic target for FLT3-ITD AML: PIM1 kinase. *Leuk. Res*. 2011; 27 [Epub ahead of print].
- [49]. Chen LS, Redkar S, Bearss D, Wierda WG, Gandhi V. Pim kinase inhibitor, SGI-1776, induces apoptosis in chronic lymphocytic leukemia cells. *Blood*. 2009; 114:4150–4157. [PubMed: 19734450]
- [50]. Warr MR, Shore GC. Unique biology of Mcl-1: therapeutic opportunities in cancer. *Curr. Mol. Med*. 2008; 8:138–147. [PubMed: 18336294]

Figure 1.
Chemical structures of GDC-0941 and sorafenib.

Figure 2. GDC-0941 inhibits cell growth in a dose-dependent manner in FLT3-ITD-mutant AML cells

MV4;11, MOLM13 and MOLM14 cells were cultured in the presence of different concentrations of GDC-0941 (1, 3, or 5 μM for MOLM13 and MOLM14, 0.1, 0.3, or 0.5 μM for MOLM14) in normoxic (21% O₂) or hypoxic (1% O₂) conditions for 48 h. The effects on cell growth (A) and on cytotoxicity (B) were determined by cell counts using the trypan blue exclusion method. Graphs show the mean ± SEM of results of three independent experiments. (C) Expression of phosphorylated Akt and S6K in MV4;11 and MOLM13 cells after 24 h treatment with GDC-0941 (1 μM) under normoxic and hypoxic conditions. Results are representative of three independent experiments. The averaged p-Akt / Akt and p-S6K / S6K levels after background subtraction were obtained using ImageJ software. Graphs show the mean ± SEM of results of three independent experiments. **P* < 0.05, ** *P* < 0.01.

Figure 3. Synergistic interaction between GDC-0941 and sorafenib in FLT3-ITD-mutant AML cells in the normoxic condition

MV4;11, MOLM13, and MOLM14 cells were cultured in the presence of indicated escalating doses of GDC-0941 and sorafenib using fixed ratios. After 48 h, the effects on cell growth were evaluated by viable cell counts using the trypan blue exclusion method for MV4;11 and MOLM13, and by the MTS assay for MOLM14 cells. Graphs show the mean $\pm$ SEM of results of three independent experiments.

(A)

(B)

(B)

(C)

(C)

Figure 4. Combination of GDC-0941 and sorafenib induces apoptosis and cell-cycle arrest in FLT3-ITD-mutant AML cells under normoxic and hypoxic and MSC co-culture conditions MV4;11, MOLM13 and MOLM14 cells were incubated for 48 h with GDC-0941 (0.5 μ M for MV4;11 and MOLM13, 0.1 μ M for MV4;11), 0.01 μ M sorafenib or both in normoxic or hypoxic conditions and with or without MSC co-culture. The DNA content (A), and annexin V-positive fractions (B) were measured by flow cytometry. The percent of viable cells compared to untreated cells in normoxic condition was measured by trypan blue exclusion assay (C). Graphs show the mean $\pm$ SEM of results of three independent experiments that utilized MSCs from different healthy BM donors. * P <0.05, ** P <0.01. (D) Primary AML cells bearing FLT3-ITD from eight patients were incubated for 48 h with 0.5 μ M GDC-0941, 1.0 μ M sorafenib or both in normoxic or hypoxic conditions and with or without MSC co-culture. The annexin V-positivity was measured by flow cytometry after electronic gating on CD34 $^{+}$ blasts.

Figure 5. Effects of GDC-0941 and sorafenib combination on cell survival, apoptosis, and cell-cycle related proteins in FLT3-ITD-mutant AML cells in normoxic or hypoxic and MSC co-culture conditions

Shown is protein expression in MV4;11 or MOLM13 cells that were treated for 24 h with 0.5μM GDC-0941, 0.01μM sorafenib or both. α-tubulin was used to confirm equal loading of proteins. Results are representative of three independent experiments.

Figure 6. Hypoxia and interactions with BM stromal cells result in stimulation of the prosurvival PI3K/Akt/mTOR pathway and Pim-1 kinase in AML cells with mutated FLT3-ITD
Hypoxia stimulates AKT and mTOR activation hence repressing inhibitory effects of class I PI3K inhibitor GDC-0941. BM stromal cells activates PI3K/AKT/mTOR signaling and interferes with FLT3-ITD inhibitor sorafenib-induced Pim-1 and Mcl-1 inhibition. Combined treatment with GDC-0941 and sorafenib inhibits FLT3-ITD-bearing AML cell growth through apoptosis induction and G₁ cell-cycle arrest by blocking the separate signaling pathways that coordinately increased Pim-1, Mcl-1, and represses p27^{Kip1} expression.

Table I

Clinical characteristics of AML patients

Patient number	source	WBC ($\times 10^9/l$)	% Blasts	Disease status	FLT3 mutation	Cytogenetics
1	PB	14.1	88	Relapsed/refractory	FLT3-ITD NPM-1 mutant	t(4;11;15)
2	PB	7.5	82	Relapsed/refractory	FLT3-ITD	t(2;12)
3	BM	26.1	86	Primary refractory	FLT3-ITD	–7
4	PB	32.6	99	Relapsed/refractory	FLT3-ITD	Complex (t(1;15),del(5), add(3),+8,del(16),add(19))
5	PB	36.8	94	Primary refractory	FLT3-ITD	Diploid
6	PB	7.2	99	Relapsed/refractory	FLT3-ITD	t(3;12)
7	PB	12.9	49	Relapsed/refractory	FLT3-ITD/ NPM-1 mutant	Diploid
8	PB	61.4	94	Primary refractory	FLT3-ITD	Complex t(12;19),del(6),add(6))

Table II

Effects of hypoxia, MSC co-cultures and GDC-0941/sorafenib on cell proliferation, AKT/mTOR activation and Pim-1 expression

Akt activation			mTOR activation			Pim-1 expression		
Hypoxia	MSC coculture		Hypoxia	MSC coculture		Hypoxia	MSC coculture	
	Normoxia	Hypoxia		Normoxia	Hypoxia		Normoxia	Hypoxia
↑	↑	-	↑	↑	↑	↑	-	-

		cell proliferation		Akt activation		mTOR activation		Pim-1 expression	
		Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
MSC(-)									
	GDC-0941	↓	-	↓	↓	↓	-	-	-
	sorafenib	↓	↓	-/↓	-/↓	↓	↓	-	↓
	GDC-0941/sorafenib	↓	↓	↓	↓	↓	↓	-	↓
MSC(+)									
	GDC-0941	↓	-	↓	↓	↓	-	-	-
	sorafenib	-	-	-/↓	-/↓	-	-	-	-
	GDC-0941/sorafenib	↓	↓	↓	↓	↓	↓	-	↓

-, no change ↑; upregulation ↓; downregulation -/↓; no change or downregulation (cell type dependent)