Anti-leukemia activity of the novel peptidic CXCR4 antagonist LY2510924 as monotherapy and in combination with chemotherapy

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Short title: LY2510924 in acute myeloid leukemia

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Key points

- In vitro, SDF-1α/CXCR4 inhibition by LY2510924 is potent and prolonged and inhibits proliferation and stromal chemoprotection of AML cells
- In vivo, LY2510924 mobilizes AML cells, has striking anti-leukemia effects as monotherapy, and strongly synergizes with chemotherapy

Abstract
Targeting the SDF-1α/CXCR4 axis has been shown to be a promising therapeutic approach to overcome chemoresistance of acute myeloid leukemia (AML). We investigated the anti-leukemia efficacy of a novel peptidic CXCR4 antagonist, LY2510924, in preclinical models of AML. LY2510924 rapidly and durably blocked surface CXCR4 and inhibited SDF-1α–induced chemotaxis and prosurvival signals of AML cells, at nanomolar concentrations, more effectively than the small-molecule CXCR4 antagonist AMD3100. In vitro, LY2510924 chiefly inhibited the proliferation of AML cells, with little induction of cell death, and reduced protection against chemotherapy by stromal cells. In mice with established AML, LY2510924 caused initial mobilization of leukemic cells into the circulation, followed by reduction in total tumor burden. LY2510924 had anti-leukemia effects as monotherapy as well as in combination with chemotherapy. Gene expression profiling of AML cells isolated from LY2510924-treated mice demonstrated changes consistent with loss of SDF-1α/CXCR4 signaling and suggested reduced proliferation and induction of differentiation, which was proved by showing the attenuation of multiple prosurvival pathways, such as PI3K/AKT, MAPK, and β-catenin, and myeloid differentiation in vivo. Effective disruption of the SDF-1α/CXCR4 axis by LY2510924 may translate into effective anti-leukemia therapy in future clinical applications.

Key words: CXCR4; SDF-1α; acute myeloid leukemia; LY2510924
Introduction

The interaction between acute myeloid leukemia (AML) cells and the bone marrow (BM) microenvironment has been postulated to be important for resistance to chemotherapy and disease relapse in AML.1 The chemokine receptor CXCR4 and its ligand, stromal cell–derived factor-1 alpha (SDF-1α, CXCL12), are key mediators of this interaction. SDF-1α is produced in the BM microenvironment, activates CXCR4 on leukemic cells, facilitates leukemia cell trafficking and homing in the BM microenvironment, and keeps leukemic cells in close contact with the stromal cells and extracellular matrix that constitutively generate growth-promoting and anti-apoptotic signals.2 Indeed, high CXCR4 expression on AML blasts is known to be associated with poor prognosis.3,4

Our group and others have tested small-molecule inhibitors against CXCR4: AMD3100 (Plerixafor), approved by the U.S. Food and Drug Administration, and its analogue AMD3465. These agents disrupted the SDF-1α/CXCR4 axis and enhanced the anti-leukemic effects of chemotherapy, markedly reducing leukemic burden and prolonging overall survival in xenograft models.5,6 Disruption of the SDF-1α/CXCR4 axis by CXCR4 antagonists is therefore an attractive investigational therapeutic approach for AML and is being tested in clinical trials. A phase I/II study recently reported that adding AMD3100 to cytotoxic chemotherapy increased response rates in patients with relapsed AML.1 However, the mobilization of leukemic blasts induced by AMD3100 is transient, and cell counts return to baseline levels within 12 hours,5 likely because of incomplete inhibition of the SDF-1α/CXCR4 axis and the short in vivo half-life (3-5 hours) of AMD3100.7 Furthermore, AMD3100 and AMD3465 did not show anti-leukemic effects as single agents in vivo,5,6 although they did have inhibitory effects on multiple cancers of non-hematologic origin.8-16

LY2510924 is a novel and potent selective peptide antagonist of CXCR4.17 A recent phase I study in advanced cancers revealed good tolerability with mostly grade 1/2 adverse events, favorable pharmacokinetics, and target engagement as indicated by dose-dependent increases in CD34+ cell mobilization.18 Here, we report preclinical studies using LY2510924 to disrupt the SDF-1α/CXCR4 axis in AML cells in vitro and in vivo.
Materials and methods

Please refer to Supplemental Methods for detailed descriptions of the methods and reagents used.

Cell lines, primary samples, and cultures

Human AML cell lines OCI-AML3, U937, and MOLM-13 (Supplemental Table S1), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 1% penicillin-streptomycin (Gibco Laboratories, Grand Island, NY). Cells were harvested during the log phase of growth and seeded at a density of 0.2×10^6 cells/mL. Peripheral blood samples from patients with AML were collected during routine diagnostic procedures after informed consent was obtained in accordance with Institutional Review Board regulations of The University of Texas M. D. Anderson Cancer Center and the Declaration of Helsinki. Mononuclear cells were separated by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO) density gradient centrifugation.

Flow cytometry

The expression of surface CXCR4 protein was analyzed by using a Gallios flow cytometer (Beckman Coulter, Brea, CA). Harvested cells were stained with antibodies against CXCR4-allophycocyanin (APC; 12G5) and CXCR4-phycoerythrin (1D9). All antibodies were purchased from BD Biosciences (San Jose, CA). The appropriate isotype-matched antibody was used as a negative control. In vivo leukemic cells were isolated by further staining of samples from mice with human CD45-APC for OCI-AML3/Luc/mCherry cells, CD45–PerCP-Cy5.5 for OCI-AML3/Luc/GFP cells,19 and CD34-PE-Cy7 and CD45-APC-H7 for primary AML cells. Flow cytometric data were analyzed with FlowJo (Version vX.0.6) software.

Chemotaxis studies

OCI-AML3 cells and primary samples from AML patients (Supplemental Table S2 and supplemental Figure S1) were subjected to chemotaxis studies in 6.5-mm-diameter Transwell culture inserts (Costar, Corning, NY) with a pore size of 5 µm. All experiments were conducted in triplicate, and results are
expressed as the percentage of migrated cells.

**Western blot analysis**

Cell lysates were separated on 12% polyacrylamide gels, transferred to nitrocellulose membranes, stained with the appropriate antibodies and infrared secondary antibodies (LI-COR Biosciences, Lincoln, NE), and quantified by the Odyssey imaging system (LI-COR Biosciences). Antibodies used were rabbit anti-human phospho-AKT (Ser473), AKT, and phospho-GSK-3β (Ser9), mouse anti-human phospho-p44/p42 MAPK (Erk1/2)(Thr202/Tyr204) (all four, Cell Signaling Technology, Beverly, MA), and mouse anti-human active-β-catenin (Millipore, Temecula, CA), Bcl-2 (Dako North America, Inc., Carpenteria, CA), ERK2 and GSK-3β (Santa Cruz Biotechnology, Santa Cruz, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-tubulin was used as the loading control.

**Co-culture with stromal cells**

OCI-AML3 cells were co-cultured with MS-5 or human mesenchymal stromal cells (hMSC). After 72 hours of incubation at 37°C in a humidified atmosphere containing 5% CO₂, co-cultured cells were harvested, and viable and apoptotic OCI-AML3 cells were quantified by flow cytometry.

**AML mouse models**

NOD/SCID/IL-2γnull (NSG) mice were used for in vivo xenograft experiments. Mice were injected with OCI-AML3 cells labeled with Luc/mCherry or Luc/GFP to establish AML. All animal experiments were done in accordance with a protocol approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center.

**Flow cytometry for detection of intracellular phospho-proteins**

Changes in AKT and ERK phosphorylation were measured in the xenograft mouse model of primary AML by multi-parametric phospho-flow cytometry in dual positive cells (human CD34 and CD45) recovered from the BM and spleen of a representative mouse from each treatment group on day 48 after transplantation.

**Gene expression profiling**
Xenotransplanted OCI-AML3 cells were harvested and isolated on days 41-43 and preserved in RNAlater solution. Total RNA was extracted, amplified, and labeled through two rounds of \textit{in vitro} transcription and then hybridized to Illumina HT12 version 4 human whole-genome arrays as described previously.\textsuperscript{20} Gene expression data were submitted to Gene Expression Omnibus as GSE64623.

\textbf{Statistical analysis}

In general, results are expressed as the mean ± standard deviation (SD) for triplicate experiments or mean ± standard error of the mean (SEM) for triplicate independent experiments. The Student paired \textit{t}-test was used to compare differences between groups. For \textit{in vivo} mouse experiments, overall survival and mean group survival times were estimated by the Kaplan-Meier method and compared with the log-rank test. Differences with \(p\) values \(\leq 0.05\) were considered statistically significant.
Results

*LY2510924 rapidly and durably blocks surface CXCR4 and inhibits SDF-1α-induced chemotaxis and prosurvival signals of leukemic cells*

To determine the effects of CXCR4 blockade by LY2510294, we used AML cell lines OCI-AML3, U937, and MOLM-13, which express high levels of CXCR4 on their cell surface. Flow cytometry results showed that LY2510924 inhibited binding of anti-CXCR4 antibody 12G5 to surface CXCR4 in a concentration-dependent fashion in AML cells (Figure 1A and 1B, Supplemental Figure S2). LY2510924 was much more potent than AMD3100 in causing CXCR4 blockade. CXCR4 occupancy by LY2510924 in OCI-AML3 cells started as early as 1 minute after treatment (Figure 1C) and continued for as long as 72 hours at 10 nM, in contrast to the short duration of blockade by AMD3100 (Figure 1D). Both LY2510924 and AMD3100 induced moderate upregulation of cell surface CXCR4 as measured by the 1D9 antibody, which binds to a different CXCR4 epitope (Supplemental Figure S3), suggesting the accumulation of CXCR4 on cell surface due to inhibition of SDF-1α-induced CXCR4 internalization.\(^1\) SDF-1α-induced migration of OCI-AML3 (Figure 1E) and primary AML cells (Figure 1F) was abolished by 1 nM LY2510924, but not by 1 nM AMD3100. Western blots showed that LY2510924 inhibited SDF-1α-induced phosphorylation of ERK and AKT (Figures 1G and 1H).

*LY2510924 inhibits proliferation of AML cells rather than causing cell death, and reverses stroma-mediated chemoresistance*

OCI-AML3 cells were cultured with LY2510924 to examine its ability to induce cell death and/or inhibit growth in *vitro*. Measurement of viable cell number over an 8-day period demonstrated that SDF-1α significantly enhanced the proliferation of OCI-AML3 cells compared to controls (at day 8; p=0.013). Proliferation was inhibited by LY2510924 (day 4, p=0.003; day 6, p=0.013; day 8, p=0.001; Figure 2A), to the same level in the presence or absence of SDF-1α; the effect of LY2510924 alone, as compared to control, was significant at days 4 (p=0.005), 6 (p<0.001), and 8 (p=0.002; Figure 2A). However, LY2510924 did not induce AML cell death in *vitro* (Supplemental Figure S4).
We then tested whether CXCR4 inhibition by LY2510924 can overcome stroma-mediated chemoresistance of AML cells in vitro by co-culturing the AML cells with MS-5 stromal cells for 3 days. As reported by another group, cytarabine significantly upregulated CXCR4 surface expression by OCI-AML3 cells in conditions reflected by measurement of antibody 12G5 (Figure 2B; monoculture, 2.6-fold increase vs. control, p=0.006; with MS-5 cells, 2.7-fold increase vs. control, p=0.002) or antibody 1D9 (Figure 2C; monoculture, 2.7-fold increase vs. control, p<0.001; with MS-5 cells, 3.3-fold increase vs. control, p<0.001). CXCR4 detection with 12G5 was blocked by LY2510924 (Figure 2B), but 1D9 showed even further upregulation of CXCR4 by cytarabine plus LY2510924 (Figure 2C), especially in cells co-cultured with MS-5 cells; this is perhaps expected, since LY2510924 alone upregulates CXCR4 (Supplemental Figure S3). Cytarabine-induced apoptosis of AML cells was significantly reduced by stromal cells (70.3%±7% vs. 43.8%±4.2%, p=0.005), but this chemoprotective effect of stromal cells was significantly inhibited by LY2510924 (61.9%±5.6%, p=0.011; Figure 2D and 2E). Similar chemoprotection by stromal cells, and its inhibition by LY2510924, were seen in OCI-AML3 cells treated with doxorubicin in hMSC co-culture (Supplemental Figure S5).

**LY2510924 monotherapy has anti-leukemia activity in OCI-AML3 xenograft models**

To test the anti-leukemia efficacy of LY2510924 in vivo, we injected sublethally irradiated NSG mice with OCI-AML3 cells (OCI-AML3/Luc/mCherry) engineered to allow bioluminescent imaging (BLI) of tumor burden as well as sorting on the basis of fluorescence. In mobilization studies, circulating leukemic cells were increased at 3 hours (3.4±1.4-fold) and further at 24 hours (24.1±15.4-fold) in three mice that received the first LY2510924 injection on day 21 after AML cell injection (Figures 3A and 3B). In two other groups (control and LY2510924) that began treatment on day 12, mice treated daily with LY2510924 had significantly less BLI signal than controls (Figures 3C and 3D; p=0.030 on day 18 and p<0.001 on day 25). Moreover, in representative mice sacrificed on day 22, immunohistochemical staining of tissues for human CD45 demonstrated that LY2510924-treated mice had less leukemic infiltration than controls (Figure 3E). Analysis of multispectral images (see
Supplemental Methods) further confirmed that controls had significantly more leukemia than
LY2510924-treated mice, in BM (84.6%±1.4% vs. 40.7%±3.2%, p<0.001), spleen (85.2%±2.2% vs.
30.2%±2.7%, p<0.001), and liver (88.5%±0.5% vs. 12.7%±1.6%, p<0.001; Figure 3E). This anti-
leukemia effect translated into significant prolongation of survival in LY2510924-treated mice
(median survival, 40 days vs. 26 days; p<0.001; Figure 3F).

**LY2510924 inhibits progression of primary AML cells in xenograft models**

To confirm the anti-leukemia efficacy of LY2510924, we injected primary AML cells derived from
patient with complex karyotype and high CXCR4 surface expression (Supplemental Figure S6) into
non-irradiated NSG mice. Mice were divided into two groups after engraftment was documented in
peripheral blood, and one group began treatment with LY2510924 on day 25. The proportion of
circulating leukemic cells was measured in five representative mice from each group after the first
LY2510924 administration. Circulating leukemia cells were significantly increased by LY2510924 3
hours after treatment compared to controls (2.1±0.2- vs. 1.4±0.1-fold increase over baseline, p=0.008)
and were increased by even more 24 hours after treatment (2.7±0.4- vs. 1.0±0.1-fold increase over
baseline, p=0.008; Figure 4A and Supplemental Figure S6). Flow cytometry showed sustained
inhibition of CXCR4 staining by 12G5 at 3 and 24 hours after the first LY2510924 injection (Figure
4B), and significant blockade after five daily treatments compared to controls (2.7±0.1- vs 10.2±1.9-
fold increase over isotype, p=0.017; Figure 4C).

On day 45, after 3 weeks of daily LY2510924 treatment, three mice were sacrificed in each group, and
flow cytometry of leukemic cells in their blood, BM, and spleens revealed significant blockade of
CXCR4 12G5 staining by LY2510924 (Figure 4D). This analysis also demonstrated that mice treated
with LY2510924 had lower leukemic cell burden in blood (50.5%±2.4% vs. 86.1%±2.9%, p<0.001),
BM (72.1%±3.6% vs. 89.7%±1.9%, p=0.012), and spleen (19.6%±0.6% vs. 60.1%±0.7%, p<0.001;
Figure 4E). Multi-parametric phospho-flow cytometry showed decreases in AKT and/or ERK
phosphorylation in leukemic cells recovered from the spleen and BM of a LY2510924-treated mouse
on day 48 (Figure 4F), which was consistent with our *in vitro* data (Figures 1G and 1H). These anti-
leukemia effects of LY2510924 alone resulted in significant prolongation of survival (median survival, 56 days vs. 44 days; overall, p<0.001, Figure 4G).

**Combination with chemotherapy enhances LY2510924 anti-leukemia effects in OCI-AML3 xenograft models**

To further explore the anti-leukemia efficacy of LY2510924 in combination with chemotherapy in vivo, we injected OCI-AML3/Luc/GFP cells into non-irradiated NSG mice. Mobilization studies were carried out in two groups of six mice each; in mice given their first LY2510924 injection on day 25, prolonged mobilization of leukemic cells into the circulation by LY2510924 was confirmed (3.1±0.7- vs. 1.3±0.3-fold increase over baseline at 3 hours, p=0.035; 5.3±1.7- vs. 1.3±0.2-fold increase over baseline at 24 hours, p=0.036; Figure 5A and Supplemental Figure S7). Four other groups began treatment on day 8: control, chemotherapy (cytarabine/doxorubicin) only, LY2510924 only, or combination (LY2510924 plus chemotherapy). BLI demonstrated significantly less tumor burden in all treated groups than in controls by day 26 (Figures 5B and 5C); leukemia progression was reduced equivalently by LY2510924 alone and chemotherapy (p=0.249) and was lowest with combination therapy. Immunohistochemical staining for human CD45 from mice sacrificed on day 27 (Figure 5D) showed findings consistent with those from BLI.

Analysis of the multispectral images (see Supplemental Methods) revealed less leukemic infiltration in all treated groups, lowest infiltration in the combination group, and no significant difference in infiltration between cytarabine/doxorubicin– and LY2510924-treated mice (BM, 3.0%±0.4% vs 2.8%±0.3%, p=0.738; spleen, 0.7%±0.1% vs 0.6%±0.1%, p=0.785; liver, 0.5%±0.2% vs 0.4%±0.1%, p=0.741). LY2510924-treated mice had prolonged survival compared to controls (median survival, 52 days vs. 40 days; p=0.006), and combination therapy extended survival even further (median survival, 62 days vs. 52 days; p=0.004; Figure 5E).

**LY2510924 induces gene expression changes in leukemic cells in vivo consistent with loss of SDF-1α/CXCR4 signaling**
To elucidate the molecular effects of inhibiting CXCR4-mediated signaling in AML cells *in vivo*, we again used the OCI-AML3/Luc/GFP xenograft model. Engraftment, assessed as sufficient involvement of the blood by leukemic cells (3.3%±1.1%), was reached in eight mice on day 40. Groups of these mice began daily treatment with LY2510924 on that day, and were sacrificed 24 hours (day 41, after one treatment; n=3) or 72 hours (day 43, after three treatments; n=2) later. Three untreated mice were sacrificed on day 42 as controls. Leukemic cells were isolated from BM, blood, and spleen of all of these by fluorescence-activated cell sorting (FACS) by human CD45 and GFP (Supplemental Figure S8). Samples from each site and time point were also analyzed for CXCR4 occupancy using flow cytometry with antibody 12G5. CXCR4 blockade by LY2510924 was delayed in BM compared to that in blood and spleen (Figure 6A), but unoccupied CXCR4 in BM was decreased to a similar level as measured in blood and spleen at 72 hours, suggesting that repeated doses are needed to affect leukemic cells in BM.

Genome-wide gene expression profiling (GEP) was performed on 24 samples of leukemic cells from various sites and treatment time points. When profiles of all samples were compared to the average profile of untreated BM, by displaying fold-change values for altered genes in a subtracted and clustered heat map (Figure 6B), there was remarkable similarity between the changes detected in leukemic cells localized in a site other than BM (i.e., blood or spleen) and changes caused by treatment with LY2510924. Furthermore, changes were greatest in samples from non-BM locations in treated mice, especially at 72 hours. GEP findings were also consistent with the finding of less CXCR4 blockade in BM at 24 hours (Figure 6A): at 24 hours, BM samples were relatively unaffected, whereas moderate changes were noted in all three spleen samples and one of three blood samples, and marked changes were noted in two of three blood samples. Assuming that SDF-1α/CXCR4 signaling is strongest in the BM, the consistency of GEP changes caused by either non-BM localization or LY2510924 treatment, and the enhancement and extension of these changes by the combination of these factors, further accentuated by treatment duration, suggest that LY2510924 is indeed a specific inhibitor of SDF-1α/CXCR4 signaling.
Biological effects of SDF-1α/CXCR4 signaling in vivo involve differentiation and proliferation of AML

GEP data were also analyzed to investigate the biological effects of SDF-1α/CXCR4 signaling in leukemic cells. To utilize GEP data for all 24 samples, we relied on the impression from Figure 6B that there was qualitative similarity in the effects of non-BM localization and/or LY2510924 treatment, assigning to each sample a score for the presumed relative degree of loss of SDF-1α/CXCR4 signaling (see Supplemental Methods). Genes were then assessed for correlation between their expression levels and this score in all samples, using the Pearson correlation r value in a metric that also considered the variance of gene expression values (Supplemental Data File S1). Genes with the highest positive values of the correlation metric, i.e., those upregulated by loss of SDF-1α/CXCR4 signaling, were predominantly associated with myelomonocytic differentiation, including CD14, TREM1, CD300a, LILRA5, LILRB3, and S100A12 (Figure 6C). This suggests that although OCI-AML3 is a cell line, it undergoes myelomonocytic differentiation in vivo, regulated by SDF-1α/CXCR4 signaling. Genes with the most negative values of the correlation metric, i.e., those downregulated by loss of SDF-1α/CXCR4 signaling, include MS4A3, a hematopoietic cell cycle regulator that is downregulated during differentiation. The effect of SDF-1α/CXCR4 inhibition on myeloid differentiation was supported by immunohistochemical analysis of tissues from OCI-AML3-Luc-mCherry xenograft model demonstrating increases of CD11c positive cells in LY2510924-treated mouse compared to an untreated mouse (BM, 60% vs. 23%; Liver, 45% vs. 18%; Figure 7). Tissues from primary AML xenograft model also showed similar findings (Supplemental Figure S9). Other notable negatively correlated genes include negative regulators of apoptosis (BCL2 and RPL23), and genes involved in proliferation (e.g., MAD2L1, BUB1, and NCAPG2; Figure 6C). We confirmed decreased Bcl-2 levels by Western blot analysis (Figure 6D).

Gene set enrichment analysis was performed, based on ranking genes by the correlation metric, to investigate further the biological effects of these changes. Among multiple gene sets that were significantly enriched (Supplemental Data File S2), positive enrichment of the gene set
JAATINEN_HEMATOPOIETIC_STEM_CELL_DN, representing genes downregulated in CD133+ cells (hematopoietic stem cells) compared to CD133- cells, implies that loss of SDF-1α/CXCR4 signaling promotes differentiation. The possibility that LY2510924-induced differentiation might have therapeutic potential was implied by positive enrichment for MARTENS_TRETINOIN_RESPONSE_UP, representing genes upregulated in acute promyelocytic leukemia NB4 cells in response to tretinoin. Also positively enriched was WHITMAN_AML_FLT3_TKDMUTATION_VS_FLT3_WT_112UPREGULATED_HUMAN, genes upregulated in FLT3-mutated de novo cytogenetically normal AML, without FLT3 internal tandem duplication, vs. those without FLT3 abnormality. The implication that loss of SDF-1α/CXCR4 signaling retards proliferation was supported by negative enrichment of multiple proliferation-associated gene sets. One of the gene set FEVR_CTNNB1_TARGETS_DN, representing genes down-regulated in intestinal crypt cells upon deletion of CTNNB1, implied that loss of SDF-1α/CXCR4 signaling inhibits Wnt/β-catenin signaling. Western blot analysis of FACS-sorted leukemic cells showed that the attenuation of phospho-ATK in vivo by LY2510924 resulted in GSK-3β kinase dephosphorylation and decreased activated β-catenin levels (Figure 6D).
Discussion

Here, we investigated the efficacy of LY2510924 in preclinical models of AML and demonstrated that effective disruption of the SDF-1α/CXCR4 axis can induce anti-leukemia effects on AML cells by this agent as monotherapy as well as in combination with chemotherapy. These findings indicate that effective CXCR4 antagonists are not simply chemosensitizers but also effective targeted anti-leukemia agents.

SDF-1α/CXCR4 inhibition appears to affect leukemia cells in several mechanisms. One is by reducing protection from apoptosis, whether spontaneous or chemotherapy induced, conferred on leukemic cells by interaction with stromal cells. LY2510924 blocked the protection of leukemic cells from chemotherapy-induced apoptosis by MS-5 stromal cells in co-culture, even though it did not induce apoptosis in monoculture, as observed by us and others for other CXCR4 antagonists.\(^5,6,27\) However, LY2510924 did have a different, measurable effect in monocultures, inhibiting basal or SDF-1α–induced leukemic cell proliferation, indicating that CXCR4 antagonism may affect proliferation and cell viability through distinct pathways. Inhibition of proliferation by LY2510924 was also implicated in vivo by our GEP studies. Another effect of SDF-1α/CXCR4 inhibition, observed with other CXCR4 antagonists\(^2,28\) and obviously specific for the in vivo setting, is the mobilization of leukemic cells into the circulation. This “physical disruption” of leukemia/stroma interactions by displacement is potentially significant in the additional context of chemotherapy, in that chemoprotective effects of the BM microenvironment, are thereby abrogated. Finally, an additional effect of SDF-1α/CXCR4 inhibition implicated by our GEP studies was the induction of myeloid differentiation, which was supported with tissues stained with CD11c from xenograft models. This is also clearly relevant by the clinical success of differentiation therapy with epigenetic modifiers in AML.\(^29\) These findings are supported by those of an earlier study of U937 cells treated in vitro with anti-CXCR4 monoclonal antibody, in which microarray data demonstrated effects on proliferation and differentiation of AML cells.\(^30\)

At a molecular level, both in vitro and in vivo experiments demonstrated that LY2510924 attenuated
SDF-1α–induced PI3K/AKT and MAPK prosurvival signaling, consistent with our previous report on AMD3465.5 PI3K/AKT and MAPK pathways are key signaling pathways promoting leukemic cell survival\textsuperscript{31,32} and are known to be linked to the SDF-1α/CXCR4 axis.\textsuperscript{33} We have reported the potential of both stromal cells and SDF-1α to activate PI3K/AKT and MAPK prosurvival pathways.\textsuperscript{6,34} There may be other effects and signaling pathways activated in AML cells by stromal cells; although these need to be identified, especially for rational combination strategies, they may be indirectly abrogated by the mobilizing effect of SDF-1α/CXCR4 inhibition. Our microarray results also suggest that inhibiting SDF-1α/CXCR4 signaling may affect Wnt/β-catenin pathway, and Western blot analysis showed that the attenuation of PI3K/ATK \textit{in vivo} by LY2510924 results in GSK-3β kinase activation by dephosphorylation, which decreases activated β-catenin levels, consistent with data in peripheral nerve sheath tumors.\textsuperscript{16} There may be additional signaling pathways and effectors of AKT and β-catenin that require further investigation.

Previous preclinical and clinical studies of SDF-1α/CXCR4 inhibition in AML have shown both promise and limitations. Small-molecule CXCR4 antagonists, such as AMD3100 or AMD3465, did not show anti-leukemia effects as monotherapy in certain studies.\textsuperscript{5,6} While this limitation may have been isolated to these particular drugs, or to the way in which they were used, our results suggest that this limitation is overcome when the SDF-1α/CXCR4 axis is inhibited more effectively, as by LY2510924. Previous studies used CXCR4 antagonists for only very short periods (less than 2 days), or with every-other-day administration, and focused on their effectiveness as chemosensitizers. In line with our results, two reports demonstrated monotherapeutic anti-leukemic effects in preclinical models for CXCR4 antagonism by a monoclonal antibody, MDX-1338,\textsuperscript{35} or a peptide inhibitor, TN140.\textsuperscript{27} MDX-1338 (given five times for 14 days) inhibited growth of the acute promyelocytic leukemia cell line HL60,\textsuperscript{35} and TN140 (given daily by osmotic pump for 7 days) reduced leukemic cell numbers in the BM and prolonged survival in primary AML xenograft models.\textsuperscript{27} In both studies, AMD3100 had no effect. In future clinical trials with CXCR4 antagonists, the use of more potent agents and extended treatment duration may be essential to fully disrupt the SDF-1α/CXCR4 axis and
induce anti-leukemia effect, in addition to optimal enhancement of chemosensitization. The combination with potential agents to disrupt the interaction of CD44, α-intergrins (VLA-4) or osteopontin to their ligands, which are important for adhesion to the stromal niche,\(^\text{36}\) may enhance the efficacy of CXCR4 inhibition.

Utilization of CXCR4 antagonists with chemotherapy might raise concerns about increased toxicity to normal hematopoiesis. However, our \textit{in vivo} data (Figure 5E) suggested that the toxicity of LY2510924 plus chemotherapy was similar to that of chemotherapy alone. Importantly, recent clinical studies with novel potent CXCR4 antagonists ulocuplumab and BL-8040 demonstrated lack of additive myelosuppression when combined with chemotherapy.\(^\text{37,38}\)

In conclusion, our study shows that LY2510924 at nanomolar concentrations rapidly and durably disrupts the SDF-1\(\alpha\)/CXCR4 axis in AML cells and has greater anti-leukemia efficacy than AMD3100. LY2510924 disrupts the SDF-1\(\alpha\)/CXCR4 axis, causes mobilization of leukemic cells into the circulation, inhibits multiple prosurvival signals generated by the SDF-1\(\alpha\)/CXCR4 axis, and induces myeloid differentiation, producing anti-leukemia effects as monotherapy or in combination with chemotherapy. CXCR4 blockade by LY2510924 may translate into effective anti-leukemia activity in future clinical applications.
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References


Figure legends

**Figure 1.** LY2510924 rapidly and durably blocks surface CXCR4 and inhibits SDF-1α–induced chemotaxis and prosurvival signals of leukemic cells. (A-B) Both OCI-AML3 (A) and U937 (B) cells were cultured with different concentrations of LY2510924 or AMD3100 for 150 minutes, and surface CXCR4 was measured by flow cytometry with antibody 12G5, which is blocked by receptor occupancy with either agent. Results are expressed as percentage change in the mean fluorescent intensity (MFI) compared with control (untreated) cells. (C) OCI-AML3 cells were cultured with 1 nM LY2510924, and surface CXCR4 12G5 binding was measured by flow cytometry at different time points. (D) OCI-AML3 cells were cultured with different concentrations of LY2510924 or AMD3100 for 72 hours, and surface CXCR4 12G5 binding was measured by flow cytometry. (E-F) OCI-AML3 (E; 0.5×10⁵) or primary AML (F; 1.0×10⁶, n=3) cells were plated onto the upper chamber of Transwell plates and exposed to 50 ng/mL SDF-1α in the lower chamber with or without 1 nM LY2510924 or AMD3100 for 2.5 hours (E) or 12 hours (F). The results are expressed as percentage of migrating cells relative to the number of input cells. (G-H) After overnight serum starvation, 1×10⁶ OCI-AML3 (G) or U937 (H) cells in RPMI medium containing 0.5% bovine serum albumin were or were not pretreated with 1 µM LY2510924 for 1 hour and were exposed to 100 ng/mL SDF-1α for 10 minutes. Phosphorylation of AKT and ERK was detected by Western blot analysis, and the intensity of the bands was quantified by densitometry and displayed as the ratio of phosphorylated protein to control phospho-protein. GAPDH was used as a loading control. All results are expressed as the mean ± SD, with the exception of (F), expressed as the mean ± SEM.

**Figure 2.** LY2510924 inhibits proliferation of AML cells and reverses stroma-mediated chemoresistance. (A) OCI-AML3 cells (3×10⁴/mL) were grown in 2% fetal bovine serum containing RPMI in the presence or absence of 100 ng/mL SDF-1α with or without daily treatment with 1 µM LY2510924 for up to 8 days. Flow cytometry using annexin V⁺/DAPI⁺ staining and counting beads was used to assess the percentage of apoptotic cells. (B-C) OCI-AML3 cells were cultured alone (monoculture) or co-cultured with stromal cells (MS-5) as indicated in Materials and Methods.
Monocultured and co-cultured cells were treated for 72 hours with 2.5 µM cytarabine in the presence or absence of 1 µM LY2510924. The surface CXCR4 12G5 (B) and 1D9 (C) staining and percentages of apoptotic cells (D) and viable cells (E) were assessed by flow cytometry. All results are expressed as the mean ± SD. * p<0.05, ** p<0.01.

Figure 3. LY2510924 monotherapy has anti-leukemia activity in OCI-AML3 xenograft models.
OCI-AML3/Luc/mCherry cells (1×10^6 per mouse) were intravenously injected into sub-lethally irradiated (250 cGy) NSG mice. Circulating OCI-AML3 cells were identified by flow cytometry (A) and percentages determined (among all nucleated cells; B) in mice (n=3) before and 3 or 24 hours after a single LY2510924 treatment on days 21-22. (C-D) Shown are serial bioluminescence images (C) and intensity quantitation (D) of five representative mice from two groups, control and LY2510924 (n=10 each), that began 3 weeks of daily treatment 12 days after cell injection. (E) Three representative mice per group were sacrificed on day 22, and tissues were fixed and sectioned for immunohistochemical analysis with anti-human CD45 antibodies to identify human leukemic cells. Analysis of the multispectral images further confirmed that leukemic cell burden was significantly reduced in LY2510924-treated mice. Original magnification, ×200. (F) Overall survival rate in each group was estimated by the Kaplan-Meier method. Results of (D), (E), and (F) are expressed as the mean ± SEM. * p<0.05, ** p<0.01.

Figure 4. LY2510924 durably blocks surface CXCR4, induces mobilization of leukemia cells, and inhibits AKT and ERK intracellular signaling in vivo, retarding progression of primary AML xenografts. Primary AML cells (0.4×10^6 per mouse) were intravenously injected into NSG mice. Mice were divided into two groups, control (n=13) and LY2510924 (n=15), after engraftment was documented in peripheral blood on day 25, and began to receive daily treatment. (A) In five representative mice of each group, percentages of circulating primary AML cells before and 3 or 24 hours after the first LY2510924 injection on days 25-26 were compared to those of untreated mice. (B) Identification of circulating leukemic cells by flow cytometry and staining for CXCR4 with antibody 12G5 (inversely reflective of receptor occupancy) at points during the initial 2 days of daily...
LY2510924 administration are shown for a representative mouse. (C) CXCR4 staining with antibody 12G5 after 5 days of LY2510924 treatment was compared in five representative mice of each group. (D-E) Three representative mice per group were sacrificed on day 45, and cells from BM, spleen, and blood in all mice (control, n=6; LY2510924, n=11) on day 45 were analyzed by flow cytometry, and dual positive cells (human CD34 and CD45) were compared between each group in terms of expression of CXCR4 12G5 (D) and proportion of leukemic cells (E). (F) AKT and ERK phosphorylation in dual positive cells (human CD34 and CD45) recovered from BM and spleen were measured by multi-parametric phospho-flow cytometry in a representative mouse from each group on day 48. The results showed that receptor occupancy by LY2510924 correlates with reduced AKT and ERK phosphorylation. (G) Overall survival rate in each group was estimated by the Kaplan-Meier method. Results of (A), (C-E), and (G) are expressed as the mean ± SEM. * p<0.05, ** p<0.01.

Figure 5. LY2510924 induces mobilization of OCI-AML3 cells in vivo and enhances anti-leukemia effects in combination with chemotherapy. OCI-AML3/Luc/GFP cells (1×10⁶ per mouse) were intravenously injected into NSG mice. (A) In mobilization studies, percentages of circulating OCI-AML3/Luc/GFP cells before and 3 or 24 hours after the first LY2510924 injection on day 25 in six mice were compared with those in untreated mice (n=6). (B-E) After confirming leukemia engraftment by bioluminescence imaging, mice were divided into four groups (10 mice per group) and began to receive treatment on day 8: control (no treatment), chemotherapy (cytarabine/doxorubicin), LY2510924, or the combination of chemotherapy with LY2510924. Chemotherapeutics were administered 3 hours after LY2510924 administration. Five representative mice from each group were subjected to serial bioluminescence images (B) and intensity quantitation (C) on days 7, 26, and 40 after leukemic cell injection. (D) Three representative mice per group were sacrificed on day 27 for immunohistochemical analysis for human CD45 to identify human leukemic cells. Analysis of the multispectral images further confirmed the significantly reduced leukemic cell burden in LY2510924-treated mice, even more so in the combination group. Original magnification, ×40. (E) Overall survival rate in each group was estimated by the Kaplan-Meier method. Results of
(A) and (C-D) are expressed as the mean ± SEM. * p<0.05, ** p<0.01.

**Figure 6. LY2510924 induces gene expression changes in leukemic cells in vivo that are consistent with loss of SDF-1α/CXCR4 signaling.** In the OCI-AML3/Luc/GFP xenograft model, engraftment was confirmed in eight mice on day 40 by proportion of leukemic cells in blood (3.3%±1.1%). Groups of these mice were then given daily LY2520924 treatment and sacrificed after 24 hours (n=3, on day 41) or 72 hours (n=2, on day 43). Control mice receiving no treatment (n=3) were sacrificed on day 42. Leukemic cells were sorted and separated from BM, blood, and spleen by FACS, using the specific markers human CD45 and GFP. (A) Samples from each site and treatment time point were also analyzed by FACS with CXCR4 antibody 12G5, as an inverse indicator of CXCR4 occupancy by LY2510924. (B) From genome-wide gene expression profiling of 24 samples (three groups, three tissue sites, two or three mice per group), log2 values of each sample’s genes from the treated mice were subtracted by the average for the corresponding gene in the control BM samples. The heat map shows subtracted values converted to fold-change, for genes with an absolute subtracted log2 value of at least 1.5 for at least six samples, with samples and genes hierarchically clustered for similarity in variation. The color bar indicates fold-change values, and the dendrogram indicates similarity between samples, which are labeled as to duration of LY2510924 treatment (Con = control) and tissue site (BM = bone marrow; SP = spleen; PB = peripheral blood). (C) Selected genes whose expression levels are highly correlated with a score of the presumed relative degree of loss of SDF-1α/CXCR4 signaling, based on non-BM localization and/or LY2510924 treatment and its duration. Score values are shown above sample names, and gene expression values are shown by fold-change from the mean, according to the color bar. The upper, positively correlated group of genes is related to myelomonocytic differentiation. The lower, negatively correlated genes are involved in differentiation, proliferation, or apoptosis. Values for genes shown twice were detected by different probes. (D) Western blot analysis was performed with leukemic cells sorted and separated from spleen and/or BM by FACS in two mice given daily LY2520924 treatment and sacrificed after 72 hours compared to two control mice. The intensity of the bands was quantified by densitometry and displayed as the ratio of...
phosphorylated protein to control phospho-protein. GAPDH and β-tubulin was used as a loading control. Results of (A) are expressed as the mean ± SEM.

**Figure 7. LY2510924 induces myeloid differentiation of AML cell in organs of OCI-AML3 xenograft model.** (A) In the OCI-AML3/Luc/mCherry xenograft model, bone marrow and liver samples of representative control and LY2510924-treated mice were fixed and sectioned for immunohistochemical analysis with anti-human CD11c antibodies to investigate myeloid differentiation of leukemic cells. Low power of bone marrow sections shows that leukemic cells almost completely efface the bone marrow medullary space in the control mouse while clusters of large pale leukemic cells are intermixed with small darker normal hematopoiesis in the LY2510924-treated mouse. Low magnification of liver sections also demonstrates that the periportal sheets of neoplastic cells are significantly larger in the control compared to the LY2510924-treated mouse. At high magnification, the leukemic cells demonstrate similar morphology in bone marrow and liver both in control and treated mice. However, anti-CD11c antibody demonstrates more differentiated cells in bone marrow and liver of a treated mouse compared to bone marrow and liver of a control mouse. (B) The CD11c expression was assessed by manually counting positive and negative signal in 200 leukemic cells, which were discriminated from mouse cells based on size and nuclear chromatin characteristics, and the bar graph demonstrates the difference between groups.
Figure 6

A

Normalized surface expression of CCR4-10G5 (MFI, %)

Bone marrow | Blood | Spleen

Control | 24 hours | 72 hours

B

Heatmap showing expression levels at different time points and tissues.

C

Heatmap of gene expression scores.

D

Western blot analysis showing protein expression levels with and without LY2510924 treatment.
Anti-leukemia activity of the novel peptidic CXCR4 antagonist LY2510924 as monotherapy and in combination with chemotherapy

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