Targeting of mTORC1/2 by the mTOR kinase inhibitor PP242 induces apoptosis in AML cells under conditions mimicking the bone marrow microenvironment

Running Title: Inhibition of mTORC1/2 in AML

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Abstract

The interactions between bone marrow (BM) microenvironment and acute myeloid leukemia (AML) is known to promote survival of AML cells. In this study, we used reverse phase-protein array (RPPA) technology to measure changes in multiple proteins induced by stroma in leukemic cells. We then investigated the potential of an mTOR kinase inhibitor, PP242, to disrupt leukemia/stroma interactions, and examined the effects of PP242 in vivo using a mouse model. Using RPPA, we confirmed that multiple survival signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR), were upregulated in primary AML cells co-cultured with stroma. PP242 effectively induced apoptosis in primary samples cultured with or without stroma. Mechanistically, PP242 attenuated the activities of mTORC1 and mTORC2, sequentially inhibited phosphorylated AKT, S6K and 4EBP1, and concurrently suppressed chemokine receptor CXCR4 expression in primary leukemic cells and in stroma cells cultured alone or co-cultured with leukemic cells. In the in vivo leukemia mouse model, PP242 inhibited mTOR signaling in leukemic cells and demonstrated a greater anti-leukemia effect than rapamycin. Our findings indicate that disrupting mTOR/AKT signaling with a selective mTOR kinase inhibitor can effectively target leukemic cells within the BM microenvironment.
Introduction

Functional interplay between acute myeloid leukemia (AML) cells and the bone marrow (BM) microenvironment is a distinct feature of this hematological malignancy. Several studies have provided evidence suggesting that proliferation, survival, and drug resistance of AML can be modulated by mesenchymal stem cells (MSCs) within the BM microenvironment\textsuperscript{1-4}. Direct contact between AML cells and BM-derived MSCs triggers a pleiotropic spectrum of proliferative and/or antiapoptotic signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR)\textsuperscript{5,6} pathway (PI3K/AKT/mTOR), which attenuates the response of AML to conventional chemotherapy. Thus, in addition to therapies that directly target AML, interruption of leukemia cell-MSC interactions should be considered when designing anti-AML therapeutic strategies.

mTOR is a critical component of PI3K/AKT signaling, forming two complexes—mTORC1 and mTORC2—that are defined by their molecular composition and substrate specificity. mTORC1 includes mTOR and raptor\textsuperscript{7}, whose downstream targets are the eukaryotic translation initiation factor 4E-binding proteins (4EBPs) and S6 kinases (S6K1 and S6K2). 4EBP1 phosphorylation by mTORC1 releases 4EBP1 from eukaryotic translation initiation factor 4E (eIF4E), allowing eIF4E to form the eIF4F complex that promotes cap-dependent mRNA translation. Phosphorylation of S6Ks by mTORC1 is an activating event that potentiates S6K-dependent phosphorylation of ribosomal S6 protein and other substrates that coordinate aspects of protein and lipid biosynthesis while opposing autophagy\textsuperscript{8}. In comparison, mTORC2 contains mTOR and rictor\textsuperscript{9}. It phosphorylates AKT at Ser473 and members of the AGC protein kinase family at hydrophobic motifs. These include protein kinase C isoforms and members of the glucocorticoid-induced kinase family\textsuperscript{10}. 
Rapamycin and its derivatives (RAD001 and CCI-779) are first generation mTOR inhibitors which showed only modest efficacy in anti-tumor clinical trials\textsuperscript{11}. These compounds affect mTORC1 more than mTORC2, especially in the initial phase of treatment, resulting in increased AKT phosphorylation through blocking negative feedback loops that limit upstream signaling by PI3K\textsuperscript{11,12}. In addition, these agents do not completely inhibit mTORC1 activity and have little effect on phosphorylation of 4EBP1 at key threonine residues (Thr37/46), resulting in weak attenuation of cap-dependent translation and little effect on overall protein synthesis\textsuperscript{13}.

PP242 is a new small-molecule protein kinase inhibitor that targets the ATP-binding site of mTOR, resulting in greater inhibition of mTORC1 and mTORC2 activity than that produced by the mTOR inhibitors discussed above\textsuperscript{14}. Compared to the other PI3K/mTOR inhibitors, such as PI-103, PP242 is more selective for leukemic cells, as evidenced by its ability to suppress PI3K/AKT/mTOR signaling in Ph\textsuperscript{+} B-cell acute lymphoblastic leukemia\textsuperscript{15,16} and T-cell lymphoma cells\textsuperscript{17}, and prolongation the survival of mice harboring these leukemias.

Microenvironment-mediated chemoresistance of AML prompted us to investigate signaling pathways activated in leukemic cells upon contact with stromal cells, and to study the anti-leukemia potency of mTOR kinase inhibitors under conditions mimicking the BM microenvironment. In this study, we report that stroma activates multiple antiapoptotic signaling through several protein-protein interactions that correlate with stroma-mediated survival in AML cells. We further show that the PP242 effectively inhibits the activity of mTORC1 and mTORC2 and their downstream targets in primary AML cells, inducing apoptosis in both primary AML blasts and CD34\textsuperscript{+} progenitor cells. Importantly, PP242 disrupts the stroma-leukemia interaction, antagonizing stroma-mediated survival by suppressing expression of CXC chemokine receptor type 4 (CXCR4) and downregulating mTOR signaling, both in primary AML cells and in
stromal cells. Furthermore, PP242 suppressed leukemia progression in a murine leukemia model driven by mutated FLT3 with constitutive activation of mTOR. Taken together, our findings indicate that the PP242 or its analogues are potentially useful therapeutic agents in AML, affecting leukemic cells within the protective BM microenvironment.

Materials and Methods

Cells and culture conditions

Peripheral blood and bone marrow samples were obtained from patients with AML or ALL after informed consent was obtained in accordance with The University of Texas MD Anderson Cancer Center Institutional Review Board regulations. Mononuclear cells were separated using Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich). Primary cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. Mesenchymal stem cells (MSCs) obtained from healthy BM donors or from patients with leukemia were cultured at a density of 5,000 to 6,000 cells/cm² in minimum essential medium (MEM) alpha supplemented with 20% FBS, 1% L-glutamine, and 1% penicillin-streptomycin as described elsewhere. 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.

Passage 3 or 4 MSCs were used for co-culture experiments. The murine stromal cell line MS-5 was provided by Dr. Itoh from Niigata University (Japan) and maintained in the 10% FBS-containing MEMα.
Cell viability

Cell viability was measured using flow cytometry with CountBright absolute counting beads (Invitrogen). The percentage of apoptosis was estimated by measuring phosphatidylserine externalization in the cells using annexin V flow cytometry (Roche). Apoptosis of bulk leukemic and leukemic progenitor cells was measured using annexin V+ after electronic gating on CD45+ leukemic blasts (CD45-APC; BD Pharmingen) or CD34+ (CD34-APC; BD Pharmingen) AML progenitor cells. The extent of apoptosis was quantified as percentage of annexin V–positive cells, and the extent of drug-specific apoptosis was assessed by the formula: % specific apoptosis = (test – control) x 100 x / (100 – control). “Control” represent the percentage of annexin V positivity in the group of cells without treatment (spontaneous apoptosis), and “Test” represents the percentage of annexin V positivity in the group of cells treated with PP242.

Flow cytometry for detection of the expression level of proteins and intracellular phosphoproteins

Murine whole blood (100 μL), harvested primary AML cells, and harvested AML MSCs were fixed with formaldehyde at a final concentration of 4% for 10 minutes at room temperature, permeabilized with 0.1% Triton X-100 at 37°C for 15 minutes, and then centrifuged at 1000g (Beckman Coulter) in cold 50% methanol for 3 minutes. The pellets were washed twice in ice-cold phosphate-buffered saline (PBS). Cells were then stained for cell surface markers and intracellular signaling markers at room temperature for 30 minutes and washed once with PBS before analysis using a Gallios flow cytometer (Beckman Coulter). The flow cytometric profiles were analyzed using the Kaluza (Beckman Coulter) or FlowJo (version 7.6.1) software programs. The antibodies against cell surface markers CD34-Cy5 and CD90-APC-Alexa750 were purchased from Beckman Coulter. The antibodies against CXCR4- phycoerythrin (PE; 12G5),
CXCR4-APC (12G5), CXC4-PE (1D9), VLA4(CD49d)-APC, CD44-APC were purchased from BD biosciences. The antibodies against intracellular signaling markers phosphorylated 4EBP1 (p4EPB1) (Thr37/43)-Alexa488, phosphorylated AKT (pAKT) (Ser473)-Alexa684, and phosphorylated S6K (pS6K) (Ser235/236)-Alexa684 were purchased from Cell Signaling Technology. Rapamycin derivative CCI-779 (temsirolimus) was purchased from LC laboratories (Woburn, MA).

The mean fluorescent intensities (MFI) of protein markers were measured by flow cytometry. The MFI ratio of a protein marker in a specific sample is calculated as MFI of protein marker/ MFI of unstained cells or isotype stained cells. Statistical analysis of changes in MFI ratios of proteins in response to different treatment or culture settings were assessed using Pearson’s correlation coefficient (ρ). The significance of the correlation was determined using Fisher’s z-transform with a false discovery rate (FDR) cutoff of 0.05.

**Leukemia mouse model**

All animal experiments were performed in accordance with protocols approved by the MD Anderson Institutional Animal Care and Use Committee. To evaluate the inhibitory effect of PP242 on mTOR in vivo, a mouse model of leukemia was generated using severe combined immunodeficient (SCID) mice. The mice were irradiated at 6 Gy and 6 hours later injected intravenously with Baf/ITD/luc/GFP cells (kindly provided by Dr. D. Small, John Hopkins)\(^{21,22}\) at a concentration of \(0.2 \times 10^6\) cells/mouse. To compare the effects of PP242 and rapamycin (Sigma-Aldrich), one group of mice (nine mice per group) was injected with rapamycin intraperitoneally at a dose of 0.5 mg/kg every other day. Mice in the other group were given PP242 (60 mg/kg, via gavage, every other day) was initiated on day 3 after the tumor cells injection. Tumor-cell engraftment and progression were then monitored using noninvasive
imaging as described previously, and flow cytometry to detect the GFP+ leukemia cells in murine peripheral blood samples. To assess the inhibitory effect of PP242 on mTOR signaling in GFP+ cells, murine peripheral blood was collected before and 2 hours after PP242 administration. Effects on mTOR signaling components p4EBP1(Thr37/43), pAKT (Ser473), and pS6K (Ser243/244) were analyzed using flow cytometry. Anti-tumor effects were compared using bioluminescent imaging.

**Western blot analysis**

For Western blot analysis of the inhibitory effects of PP242 on mTOR, primary AML samples were lysed in a phosphoprotein lysis buffer (150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1% Triton X-100, 10 mM iodoacetamide, 1 mM Na3VO4, 0.1% NaN3, 3 mM phenylmethylsulfonyl fluoride). The buffer was supplemented with a protease inhibitor cocktail (Roche). Lysates were then separated on a 10% or 12% polyacrylamide gel, transferred to Hybond-P membranes (Amersham Pharmacia Biotech), probed with the appropriate antibodies, and visualized using an ECL plus kit (Amersham Pharmacia Biotech). Western blots were analyzed using a Storm 860 phosphorimager with the ImageQuant software program (Molecular Dynamics). Antibodies against human pAKT(Ser473), pAKT(Thr308), phosphorylated FoxO1a(Thr24)/FoxO3a (Thr32) (pFoxO1a(Thr24)/FoxO3a(Thr32)), FoxO3a, pS6K(Ser240/244), phosphorylated PRAS40 (pPRAS40(Ser246)), p4EBP1(Thr37/46), AKT, S6K, and 4EBP1 were purchased from Cell Signaling Technology.
Reverse-phase protein array

Two AML cell lines (OCI-AML3 and U937) and 20 blood samples obtained from patients with primary AML(19) or ALL(1) with more than 50% blasts were cultured alone or co-cultured with mouse stromal MS-5 cells for 24 hours. Cells were lysed in a protein lysis buffer (2×; 0.5 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 4% β-mercaptoethanol), then heated at 95°C for 5 minutes and stored at -80°C. To verify lack of significant contamination in collected leukemic cells, anti-human CD45-APC and CD34-APC were measured by flow cytometry to discriminate between human leukemic cells and mouse stroma cells (MS5).

Proteomic profiling of the samples was performed using reverse-phase protein arrays (RPPA). The resulting comprehensive profiles included measures of 51 proteins (Supplementary table 1) representing the level of activation and expression of proteins in signal transduction pathways and apoptosis networks in response to different culture conditions. The methods for using and validating RPPAs were fully described previously24. Briefly, protein lysates obtained from primary samples were printed onto slides, together with normalization and expression controls, in five serial dilutions. The slides were probed with strictly validated primary antibodies against total or phosphorylated protein (as described in the supplementary section) and a secondary antibody was used to amplify the signal. The stained slides were scanned using a scanner and the images were analyzed using the MicroVigene software program (VigeneTech, Carlisle, MA) to produce quantified data.

The “Supercurve” algorithm25 was used to estimate the sample-specific protein expression levels. The RPPA data were normalized by median centering the results for each sample across all antibodies. The expression levels of proteins were examined under two different culture settings (with or without MS5), and the effect of culture setting changes in each
protein was estimated using ANOVA. To account for multiple testing, we fit the distribution of p-values (raw p-values) for the contrast tests with a beta-uniform mixture (BUM) model\textsuperscript{26} and checked whether any proteins showed significant changes at an FDR of 0.05. For each protein, we also compared the number of samples showing detectable upregulation to the number of samples showing detectable downregulation using binomial tests (p $\leq$ 0.05), and then calculated significance using an FDR with a cutoff of 0.05. FDR was defined in the flow cytometry section above.

**Quantitative real-time PCR**

Total RNA was extracted using Trizol (Invitrogen, Grand Island, NY) as directed by the manufacturer; cDNA was then prepared using SuperScript III primed by random hexamers as described by Andreeff \textit{et al.} (2008).\textsuperscript{27} The level of expression of CXCR4 was assessed by real time PCR using the following TaqMan gene expression assays in triplicate as directed by the manufacturer (Applied Biosystems, Carlsbad, CA): CXCR4 (Hs00607978_s1) and ABL1 (Hs00245445_m1). The abundance of CXCR4 transcript relative to that of ABL1 was determined by the DDCt method with RQ Manager version 1.2 software (Applied Biosystems).

**Leukemia/MSC/MS-5 co-culture**

Primary leukemic cells were co-cultured with either primary MSCs or MS-5 cells at a ratio of 100:1. In the direct cell-to-cell contact co-culture setting, MS-5 cells or primary MSCs were plated in either six-well plates or 25-cm\textsuperscript{2} culture flasks in 10% FBS-containing MEM\textsubscript{α} for 4 hours. Primary leukemic cells were then added on top of the stromal cells for 4 hours, after which cells were treated with PP242 for the indicated time interval. Non-attached leukemic cells were then harvested by collecting the floating cells and some of the detached cells after washing.
twice with 1× PBS. The viability of the collected cells was estimated, and the cells were lysed for Western blot analysis. In the transwell-based co-culture setting, a transwell membrane with 0.4-μM pore diameter (Costar; Corning) was used to prevent suspended cells from direct cellular contact with MSCs. Primary AML MSCs were first seeded at a concentration of $3.5 \times 10^4 /0.5\text{ml}$ on the backside of the transwell membrane for 4 hours. After attachment, the transwells were inverted, and the primary AML cells were seeded on top of the transwells. These transwells harboring the cells on both sides of the insert membrane were placed in 1 mL of 10% FBS-containing MEMA for 4 hours before initiating treatment. At the indicated time points, AML cells were harvested and lysed for Western blot analysis.

**Results**

**Stroma upregulates multiple survival signaling in primary leukemic cells**

To investigate stroma-mediated signaling pathways in AML cells, we performed RPPA profiling of 51 proteins along with their phosphorylated forms in 19 primary AML, one ALL and two AML cell lines cultured alone or co-cultured with murine stromal MS-5 cells commonly used for support of human hematopoietic stem cells. Co-culture with MS-5 stromal cells significantly diminished spontaneous apoptosis of leukemic cells in 10 of 12 patient samples (AML cells alone, 33.7% ± 3.8% annexin+ cells; AML+MS-5, 19.6 % ± 3.1% annexin+ cells; $P = .006$) (Supplementary Figure 1), consistent with our published data. We statistically compared the expression levels (average density) for each protein in 20 primary samples and 2 AML cell lines under two culture conditions (samples cultured alone versus samples co-cultured with MS-5 cells); and the frequency of changes in protein expression in response to the co-culture with stroma. Stroma upregulated p4EBP1 (Thr37/46), p4EBP1(Thr70), pmTOR(Ser2448),
pS6K(Ser235/236) and pS6K (Ser240/244) in majority of the samples, and significantly modified the phosphorylation of four proteins in leukemic cells in the co-culture setting (Table 1). Specifically, it upregulated pAKT (Thr308), phosphorylated extracellular signal-regulated pERK (Thr202/204), and pSTAT3 (Thr727), and downregulated pβ-catenin (Ser33/37/Thr41) expression.

Furthermore, six proteins were altered in the majority of the samples when co-cultured with stroma. Co-culture with stroma upregulated pAKT (Thr308) in 18 of the 22 samples, mTOR in 17 samples, pERK (Thr202/204) in 14 samples, and pSTAT3 (Ser727) in 12 samples and decreased the expression of pβ-catenin (Ser33/37/Thr41) in 15 samples, and of mTORC1 substrate PRAS40 in 13 samples (Table 1). These findings indicate that stroma triggers activation of multiple parallel pro-survival signaling pathways in leukemic cells.

**PP242 overcomes protection by stroma and induces apoptosis in primary AML cells**

Since activation of pAKT (Thr308) and upregulation of mTOR were seen in primary AML cells co-cultured with stroma, we wanted to assess the anti-leukemic efficacy of PP242 under stromal co-culture conditions. To this end, we cultured cells from 9 AML samples (Supplementary Table 2) alone or co-cultured with MS-5 cells, in the presence or absence of the indicated concentrations of PP242 for 48 hours. PP242 induced apoptosis in a dose-dependent manner in primary AML cells cultured alone or co-cultured with stroma, as determined by Annexin V flow cytometry (Figure 1A). In addition, PP242 induced apoptosis in CD34+ AML progenitor cells cultured under these conditions (Supplementary Figure 2), although stroma exerted some protective effect in sample#2. We have previously reported that rapamycin derivatives upon prolonged exposure suppress both mTORC1 and mTORC2 signaling in AML. In direct
comparison, PP242 has more potent inducer of apoptosis than rapamycin dirative temsirolimus in OCI-AML3 cells and in primary AML samples tested, in particular, under conditions of stromal co-cultures (Figure 1B). Taken together, these results indicate that blockade of mTOR activity with PP242 induces apoptosis in leukemic cells, and overcomes protective signals of stromal cells. Importantly, the significant apoptosis was observed even at lower concentration of PP242 in the range from 0.1 μM to 0.6 μM, suggesting that PP242 is highly selective for mTOR signaling in the cells.

We next examined effects of PP242 on intracellular signaling of AML cells cultured under conditions mimicking the BM microenvironment, i.e. in MS-5 co-cultures. Consistent with our RPPA findings, stroma stimulated PI3K/mTOR/AKT signaling by significantly upregulating phosphorylation of AKT (Ser473), S6K (Ser240/244), PRAS40 (Thr246), and 4EBP1 (Thr37/46) in AML cells. PP242 exerted an inhibitory effect on mTORC1 and mTORC2 and partially abrogated stroma-induced activation of their direct downstream substrates (Figure 1B).

**PP242 impairs interactions between primary AML cells and AML MSCs through inhibition of mTOR signaling in both cell types**

Several studies have demonstrated that leukemia cells reside in close contact with stromal cells in BM microenvironments and that direct cell-cell contact plays an important role in the activation of intracellular signaling. One of the challenges in studying leukemia-stroma interactions is identifying and separating the different cell types in co-culture. To avoid physical cell separation while assessing the rapid signaling events upon direct leukemia-stroma cell-cell contact, we utilized phospho-specific multiparameter flow cytometry. To this end, we isolated primary BM MSC from 11 AML samples and one normal healthy donor. Using a co-culture
system of primary AML (n=16, Supplementary Table 2) with allogenic leukemia or normal BM-derived MSC, we collected (1) floating AML cells that did not attach to the stromal cells; and (2) AML cells that were tightly attached to the stromal cells (by trypsinization). We then fixed and permeabilized cells prior to staining with the cell surface markers CD90, CD34 and intracellular signaling markers pAKT (Ser473), pS6K (Ser235/236), and p4EBP1 (Thr37/46). We discriminated between leukemic cells and MSC by electronic gating on CD90+ cells for leukemia-derived MSC and on CD34+ cells for AML progenitors (Figure 2A-C).

Using this technology, we investigated the effects of PP242 on intracellular signaling in both primary AML cells and MSC, cultured alone or co-cultured (Figures 3A-D, Supplementary Fig 3). PP242 significantly inhibited p4EBP1 (Thr37/46) expression in both bulk leukemic cells and CD34+ AML progenitor cells in all 16 samples cultured in medium (P = .01, P = .01, respectively, Fig. 3A “alone”). It also downregulated pAKT (Ser473) expression in 12 of 16 samples (P = .01), consistent with inhibition of mTORC2 complex (Fig. 3C “alone”). Co-culture with stroma significantly increased the phosphorylation of 4EBP1 (Thr 37/46) in all 16 floating AML cells (P = .03; n = 16) (Fig. 3A dashed line), and activated pS6K (Ser235/236) in majority of AML (P = .03; n = 15, dashed line) and CD34+ progenitor cells attached to stroma in 14 of 16 samples (P = .04; n = 14) (Fig. 3B dashed line). Treatment with PP242 effectively suppressed the phosphorylation of 4EBP1 (Thr37/46) (P < .001; n = 16), AKT (Ser473) (P = .02; n = 12), and S6K (Ser235/236) (P = .05; n = 15) in floating leukemia cells, and downregulated the phosphorylation of AKT (Ser473) in majority of floating CD34+ AML progenitor cells (P = .05, n = 12). Importantly, PP242 exerted its inhibitory effect on p4EBP1 (Thr37/46) and pAKT (Ser473) in both attached AML cells (P = .03, n = 16; P = .01, n = 12, respectively) and most of attached CD34+ AML progenitor cells (P = .02; n = 16; P = .02; n = 12, respectively) and
abrogated stroma-mediated upregulation of pS6K (Ser235/236) in 14 of 16 attached CD34+ AML progenitor cells ($P = .05, n = 14$). In BM-derived MSC, PP242 significantly downregulated p4EBP1 (Thr37/46) ($P = .002; n = 15$) and pS6K (Ser235/236) ($P = .002; n = 15$) in most of MSCs cultured alone and potently inactivated both proteins in most of attached MSCs in the co-culture setting ($P = .004; n = 15$ [p4EBP1 (Thr37/46)]; $P = .007; n = 15$ [pS6K (Ser235/236)]) (Figure 3D). Unlike in primary AML cells, PP242 modestly increased mTORC2-mediated phosphorylation of AKT in the majority of MSCs cultured alone or co-cultured ($P = 0.88$, data not shown), indicating that PP242 affects mTORC2 sensitivity in a cell context-dependent manner.

To validate the findings obtained by flow cytometry, we analyzed expression and phosphorylation of proteins by immunoblotting. To eliminate cross-contamination of the primary AML cells and MSCs in the co-culture setting, we co-cultured cells from primary AML cells and leukemia-derived MSCs on each side of the Transwell membrane, as described under Materials and Methods. The Transwell membrane pores prevent migration of leukemia cells through the membrane, and yet allow direct contact of AML/MSC cell surface receptors. In agreement with flow cytometry data, Western blot analysis showed that PP242 completely suppressed mTORC1 direct targets p4EBP1 (Th37/Thr46) and pS6K (Ser240/244) and reduced mTORC2/AKT activity as evidenced by decreased phosphorylation of FoxO1a(Thr24)FoxO3a(Thr32) in AML cells cultured alone (Figure 3E). Co-culture of primary AML cells and MSCs increased the activity of mTORC1 and mTORC2 in primary AML cells, and this was partially abrogated by PP242. Taken together, these results indicate that treatment with PP242 effectively interferes with the function of both mTORC1 and mTORC2 in primary AML cells, AML progenitor cells, and primary AML MSCs; and partially antagonized MSC-mediated activation of
PI3K/mTOR/AKT signaling in primary AML cells and AML progenitor cells in the co-culture setting.

**PP242 interrupts leukemia-stroma interactions by suppressing surface expression of CXCR4 and simultaneously inhibiting intracellular mTOR signaling in primary AML cells and MSCs**

Our findings of PP242-mediated inhibition of mTOR signaling in AML co-cultured with stroma prompted us to examine the direct effects of PP242 on adhesion of AML cells to MSC. To this end, we counted primary AML cells directly attached to MSC. As shown in a representative example (Figure 4A, left), in the control co-culture of primary AML cells and MSCs, 5,746 of 51,966 CD34+ AML cells (11%) were attached to the AML MSCs, whereas only 1,718 of 31,643 (5.4%) were attached in the sample treated with 2.5µM PP242 for 72 hours. We further confirmed this finding using light microscopy (Figure 4A, right). Overall, treatment with PP242 reduced the proportion of CD34+ AML progenitor cells attached to AML MSCs in 12 of 16 patients (Student t-test $P = .0006$) (Figure 4B), and in 8 of these the % decrease in the fraction of attached cells exceeded the % decrease in the total cell number (not shown). These results demonstrated that PP242 effectively disrupted the interactions between leukemia and stroma by reducing the number of leukemic blasts attached to the MSC.

CXCR4, a G-protein–coupled chemokine receptor, plays an important role in leukemia-stroma interactions through regulation of migration and adhesion$^{5,23,30}$. We therefore investigated whether the observed decrease in the proportion of attached cells is mediated by inhibition of CXCR4. Notably, expression of CXCR4 increased significantly in both primary AML cells and CD34+ progenitor cells that attached to stroma in samples from 8 of 9 patients examined ($P =$
.01 and .02, respectively) (Figures 5Aa). In floating cells, the increase was observed only in 4 of the 9 samples (P = 0.46, data not shown). In MSC, CXCR4 levels were overall higher than in AML cells (Figure 5Ab) and were induced by co-cultures only in 3 samples (not shown). In attached cells, the increased expression of CXCR4 was significantly associated with the increased activity of mTORC1, mTORC2 or both as indicated in Table 2. Treatment with PP242 reduced the expression of CXCR4 in the majority of AMLs, AML CD34+ cells and MSCs, cultured alone or co-cultured (Fig. 5Ab and Supplementary Fig. 4). Downregulation of CXCR4 by PP242 significantly correlated with suppression of the intracellular proteins, p4EBP1 (Thr37/46) in attached primary AML cells, AML CD34+ cells and primary MSCs; and inhibition of pS6K (Ser235/236) and pAKT (Ser473) in attached primary MSCs (Supplementary Figure 5 A-C). The circos diagram (Figure 5B) further illustrates the correlations and the degree of inhibition in co-cultured AML CD34+ cells and in their associated MSCs. We observed similar correlations of altered expression of CXCR4 and changes in intracellular levels of p4EBP1 (Thr37/43) and pAKT(Ser473) in AML cells cultured alone (Supplementary Figure 5D); and of p4EBP1 (Thr37/43), pS6K (Ser235/236) and pAKT(Ser473) in AML MSC cultured alone (Figure 5E). These data suggest that PP242 disrupts leukemia-stromal interactions by reducing expression of CXCR4 in both cell types, cultured alone or co-cultured. To assure that the observed changes in CXCR4 are not secondary to PP242-induced cell death, we evaluated CXCR4 expression in attached live CD34+ primary AML cells, and confirmed decrease of CXCR4 levels upon 48-hr PP242 exposure (Supplementary Figure 6A-B). We further examined the surface expression of CXCR4 and other adhesion molecules, CD44 and VLA4 in OCI-AML3 cells at short time intervals of PP242 treatment, preceding alterations in cell viability. CXCR4 cell surface expression detected by antibodies against two different isotopes (12G5 and 1D9,
please refer to Figure legend) diminished after only 2 hrs of PP242 treatment, with further decrease after 4 hr and 8 hr exposure (Figure 5C). On the contrary, PP242 minimally affected VLA4 and CD44 expression, suggesting specificity of changes in CXCR4 levels. However, PP242 at 30 and 60 min had no effect on CXCR4 expression, while it inhibited both mTORC1/C2 functions via inactivating the phosphorylation of S6K by 70%, and of AKT (Ser 473) by 50% (Supplementary Figure 6C). To evaluate the possibility of PP242 affecting internalization of surface CXCR4, we measured CXCR4 levels using 12G5 and 1D9 antibodies in live (surface) vs. fixed/permeabilized (intracellular) cells and found comparable decrease, arguing for changes in total protein levels rather than redistribution phenomenon (Supplementary Figure 7A, B). In turn, PP242 downregulated CXCR4 mRNA levels measured by quantitative RT-PCR in three AML cell lines as early as 6 hrs, with persistent downregulation at 24hrs, and in primary AML samples at 6 hrs (sample#2) or 24 hrs (samples#1-3). (Supplementary Figure 8A, B). Twenty-four hour exposure to temsirolimus did not affect cell viability and caused modest decrease of cell surface and intracellular CXCR4 levels (Supplementary Figure 7A, B). Taken together, these results indicate that PP242 selectively decreases CXCR4 expression following blockade of the intracellular mTOR signaling. Importantly, this effect is seen in primary AML cells and MSCs, cultured alone or co-cultured.

**PP242 reduces leukemia burden and extends survival in a mouse model of leukemia**

We further evaluated the anti-leukemic potential of PP242 in vivo using a mouse model of leukemia generated by Baf3/ITD-GFP/luc in NOD/Scid mice\textsuperscript{21,22,31}. We monitored leukemia burden in murine peripheral blood using flow cytometry gated on GFP+ circulating cells. As shown in Figure 6A, 2 hours after PP242 administration, PP242 inactivated the mTORC1 downstream targets p4EBP1 (Thr37/46) and pS6K (Ser235/236) and mTORC2 direct target
pAKT (Ser473) in circulating FLT3-ITD-GFP leukemic cells. PP242 at 60 mg/kg reduced leukemia burden as measured by bioluminescence imaging (Figure 6B a-b). The anti-leukemia effect of PP242 was greater than that of rapamycin used at 0.5 mg/kg, the tolerable dose that was previously shown to inhibit mTOR signaling.

Discussion

The interaction of AML cells with stroma within the BM microenvironment attenuates the response of AML cells to conventional chemotherapeutic agents and promotes chemoresistance. Stromal cells can trigger activation of multiple signaling pathways in leukemic cells through secreted growth factors/cytokines and by means of direct cell-cell interactions, such as ligation of integrins and other cellular receptors. In this study, using RPPA, we showed the complex, multidimensional stroma-mediated survival signals conveyed in leukemia, including signals affecting the PI3K/mTOR/AKT pathway. Our results support the notion that AML-stroma interaction is an important factor that must be considered when designing investigational anti-AML therapeutics. Furthermore, our results emphasize the utility of the selective mTOR kinase inhibitors as potential therapeutic adjuncts in AML therapy, particularly under conditions mimicking the BM microenvironment.

PP242, an ATP-competitive inhibitor of mTOR kinase, is one of the new generation of mTOR inhibitors. Compared with rapamycin, PP242 more efficiently inhibits mTORC1 as evidenced by diminished 4EBP1 phosphorylation and cap-dependent protein translation. Furthermore, PP242 is more effective in preventing the occurrence of negative feedback loops by enhancing its inhibitory effect on mTORC2. Consequently, PP242 is a more potent inhibitor of protein synthesis, metabolism, and proliferation of cancer cells. In this study, PP242 exhibited
significant antileukemic effects in vitro by inducing dose-dependent apoptosis of both, bulk primary AML cells and AML progenitor cells. Most importantly, PP242, but not rapamycin derivative temsirolimus, induced cell death in stromal co-cultures, which otherwise reliably support viability of leukemic blasts (Supplementary Fig. 1) and reduce efficacy of traditional anti-AML chemotherapy agents. These data support the recently reported evidence of the superior anti-leukemic potency of mTOR kinase inhibitors or dual PI3K/mTOR kinase inhibitors through suppression of rapamycin-resistant mTORC1 and mTORC2 complexes compared with allosteric mTOR inhibitors. These findings indicate that mTOR signaling plays an essential role in the stroma-leukemia cell interaction, and its blockade is sufficient to abrogate the stroma-mediated survival advantage of leukemic cells.

Consistent with findings in other cancer cell types, PP242 potently inhibited phosphorylation of 4EBP1 (Thr37/46) and S6K (Ser235/236), two direct targets of mTORC1, in AML cells. Importantly, this inhibition is preserved in AML/stromal cells in direct physical contact. These data demonstrate for the first time that PP242 can disrupt AML-stroma interactions by directly targeting not only the intrinsically activated mTOR signaling in AML cells but also additional stroma-induced activation of this pathway. We have further observed suppression of the mTORC2 target pAKT (Ser473) in bulk AML cells, AML progenitor cells and floating and attached AML cells in the co-culture setting. Inhibition of mTORC2 was further supported by inactivation of FoxO1a/FoxO3a, direct targets of AKT, in co-cultured AML cells after treatment with PP242. Importantly, PP242 also suppressed mTOR activity in stromal cells of supporting microenvironment (MSC). However, PP242 modestly activated pAKT (Ser473) in AML MSCs under both culture-alone and co-culture conditions. Recent findings by Rosen et al. demonstrated that mTOR kinase inhibitors may cause activation of PI3K and rephosphorylation
of AKT T308 through unleashed receptor tyrosine kinase signaling, at least in breast cancer cells with constitutive activation of HER2/EGFR\textsuperscript{39}. Although this mechanism is unlikely operational in untransformed bone marrow stromal cells, these observations argue for potential advantage of combined PI3K/mTOR blockade to fully inhibit leukemia/stroma interactions. Further studies are required to compare mTOR kinase inhibitors and dual PI3K/mTOR inhibitors in the co-culture setting.

In the present study, mTOR blockade with PP242 decreased the number of AML progenitor cells attached to MSCs in the co-culture setting. This prompted us to examine the effect of PP242 on chemokine receptor CXCR4, which plays a key role in chemotaxis and adhesion of hematopoietic cells to cells of their microenvironment. We previously reported that CXCR4 inhibition interferes with stromal/leukemia cell interactions and in part overcomes stroma-mediated resistance to chemotherapy and FLT3 inhibitors\textsuperscript{23,30}. While stroma co-culture promoted CXCR4 expression in AML cells, PP242 decreased CXCR4 levels in the majority of primary AML samples and in MSCs, cultured alone and co-cultured. PP242-altered surface expression of CXCR4 occurred within hours subsequent to the blockade of intracellular mTOR signaling. Overall, we identified a novel function of PP242 in that it abrogates, at least partially, AML cell-MSC interactions by targeting mechanisms by which AML cells communicate with cells in their microenvironment. Of note, recent ribosome profiling efforts have identified pro-invasion mRNA networks translationally controlled by mTOR that were effectively blocked by mTOR ATP site inhibitors\textsuperscript{40}. While further studies are warranted to dissect the mechanisms responsible for CXCR4 downregulation in our system, our preliminary data indicate effect of PP242 on mRNA transcription (Supplementary Figure 8). Notably, prolonged exposure to allosteric mTOR inhibitor temsirolimus also resulted in downregulation of CXCR4 levels; albeit
at a lesser extent compared with PP242. These results would suggest that ATP kinase mTOR inhibitors may have far superior anti-leukemic effects by virtue of their ability to fully inhibit rapamycin-insensitive TORC1/TORC2 complexes and target leukemia/stroma interactions, similar to recent findings with INK128 in metastatic prostate cancer models\textsuperscript{40}. It is plausible to suspect that PP242 may also target other chemokines, cytokines, and growth factors that mediate the interaction between AML and stroma, and identification of these mediators require further study. We have further examined anti-leukemia effects of PP242 in a FLT3-driven mouse model of leukemia. This in vivo model was selected based on our published data indicating the prominent role of BM microenvironment in survival of FLT3-mutated AML cells, mediated in part through CXCR4 signaling\textsuperscript{23}. In this model, PP242 suppressed mTOR signaling in mouse leukemia cells and reduced leukemia burden without evidence of toxicity, confirming therapeutic efficacy of this agent.

In summary, we have identified several signaling pathways activated by stroma in AML cells that promote leukemia cell survival within its protective microenvironment. Our studies support the key importance of the PI3K/AKT/mTOR pathway in microenvironment-mediated resistance, justifying the use of the selective inhibitors of this pathway as adjunct agents that target leukemia-stroma interactions. We further showed that PP242 has antileukemia activity in vivo, providing a compelling rationale for testing this inhibitor in clinical trials.

Acknowledgments

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Institutes of Health through MD Anderson's Cancer Center Support Grant CA016672.

**Author Contributions**

Zhihong Zeng: designed, performed the experiments, wrote the paper

Yue Xi Shi: performed the in vivo study

Twee Tsao: performed RPPA study

YiHua Qiu: performed RPPA study

Steven M. Kornblau: supervised RPPA study, wrote the paper

Keith A. Baggerl: supervised and performed statistical analysis; wrote the paper

Wenbin Liu: performed statistical analysis

Katti Jessen: provided basic information on the compound

Yi Liu: provided basic information on the compound, designed the experiments and wrote the paper

Hagop Kantarjian: supervised data analysis and wrote the paper

Christian Rommel: provided basic information on the compound, designed the experiments and wrote the paper

David A. Fruman: designed the experiments, supervised data analysis, wrote the paper

Michael Andreeff: supervised data analysis, wrote the paper

Marina Konopleva: designed the experiments, supervised data analysis, wrote the paper
Disclosures of Conflict of Interest

K.J., Y.L., and C.R. are employed by Intellikine.

Reference List


(7) Kim DH, Sarbassov DD, Ali SM et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 2002;110(2):163-175.


Table 1. Comparison of the average protein-density changes and the frequency of protein-expression changes in leukemic samples cultured alone or co-cultured with MS-5 cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average density change</th>
<th>$P$</th>
<th>↑ Expression (n)</th>
<th>↓ Expression (n)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAKT (Thr308)</td>
<td>+0.880</td>
<td>0.001</td>
<td>18</td>
<td>2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pβ-Catenin (Ser33/37/Thr41)</td>
<td>-0.830</td>
<td>0.002</td>
<td>3</td>
<td>15</td>
<td>.035</td>
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<tr>
<td>pERK (Ser42/44)</td>
<td>+0.660</td>
<td>0.002</td>
<td>14</td>
<td>3</td>
<td>.039</td>
</tr>
<tr>
<td>mTOR NS</td>
<td>NS</td>
<td>NS</td>
<td>17</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PRAS40 NS</td>
<td>NS</td>
<td>NS</td>
<td>0</td>
<td>13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pSTAT3 (Ser727)</td>
<td>+0.298</td>
<td>0.013</td>
<td>12</td>
<td>2</td>
<td>.039</td>
</tr>
</tbody>
</table>

First column: proteins with significant density changes and/ or frequent alteration in response to co-culture. NS, not significant.

Second column: changes in the average protein density in all samples (see Materials and Methods for details). The positive sign represents an increase, whereas the negative sign represents a decrease in the average protein density.

Third column: adjusted $P$-values (see Materials and Methods). The cutoff $P$ value for significance was less than or equal to 0.05.

Fourth column: number of primary samples with upregulation of expression of the indicated protein (please refer to materials and Methods for statistical analysis).

Fifth column: number of primary samples with downregulation of expression of the indicated protein.
Sixth column: comparison of the numbers of samples with detectable upregulation and
downregulation of protein expression using conditional binomial tests ($P$ value) to confirm that
alteration was heavily skewed ($P \leq .05$).
Table 2. Pearson Correlation Coefficient (\(\rho\))-defined association of stroma-altered expression of CXCR4 with changes in intracellular mTOR signaling in nine pairs of co-cultured primary AML cells and MSCs. Raw and adjusted \(P\) values were calculated as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Intracellular signaling</th>
<th>CXCR4 in attached MSC</th>
<th>CXCR4 in attached CD34+ cells</th>
<th>CXCR4 in attached leukemic cells</th>
</tr>
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<tbody>
<tr>
<td>N = 9</td>
<td>(\rho)</td>
<td>(p) value</td>
<td>(\rho)</td>
</tr>
<tr>
<td>p4EB1 (TORC1)</td>
<td>0.999</td>
<td>&lt;0.001</td>
<td>0.724</td>
</tr>
<tr>
<td>pS6K (TORC1)</td>
<td>0.999</td>
<td>&lt;0.001</td>
<td>0.123</td>
</tr>
<tr>
<td>pAKT (TORC2)</td>
<td>0.995</td>
<td>&lt;0.001</td>
<td>0.687</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1. PP242-induced apoptosis in primary AML cells.**

(A) Nine samples obtained from patients diagnosed AML with high blast count were treated with PP242 at indicated concentrations in the presence or absence of MS-5 stromal cells for 48 hours. Apoptotic cells were detected using annexin V+ flow cytometry. The percentage of specific annexin V+ apoptosis was calculated as described under Materials and Methods. Data represent average +/- SEM of specific apoptosis in 9 AML samples. (B) Comparison of pro-apoptotic effects of PP242 and temsirolimus. OCI-AML3 cells (top panel) and two primary AML samples (bottom panel) were treated with PP242 and temsirolimus with or without MS-5 co-cultures for 72 hrs, and the percentage of apoptotic cells (Annexin V+/DAPI+/CD45-) was detected by flow cytometry. (C) AML cells from samples of pt #1 and #2 co-cultured with MS-5 were treated with 2.5 µM PP242 for 24 hours. Cells were harvested, and lysates subjected to immunoblotting with indicated antibodies to probe mTOR signaling.

**Figure 2. Flow cytometry-based discrimination of primary AML cells and MSCs cultured alone or co-cultured.** Primary AML cells (A) or AML MSC (B) cultured alone and used as controls; or harvested from AML/MSC co-culture (C) were fixed, permeabilized, and stained with the indicated cell surface markers. (C), Contour blot depicting different population of attached AML and MSC cells (left) and floating AML cells (right) detected by flow cytometry in the co-culture setting. Blue, CD90(-) AML cells; green CD34+ AML progenitor cells; red, CD90+ AML MSC.
Figure 3. PP242-mediated intracellular mTOR signaling in primary AML cells and MSCs cultured alone or co-cultured. Measurement of the mean fluorescent intensity (MFI) of intracellular p4EBP1 (Thr37/46) (A), pAKT (Ser473) (C), and pS6K (Ser235/236) (B) in primary AML cells, AML CD34+ progenitor cells, and primary AML MSCs (D) cultured alone and in co-cultures using flow cytometry. The gray bars represent the MFIs of the phosphorylated proteins before, and the black bars after treatment with PP242 for 72 hours. Cells were cultured in medium only (‘alone’), or co-cultured with MSC (‘floating’ or ‘attached’ cells, respectively). Data represent average +/- SEM of MFI of the phospho-proteins measured in the indicated number of primary samples. Paired two-sample t-test was used to determine reported p-values. (E) Primary AML cells and MSCs in a Transwell setting (described in Materials and Methods) were treated with PP242 for 24 hours. Cells were then lysed, and mTOR signaling targets in the AML cells were detected by immunoblotting.

Figure 4. PP242 reduced the proportion of the leukemic cells attached to primary MSC in co-culture condition. (A). Contour blot depicting different population of attached cells detected by flow cytometry in the co-culture setting. Leukemic cells (blue), CD34+ AML cells (green) and MSC (red). Right, microscopic images display these co-cultured AML cells and MSCs prior to and after PP242 treatment. (B) Percentage of AML CD34+ progenitor cells attached to the primary AML MSCs in the co-culture setting before and after PP242 treatment for 72 hours. In samples from 8 patients depicted by solid lines, the % decrease in the attached cell number exceeded the % decrease in the total number of cells after the PP242 treatment.
Figure 5. PP242-altered expression of CXCR4 and intracellular signaling in primary AML cells and MSCs cultured alone and co-cultured. (A) (a) Comparison of CXCR4 expression in primary AML and AML CD34+ progenitor cells cultured alone and co-cultured with stroma. Results represent the ratio of mean fluorescent intensity calculated as described in the Materials and Methods. P-value was calculated using paired two-sample t-test. (b) Contour dot plot and histograms demonstrating effects of PP242 on CXCR4 expression in a representative sample. (B) Circos diagram displays the inhibitory effect of PP242 on CXCR4 expression and intracellular signaling in attached AML CD34+ cells and corresponding MSC samples in co-cultures. The fold inhibition was calculated by MFI of untreated cells divided by MFI of treated cells. I – IV: surface marker and intracellular markers measured by flow cytometry. I: 4EBP1 (cyan), II: pAKT (blue); III: pS6K (purple); IV: CXCR4 (magenta). A – H: 8 AML CD34+ samples and 8 MSCs in co-cultures. Left panel are the attached AML CD34+ cells, right panel are their associated MSCs in co-cultures. Each ribbon connects a marker and an individual patient. The width of a ribbon represents the degree of inhibition: the wider the ribbon, the greater is the degree of inhibition. The color of the ribbon represents an individual marker. (C) The inhibitory effects of short-term PP242 treatment on CXCR4 expression and on mTOR signaling in OCI-AML3 cells. Surface expression of CXCR4, CD44, VLA4 was examined by flow cytometry in OCI-AML3 cells treated with 2.5µM PP242 for the indicated time period. Data represent the MFI ratios (a). Histograms of the triplicate experiments measuring changes in CXCR4 levels with 12G5 antibody recognizing the extracellular loop two (EC2) domain of CXCR4, and 1D9 antibody recognizing the N-terminal of CXCR4, are shown in (b).

Figure 6. PP242 suppression of AML cell expansion in Baf3-FLT3/ITD-GFP/luc mouse model of leukemia. (A) Peripheral blood obtained from retro-orbital plexus before and 2 hours
after PP242 administration. Whole blood was fixed and permeabilized as described in Materials and Methods. The cells were then stained with antibodies against p4EBP1 (Thr37/46), pAKT (Ser473), and pS6K (Ser235/236), and the MFI of each intracellular marker in GFP+ leukemic cells was assessed using flow cytometry after gating on GFP+ cells. (B a-b) The leukemia burden was monitored using bioluminescent imaging on days 5 and 14 following leukemia-cell inoculation (a). The bioluminescent intensities were averaged the peak light-emitting exposure in each group of mice and displayed as photons/second in the bar graphs (b).
Figure 2

A

AML cells

Unstained

CD34+

FS

B

AML MSC

Unstained

debris

CD90+

FS

C

AML Cells and AML MSC

Attached cells

CD90+

CD34+

debris

Attached leukemic cells and MSC in co-culture

Floating cells

CD34+

debris

Floating leukemic cells in co-culture
**Figure 3**

E: Co-culture (Transwell both sides growing cells)

- FoxO1a (Thr24)/3a (Thr32)
- FoxO3a
- pAKT (Thr308)
- Total AKT
- pS6K (Ser240/244)
- Total S6K
- p4EPB1 (Thr37/46)
- Total 4EBP1
- Tubulin

1. Control
2. PP242 (5 μM)
3. Control in transwell co-culture
4. PP242 (5 μM) in transwell co-culture
Figure 4
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>Leukemic cells</th>
<th>CD34+ leukemic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>.01</td>
<td>.02</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Ratio of CXCR4 MFI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>attached</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b

CXCR4

MSC Control | CD34+ Control | MSC-PP343 | CD34+ PP242 |

FS

CD90

C

CXCR4 (1D9)  CXCR4 (12G5)  CD44  VLA4

<table>
<thead>
<tr>
<th>MFI ratio (treated/Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

b

CXCR4 (1D9)  CXCR4 (12G5)

Control  2hr  4hr  8hr  16hr
Targeting of mTORC1/2 by the mTOR kinase inhibitor PP242 induces apoptosis in AML under conditions mimicking the bone marrow microenvironment

Zhihong Zeng, Yue Xi Shi, Twee Tsao, YiHua Qiu, Steven M. Kornblau, Keith A. Baggerly, Wenbin Liu, Katti Jessen, Yi Liu, Hagop Kantarjian, Christian Rommel, David A. Fruman, Michael Andreeff and Marina Konopleva