

Inhibition of CXCR4 with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias

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Abstract

The chemokine receptor CXCR4 mediates the migration of hematopoietic cells to the stroma-derived factor 1 α (SDF-1 α)–producing bone marrow microenvironment. Using peptide-based CXCR4 inhibitors derived from the chemokine viral macrophage inflammatory protein II, we tested the hypothesis that the inhibition of CXCR4 increases sensitivity to chemotherapy by interfering with stromal/leukemia cell interactions. First, leukemic cells expressing varying amounts of surface CXCR4 were examined for their chemotactic response to SDF-1 α or stromal cells, alone or in the presence of different CXCR4 inhibitors. Results showed that the polypeptide RCP168 had the strongest antagonistic effect on the SDF-1 α – or stromal cell–induced chemotaxis of leukemic cells. Furthermore, RCP168 blocked the binding of anti-CXCR4 monoclonal antibody 12G5 to surface CXCR4 in a concentration-dependent manner and inhibited SDF-1 α –induced AKT and extracellular signal-regulated kinase phosphorylation. Finally, RCP168 significantly enhanced chemotherapy-induced apoptosis in stroma-cocultured Jurkat, primary chronic lymphocytic leukemia, and in a subset of acute myelogenous leukemia cells harboring FIt3 mutation. Equivalent results were obtained with the small-molecule CXCR4 inhibitor AMD3465. Our data

therefore suggest that the SDF-1 α /CXCR4 interaction contributes to the resistance of leukemia cells to chemotherapy-induced apoptosis. Disruption of these interactions by the peptide CXCR4 inhibitor RCP168 represents a novel strategy for targeting leukemic cells within the bone marrow microenvironment. [Mol Cancer Ther 2006;5(12):3113–21]

Introduction

We have reported that cells of the bone marrow microenvironment provide a sanctuary in which subpopulations of acute myelogenous leukemia (AML) can evade chemotherapy-induced death and acquire a drug-resistant phenotype (1). Similar findings have been noted in chronic lymphocytic leukemia (CLL; ref. 2). Although the mechanisms of stroma-mediated protection involve a complex interplay among stroma-produced cytokines, chemokines, and adhesion molecules, stroma-secreted chemokine stroma-derived factor 1 α (SDF-1 α) and its cognate receptor CXCR4 have recently emerged as critical mediators of stroma/leukemia cell interactions (3). SDF-1 α , a growth factor for B-cell progenitors and a chemotactic factor for T cells and monocytes, is essential for B-cell lymphopoiesis and bone marrow myelopoiesis (4), and mice lacking expression of either CXCR4 or SDF-1 die perinatally due to the virtual absence of bone marrow hematopoiesis (5, 6). The SDF-1 α /CXCR4 axis is thus believed to play a critical role in fetal development, the mobilization of hematopoietic stem cells, and the trafficking of normal lymphocytes (3). In contrast to other chemokine receptors, the stimulation of CXCR4 can induce the prolonged activation of the extracellular signal-regulated kinase (ERK) and phosphoinositol 3-kinase pathways (7), and the optimal chemotactic response of T cells to SDF-1 α requires the activation of both class IA and class IB phosphoinositol 3-kinases (8). Signaling via CXCR4 also enhances the association of components of focal adhesion complexes, such as paxillin and nuclear factor- κ B activity in nuclear extracts (9). In contrast, chemokines, including CXCR4, modulate adhesion by activating integrins, and Rap1 plays a pivotal role in these responses (10).

Intracellular CXCR4 levels are significantly elevated in B-CLL (11), B-cell but not T-cell acute lymphoblastic leukemia (12, 13), multiple myeloma (14, 15), and some AMLs (16, 17). Leukemic cells expressing CXCR4 show SDF-1 α –induced calcium flux, integrin-mediated cell adhesion, chemotaxis, and migration. Furthermore, CXCR4 mediates the homing to and engraftment of AML (18) and pre-B acute lymphoid leukemia cells to the bone marrow of nonobese diabetic/severe combined immunodeficient mice (19). CXCR4/SDF-1 α interactions

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not only protect CLL cells from apoptosis (2) but also allow the spontaneous migration of malignant cells beneath bone marrow stromal cells, suggesting that CLL cells use this mechanism to infiltrate the marrow (20).

In this study, we tested the hypothesis that CXCR4 inhibition interferes with stromal/leukemia cell interactions and hence increases the sensitivity of CLL or AML cells to chemotherapy. In particular, we investigated the ability of the novel peptide CXCR4 inhibitors from the family of synthetically and modularly modified chemokines to prevent SDF-1 α - and stromal cell-induced chemotaxis and SDF-1 α /CXCR4 signaling. Our results identified the synthetically and modularly modified chemokine RCP168 as a potent antagonist of SDF-1 α /CXCR4 signaling, which acts by blocking SDF-1 α -induced chemotaxis and suppressing survival signaling pathways at submicromolar concentrations. Most importantly, our findings indicate that CXCR4 inhibition by RCP168 can overcome the protection conferred by stromal cells and enhance the sensitivity of CLL and AML cells to chemotherapy.

Materials and Methods

Cell Lines

HL-60, KG-1, U937, TF-1, CEM, and Jurkat cells were purchased from the American Type Culture Collection (Rockville, MD). KBM5 cells were derived from a patient in the myeloid blastic phase of chronic myelogenous

leukemia; these cells contain multiple copies of the Philadelphia chromosome but lack the normal *ABL* gene. KBM5 cells resistant to imatinib (KBM5-ST1) were derived by Ricci et al. (21) through the chronic exposure of KBM5 cells to imatinib. NB4 cells were obtained from Lanotte et al. (22), and OCI-AML3 cells were obtained from Dr. M.D. Minden (Ontario Cancer Institute, Toronto, Ontario, Canada). Cell lines were maintained in RPMI 1640 containing 10% FCS (Gemini Bio-Products, Woodland, CA) and 1% penicillin-streptomycin (Life Technologies Laboratories, Grand Island, NY). The mouse stromal cell line MS-5 was kindly provided by Itoh et al. (23).

Patients

Peripheral blood samples from patients with CLL or 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 (Houston, TX). Mononuclear cells were separated by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density-gradient centrifugation.

Chemokines, Peptides, and Antibodies

The recombinant human chemokine SDF-1 α was purchased from R&D Systems (Minneapolis, MN). Anti-human CXCR4 (12G5) was purchased from BD PharMingen (San Diego, CA); antibodies to human AKT, Ser⁴⁷³-phosphorylated AKT, and phosphorylated ERK were purchased from Cell Signaling Technology (Beverly,

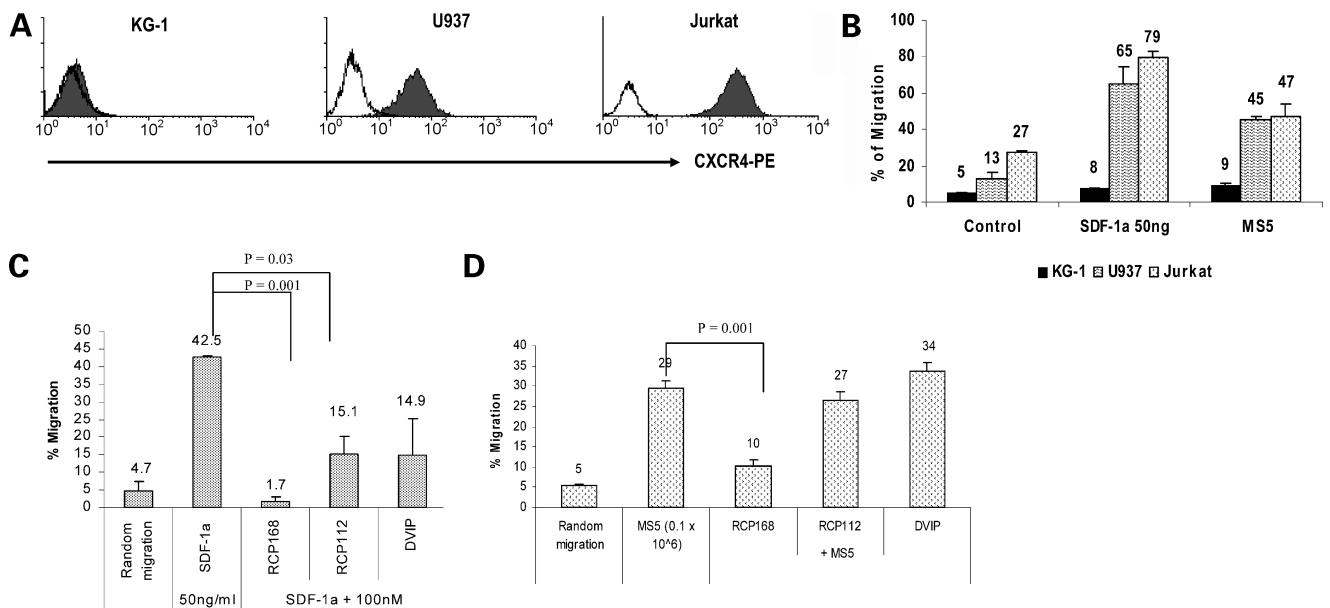


Figure 1. Surface expression of CXCR4 and stroma- or SDF-1 α -induced chemotactic response in leukemia cell lines. **A**, histogram shows surface CXCR4 expression on Jurkat, U937, and KG-1 leukemic cell lines (white, IgG control; black, CXCR4-PE). **B**, chemotactic responses of three cell lines to either 0.1×10^6 MS-5 or 50 ng/mL SDF-1 α . **C** and **D**, effect of different CXCR4 inhibitors on the SDF-1 α - and MS-5-induced chemotactic response. Jurkat cells (0.5×10^6) were plated on the upper chamber of Transwell plates and then exposed to 50 ng/mL SDF-1 α and different CXCR4 inhibitors in the lower chamber for 24 h (**C**), or exposed to (**D**) 0.05×10^6 MS-5 cells preplated in the lower chamber with or without different CXCR4 inhibitors. Migrating cells were counted after 24 h of incubation. Expressed as percentage of migrating cells relative to the numbers of input cells.

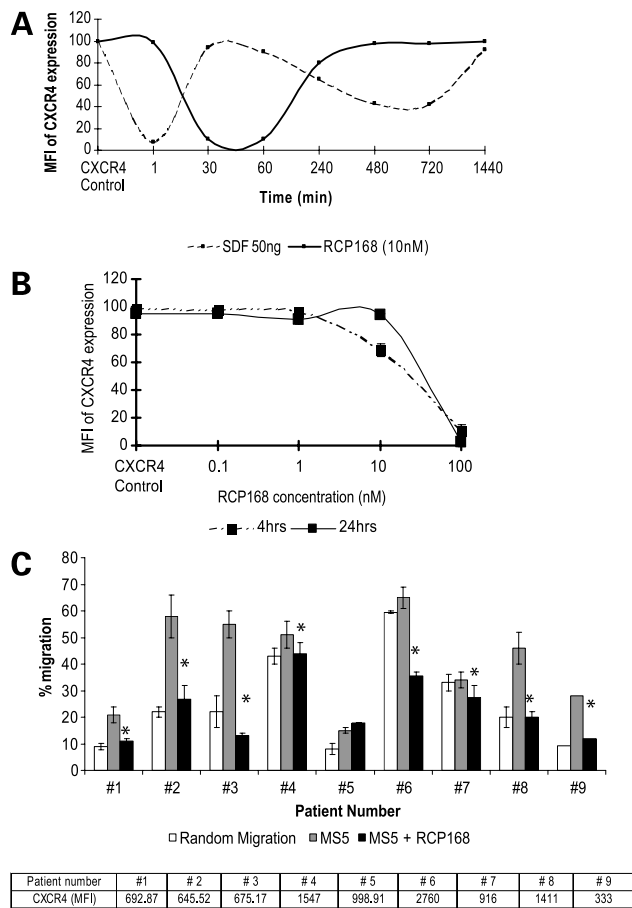


Figure 2. RCP168 suppresses binding of 12G5 anti-CXCR4 antibody to cell surface CXCR4. **A**, Jurkat cells were treated with 10 nmol/L RCP168 or 50 ng/mL SDF-1 α . Cells were harvested at the indicated time points, and surface expression of CXCR4 was detected by flow cytometry. **B**, Jurkat cells treated with different doses of RCP168 for 4 or 24 h; surface expression of CXCR4 was detected by flow cytometry. **C**, RCP168 suppresses MS-5-induced migration of CLL cells. *, significant inhibition of MS-5-stimulated cell migration.

MA). Cytarabine (AraC) was from Skye Pharmaceutical, Inc. (San Diego, CA). Fludarabine (9- β -D-arabinofuranosyl-2-fluoroadenine) was provided by Dr. W.K. Plunkett (M.D. Anderson Cancer Center; ref. 24). AMD3465, a second-generation small-molecule reversible inhibitor of SDF-1 α /CXCR4 with an IC₅₀ SDF-1 binding of 42 ± 2 nmol/L was provided by Dr. G. Bridger (AnorMED, Inc., Langley, British Columbia, Canada).

Chemotaxis Study

For the chemotaxis study, cells from leukemia cell lines or primary samples were treated with the indicated concentrations of CXCR4 inhibitors in 10% FCS-containing RPMI at 37°C for 30 min. A total of 0.5×10^6 treated cells in a volume of 200 μ L were added to the top chamber of 6.5-mm diameter Transwell Culture Inserts (Costar, Corning, NY) with a pore size of 6.5 μ m. Inserts were placed in wells containing 800 μ L of 10% serum RPMI or 10% serum RPMI with the indicated concentra-

tion of SDF-1 α or the indicated number of MS-5 cells. Chemotaxis assays were done at 37°C for 4 or 24 hours. Cells that migrated were counted on a hemacytometer (in triplicates).

Viability Assay

For the cell viability assay, 0.1×10^6 MS-5 cells were plated in six-well plates in α -MEM supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and penicillin-streptomycin at 37°C in 5% CO₂ in a humidified incubator for 3 h. Leukemia cells were incubated for 1 h at 37°C either in 10% serum RPMI alone or in the same medium containing the indicated concentrations of RCP168 at a density of 1×10^6 per mL; seeded on the top of MS-5 stromal cells, which had been washed to remove 10% serum α -MEM; and cocultured with MS-5 cells in 10% serum RPMI for 3 h before the addition of 10 μ mol/L fludarabine or 0.5 μ mol/L cytarabine. After 48 or 72 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, cells were harvested with trypsin/EDTA, washed, and resuspended in binding buffer containing Annexin V (Roche Diagnostic Corp., Indianapolis, IN). Cells were counterstained with CD45-PE (BD PharMingen) or with respective isotype control antibody and analyzed by flow cytometry after electronic gating on CD45⁺ leukemia cells.

Western Blot

For the Western blot analyses, cells were lysed in phosphoprotein lysis buffer (150 mmol/L NaCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 10 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 10 mmol/L β -glycerophosphate, 1% Triton X-100, 10 mmol/L iodoacetamide, 1 mmol/L Na₂VO₄, 0.1% NaN₃, and 3 mmol/L phenylmethylsulfonyl fluoride). Lysis buffer was supplemented with a protease inhibitor cocktail (Roche Diagnostic). Lysates were then separated on a 12% polyacrylamide gel, transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, England), probed with the appropriate antibodies, and visualized using an enhanced chemiluminescence plus kit (Amersham Pharmacia Biotech). Western blots were analyzed on a STORM-860 system by using Imagequant software (Molecular Dynamics, Sunnyvale, CA).

CXCR4 Receptor Expression Studies

For the CXCR4 receptor expression studies, Jurkat or primary CLL cells were adjusted to a density of 0.5×10^6 per mL in RPMI 1640 with 0.5% bovine serum albumin. The cells were cultured with CXCR4 inhibitors either at various concentrations for 1 h or at a constant concentration for the indicated time at 37°C in 5% CO₂ in air. Cells were washed with a 20-fold volume of ice-cold buffer without FCS, stained at 4°C with saturating concentrations of phycoerythrin-conjugated anti-CXCR4 monoclonal antibody, and then analyzed by flow cytometry.

Data and Statistical Analysis

Results are shown as the mean \pm SD or SE of at least three experiments each. Paired data were analyzed using the paired Student's *t* test or Wilcoxon matched paired test. We calculated the Spearman correlation between two variables and tested whether the correlation was

significantly different from 0 with a *t* test. *P*s < 0.05 were considered statistically significant. Flow cytometry data were analyzed using WinMDI flow software (version 2.8).

Results

Surface Expression of CXCR4 in Leukemia Cell Lines and Its Correlation with SDF-1 α - or MS-5-Induced Chemotaxis

Published studies have shown the correlation of surface CXCR4 expression and cell migration in response to SDF-1 α (11, 25). In our studies, we used the murine stromal line MS-5, which is known to provide long-term support for primitive hematopoietic progenitors (26) and has been shown to produce significant amounts of SDF-1 α (27). Notably, MS-5 cells produce SDF-1 α levels comparable with SDF-1 α secretion by human bone marrow stromal cells (28). To determine the relation between MS-5-induced migration of leukemia cells and surface CXCR4 levels, we analyzed SDF-1 α - and MS-5-induced migration in six leukemia cell lines. The histograms show three representative results from the cell lines studied (Fig. 1A). CXCR4 was highly expressed on the surface of acute lymphoid leukemia CEM and Jurkat cells and AML OCI-AML3 and U937 cells. In contrast, AML NB4 and TF-1 cells expressed low levels of CXCR4 on their surfaces, and AML KG-1 and HL-60 leukemia cells expressed no CXCR4 on their surfaces. The chemotaxis studies on Jurkat, U937, and KG-1 cells showed that SDF-1 α (50 ng/mL) and MS-5 cells (0.1×10^6) induced the significant migration of Jurkat and U937 cells ($79 \pm 3.4\%$ and $47 \pm 6.3\%$, $65 \pm 8.1\%$ and $45 \pm 1.3\%$ respectively) but not of KG-1 cells (Fig. 1B). We used the Spearman rank correlation to assess the association between levels of CXCR4 expression and migration. The Spearman rank correlation for CXCR4 expression and MS-5-induced migration was 0.741 (*P* = 0.0382), and the Spearman rank correlation for CXCR4 and SDF-1 α -induced migration was 0.900 (*P* = 0.0117). These results suggest that the chemotactic response induced by SDF-1 α and by MS-5 cells requires the surface expression of CXCR4.

Inhibition of SDF-1 α - or MS-5-Induced Chemotaxis by CXCR4 Inhibitors

Next, the effects of three CXCR4 inhibitors (see below) on the SDF-1 α - or stromal-induced migration of Jurkat cells were examined. RCP168 is a new analogue of the viral macrophage inflammatory protein II in which the first 10 amino acids in the NH₂ terminus have been replaced with D stereoisomers. RCP168 is a prototype molecule for a novel family of synthetically and modularly modified chemokines that are highly selective and potent CXCR4 inhibitors compared with natural chemokines. It has been reported that RCP168 and SDF-1 α bind overlapping yet distinct sites on the CXCR4 receptor. This pattern of RCP168 binding disrupts HIV infection much more potently than SDF-1 α signaling through CXCR4 (nanomolar versus micromolar concentrations), albeit both effects are mediated by modulating the function of

this receptor (29). RCP112 is an analogue of viral macrophage inflammatory protein II in which the first 10 amino acids in the NH₂ terminus have been deleted; it has shown a significantly reduced affinity to CXCR4 (30). DVIP is a 21 D-amino acid peptide of NH₂ terminus viral macrophage inflammatory protein II with a higher biological stability (31). Cell migration experiments showed that all three peptides significantly inhibited SDF-1 α -induced migration, although RCP168 was the most potent (*P* = 0.0012), essentially blocking migration completely (Fig. 1C). In contrast, DVIP and RCP112 did not significantly affect MS-5-induced migration, whereas RCP168 significantly (*P* = 0.0013) inhibited it (Fig. 1D). Based on these results, RCP168 was selected for further functional studies.

Amino acid sequence of three CXCR4 inhibitors

Inhibitor name	Amino acid sequences
RCP112	CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVI FLTKRGRQVCADKSKDWVKKLMQQLPVTAR
RCP168	LGASWHRPDKCCLGYQKRPLPQVL LSSWYPTSQLCSKPGVIFLTKRGRQV- CADKSKDWVKKLMQQLPVTAR
DVIP	LGASWHRPDKCCLGYQKRPLP

Effect of RCP168 on the Surface Expression of CXCR4

The ability of DV1 and related peptides to compete for CXCR4 binding has been shown in Sup-T1 cells using the anti-CXCR4 antibody 12G5 (31). We therefore used 12G5 to examine the dose- and time-related effects of 10 nmol/L RCP168 on the surface expression of CXCR4. Jurkat cells treated with RCP168 displayed markedly reduced CXCR4 levels (~100% loss) at 40 min that returned to basal levels by 240 min. In contrast, SDF-1 α induced a biphasic decrease in the surface expression of CXCR4 that was observed as early as 1 min after exposure (90% loss), returned to baseline levels at 45 min, and then decreased a second time, reaching a plateau at 700 min (60% loss), after which it gradually returned to baseline levels by 1,440 min (24 h; Fig. 2A). These results indicate that the effect of RCP168 on the surface expression of CXCR4 differs from that of SDF-1 α . To determine whether the transient nature of the down-modulating effects of RCP168 on CXCR4 expression could be overcome by increasing concentrations of RCP168, we examined the effects of higher doses of RCP168 on the surface expression of CXCR4 in Jurkat cells after treatment for 24 h. Our results indicated that RCP168 at concentrations >100 nmol/L completely blocked CXCR4 expression for 24 h, which suggests that increasing concentrations of this peptide inhibitor sustain the inhibition of SDF-1 α /CXCR4 interactions (Fig. 2B).

Next, we examined the inhibitory effects of RCP168 on the MS-5-induced migration of primary CLL cells. CXCR4

was highly expressed in all CLL samples, with mean fluorescence intensity ranging from 333 to 2,760 (relative units). RCP168 significantly inhibited the MS-5-induced migration of cells in 6 of the 9 CLL samples examined. Specifically, compared with random migration (mean, 28%; 95% confidence interval, 14–36%), MS-5 stromal cells induced 41% of CLL cells to migrate (95% confidence interval, 30–52%), and RCP168 significantly inhibited this effect (mean, 28%; 95% confidence interval, 16–30%; $P = 0.00183$; Fig. 2C). These results suggest that RCP168 effectively inhibits MS-5-induced migration of CLL cells.

Inhibition of Endogenous and SDF-1 α -Induced AKT and ERK Signaling by RCP168

SDF-1 α /CXCR4 interactions have been shown to trigger AKT and ERK signaling, which accounts for the survival advantage conferred by SDF-1 α (32). We therefore did Western blot analyses of the activation of ERK (phospho-ERK) and AKT (phospho-AKT) in Jurkat cells treated with SDF-1 α alone or in combination with this peptide inhibitor. The results showed that RCP168 down-regulated the basal phospho-AKT level and abrogated the robust phosphorylation of AKT in cells treated with SDF-1 α (Fig. 3A). In contrast, no significant changes in the expression of phospho-ERK were observed (Fig. 3A1). Similarly, RCP168 down-regulated SDF-1 α -induced phospho-AKT levels in a primary CLL sample and also decreased SDF-1 α -induced phospho-ERK expression (Fig. 3B and B1). Similar results were obtained in two additional CLL samples tested (data not shown). These results show that SDF-1 α signaling through CXCR4 activates AKT and ERK and that RCP168 inhibits these responses.

RCP168 Partially Inhibits Stroma-Mediated Resistance of Leukemia Cells to Chemotherapy

The effects of RCP168 on chemosensitivity were investigated in Jurkat cells cocultured with a stromal feeder layer (Fig. 4A). Stromal cells significantly protected these cells from cytarabine-induced apoptosis: $71 \pm 2.0\%$ Jurkat cells treated with $0.5 \mu\text{mol/L}$ cytarabine alone for 48 h displayed Annexin V positivity, whereas only $35 \pm 1.8\%$ of Jurkat cells cocultured with stroma showed Annexin V positivity. Pretreatment with RCP168 partially abrogated stroma-induced protection from cytarabine cytotoxicity, with $53 \pm 1.6\%$ cells being Annexin V positive ($P = 0.0017$). However, RCP168 failed to increase the sensitivity of Jurkat cells to cytarabine in the absence of MS-5 cells, indicating that the effects of RCP168 are specific to the interaction of leukemia cells with the stromal feeder layer.

Next, we examined the ability of stromal cells to support the survival of cells from primary CLL samples. Stromal cells significantly protected primary CLL cells from spontaneous apoptosis ($42.5 \pm 5.7\%$ versus $20.7 \pm 1.7\%$, $P < 0.001$, $n = 23$) and drastically inhibited fludarabine-induced apoptosis ($73.1 \pm 4.3\%$ versus $44.3 \pm 4.0\%$, $P < 0.001$; Fig. 4B). These data suggest that MS-5 stromal cells protect leukemia cells from spontaneous and chemotherapy-induced apoptosis, in agreement with previously published findings (1). Therefore, we investigated whether disrupting SDF-1 α /CXCR4 interactions with RCP168 would sensitize CLL cells to chemotherapy.

Figure 5A shows the effects of RCP168 in cells from a primary CLL sample expressing high levels of CXCR4.

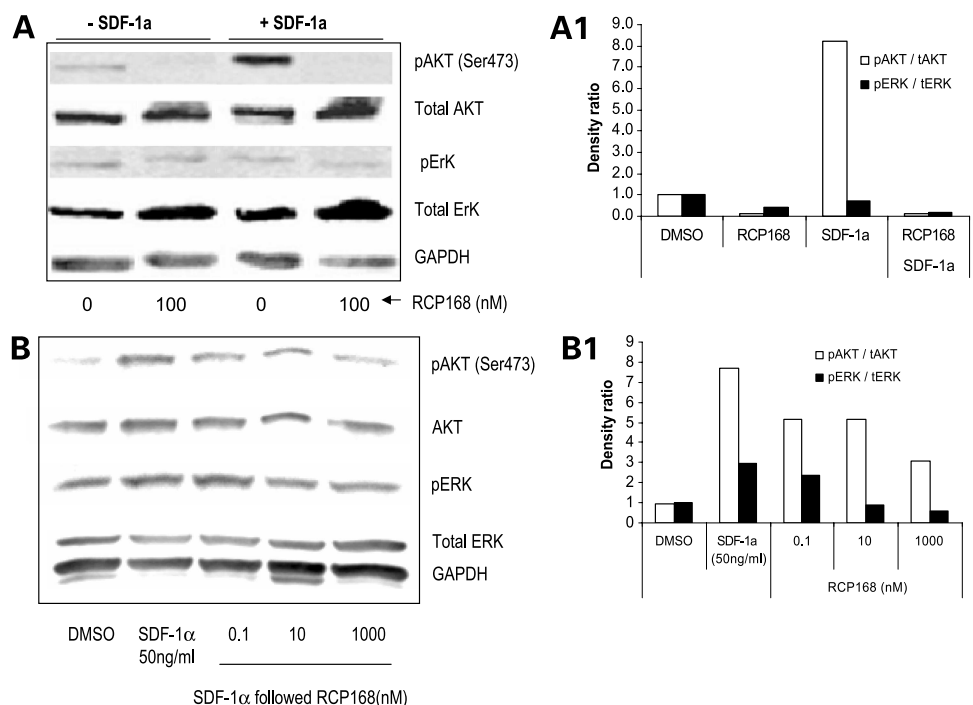


Figure 3. RCP168 down-regulated SDF-1 α -activated signaling pathways. Jurkat cells (**A**) or primary CLL cells (**B**) were left untreated or exposed to 50 ng/mL SDF-1 α , alone or in combination with the indicated concentrations of RCP168 for 4 h. Phosphorylation of AKT and ERK was detected by Western blot, and the intensity of the bands was quantified by densitometry and displayed as ratios of phospho-proteins to total proteins. pAKT, phospho-AKT; pErk, phospho-ERK; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

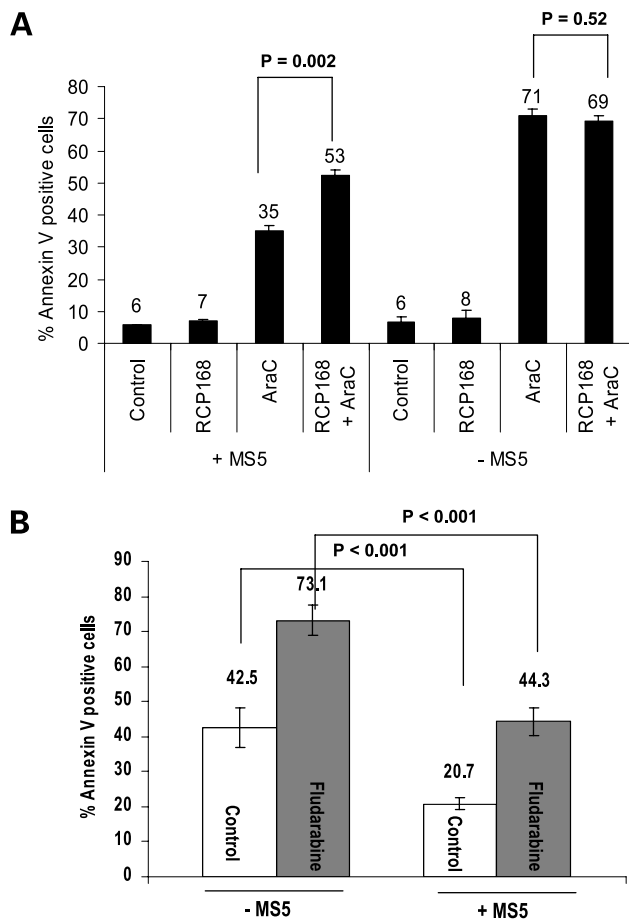


Figure 4. Stroma protects leukemic cells from spontaneous and chemotherapy-induced apoptosis. **A**, RCP168 enhances chemotherapy-induced apoptosis in Jurkat cells cocultured with MS-5 cells. Jurkat cells at a density of 1×10^6 were pretreated with $1 \mu\text{mol/L}$ RCP168 for 1 h and then either cocultured with 0.1×10^6 MS-5 for 3 h followed by $0.5 \mu\text{mol/L}$ cytarabine treatment for 48 h or cultured in medium only with cytarabine for 48 h. Apoptotic cells were detected by Annexin V flow cytometry. **B**, stromal cells protect primary CLLs from spontaneous and chemotherapy-induced apoptosis. Percentage of Annexin V⁺ cells from 23 primary CLL samples cultured alone or with MS-5 cells for 72 h.

Coculture with stromal cells significantly protected the cells in this sample from spontaneous and fludarabine-induced apoptosis. Notably, 58% of CLL cells pretreated with RCP168 followed by fludarabine treatment were apoptotic; in contrast, only 35% of cells treated with fludarabine alone were apoptotic. However, no such difference was seen in primary CLL cells expressing low levels of CXCR4 (Fig. 5B), indicating that CXCR4 surface expression is critical for RCP168 to abrogate stroma-mediated chemoresistance.

Altogether, the effects of RCP168 on chemosensitization were examined in 23 primary CLL samples. In these samples, CXCR4 was expressed at high levels (>80% positive cells compared with isotype control) in 13 samples, at moderate levels ($\leq 80\%$ and $>10\%$) in 8 samples, and at low levels (<10%) in 2 samples. Data shown in Fig. 5C

show that RCP168 did not significantly modulate the degree of spontaneous (without fludarabine) apoptosis in stromal cocultures. In contrast, cells cocultured with stromal cells and pretreated with RCP168 were significantly more sensitive to fludarabine than cells treated with fludarabine alone. Specifically, the inhibition of CXCR4 by RCP168 produced chemosensitization in 22 of the 23 CLL samples, with an average of 16.4% more cells showing apoptosis compared with cells treated with fludarabine alone.

CXCR4 Inhibitors RCP168 and AMD3465 Enhance Sensitivity to Chemotherapy in AML

We have previously shown the expression of CXCR4 in a subset of primary AML progenitor cells (1) and have now studied the consequences of CXCR4 inhibition in AML. We first examined CXCR4 expression in 14 of 15 primary AML samples. CXCR4 was expressed in all 14 samples, with an average mean fluorescence intensity of 72.4 (95% confidence interval, 14.7–130.1), which was significantly less than the CXCR4 levels in CLL cells ($P < 0.001$).

Consistent with data in CLL and our published results, MS-5 significantly inhibited spontaneous or cytarabine-induced apoptosis in 15 primary AML samples ($P = 0.030$ and 0.035 , respectively; data not shown). Furthermore, RCP168 decreased stroma-mediated protection from cytarabine-induced apoptosis in 9 of 15 primary AML samples (Fig. 6A, left). We next compared the effects of RCP168 and AMD3465, the second-generation small-molecule reversible inhibitor of SDF-1 α /CXCR4 with IC_{50} SDF-1 binding of $42 \pm 2 \text{ nmol/L}$.⁶ AMD3465 inhibited stroma-mediated chemoresistance and enhanced cytarabine-induced apoptosis in 8 of 15 samples (Fig. 6A, right). Interestingly, FLT3/ITD mutation was present in 7 of 8 samples that were sensitized to cytarabine by AMD3465 and RCP168, and in the remaining sample, FLT3/D835 point mutation was found. Moreover, the ability of these two structurally different CXCR4 antagonists to enhance the efficacy of cytarabine in stromal cocultures was strongly correlated ($P < 0.01$). An example of the flow cytometry profile in a primary AML sample with FLT3/ITD exposed to cytarabine in combination with RCP168 and AMD3465 is shown in Fig. 6B. In contrast to RCP168, however, AMD3465 also ameliorated the stroma-induced protection of AML cells from spontaneous apoptosis in this sample (18% and 17% apoptotic cells in control or RCP168-treated samples in the MS-5 coculture and 37% apoptotic cells in AMD3465-treated samples in the MS-5 coculture), and the same observations were made in five additional samples (data not shown).

Discussion

In our study, coculture with the murine stromal cell line MS-5 significantly protected CLL cells from spontaneous and fludarabine-induced apoptosis, and the CXCR4

⁶AnorMED, unpublished.

inhibitor RCP168 partially abrogated this protective effect and sensitized the cells to chemotherapy. Hence, our data indicate that disrupting the interaction of CXCR4 and SDF-1 α through the use of a CXCR4 inhibitor is an effective way to attenuate stroma-mediated resistance to apoptosis. These chemosensitizing effects were observed in the majority of primary CLL samples. Notably, RCP168 did not affect sensitivity to fludarabine in CLL cells lacking CXCR4 expression, suggesting that the observed effects were specific to cells expressing CXCR4. Nonetheless, our results illustrate that in heterogeneous primary leukemia populations, not only the levels of CXCR4, but also other components, such as FLT3, critically regulate the sensitivity of these cells to the inhibitory effects of synthetically and modularly modified chemokines. Interestingly, published reports have shown the presence of CXCR4 mutations (33, 34), and Schabath et al. have shown that CD24, a small glycosylphosphatidylinositol-anchored cell surface protein that localizes in lipid rafts, negatively modulates the function of CXCR4, indicating that the functionality of this chemokine receptor and other proteins and/or cell membrane components influence the activity of the SDF-1 α /CXCR4 axis (35). Further studies are thus required to elucidate the critical determinants of this response, including the SDF-1 α -induced activation of intracellular signaling pathways, CXCR4 polymorphisms, the augmentation of integrin-mediated adhesion, and the intracellular localization of CXCR4 itself.

Our results suggest that SDF-1 α triggers a prosurvival pathway in leukemia cells by activating phosphoinositol 3-kinase/AKT and mitogen-activated protein kinase signaling. Both are key signaling pathways for promoting and enhancing leukemia cell survival, proliferation, and protection against apoptosis (36, 37). Albeit we observed that RCP168 moderately decreased basal AKT phosphorylation in leukemia cells alone, supporting the notion of an active autocrine SDF-1 α /CXCR4 axis (38, 39), this agent induced a more striking abrogation of AKT and ERK phosphorylation in response to SDF-1 α in leukemic cell lines and primary patient samples. Consistent with our observations, interactions of CLL cells with a murine fibroblast cell line were reported to sustain basal levels of AKT and nuclear factor- κ B activities that depended on phosphoinositol 3-kinase (40). This was associated with the lack of down-regulation of the prosurvival Bcl-2 family protein Bcl-X_L, the caspase inhibitor proteins FLIP-L and XIAP, and consequently with caspase-3 activation and apoptosis, suggesting a proximal role for the phosphoinositol 3-kinase pathway in stroma-regulated B-CLL cell survival and growth.

In our study, we used the RCP168 peptide from the novel family of synthetically and modularly modified chemokines. Our results showed that this peptide is a potent antagonist of SDF-1 α and a CXCR4 inhibitor, in that it inhibited stromal cell-induced migration and blocked the binding of the anti-CXCR4 12G5 antibody to cell surface CXCR4. Notably, the concentrations of RCP168 sufficient to inhibit SDF-1 α /CXCR4 interactions compares

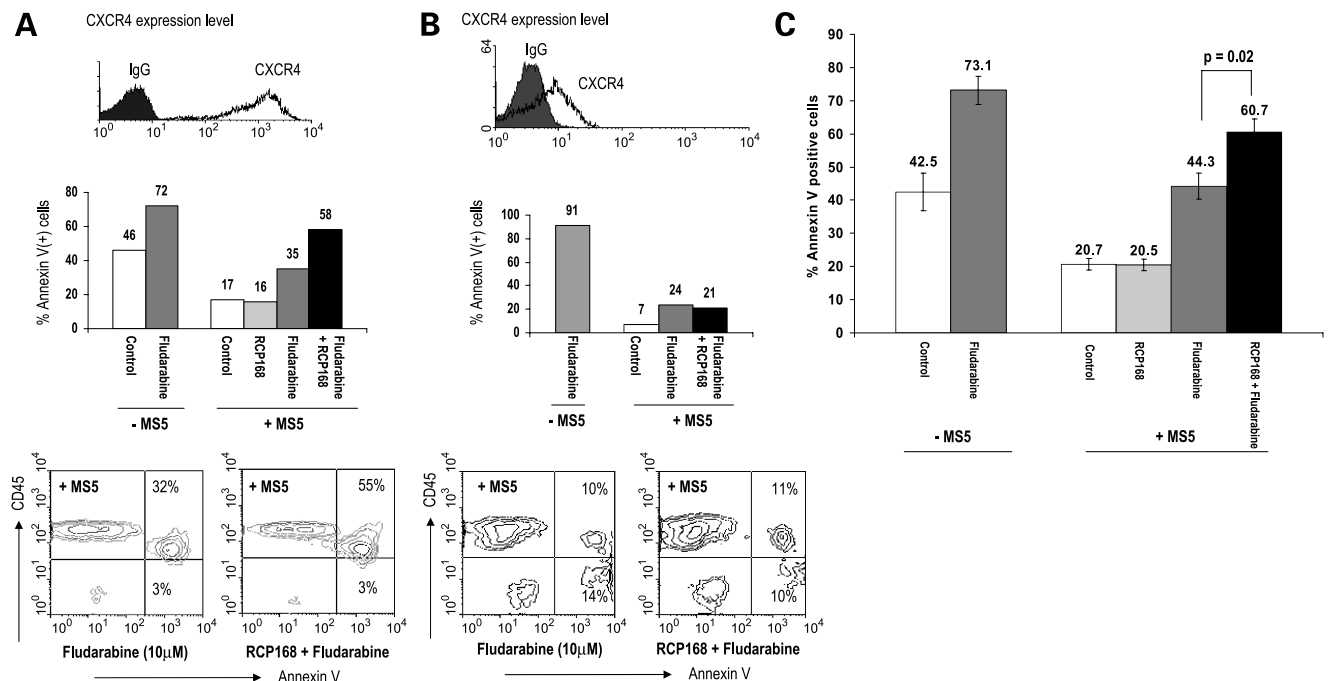


Figure 5. RCP168 sensitizes CLL cells to chemotherapy by disrupting SDF-1 α /CXCR4 interaction. Primary CLL cells with high (A) or low (B) surface expression were treated with either 10 μ M/L fludarabine alone or pretreated with 1 μ M/L RCP168 followed by fludarabine in MS-5 coculture. Middle, % apoptotic cells detected by Annexin V flow cytometry after gating on CD45⁺ leukemia cells; bottom, contour dot plots of flow cytometry results. C, average percentage of apoptotic cells from 23 primary CLL samples induced by fludarabine alone or combined with RCP168.

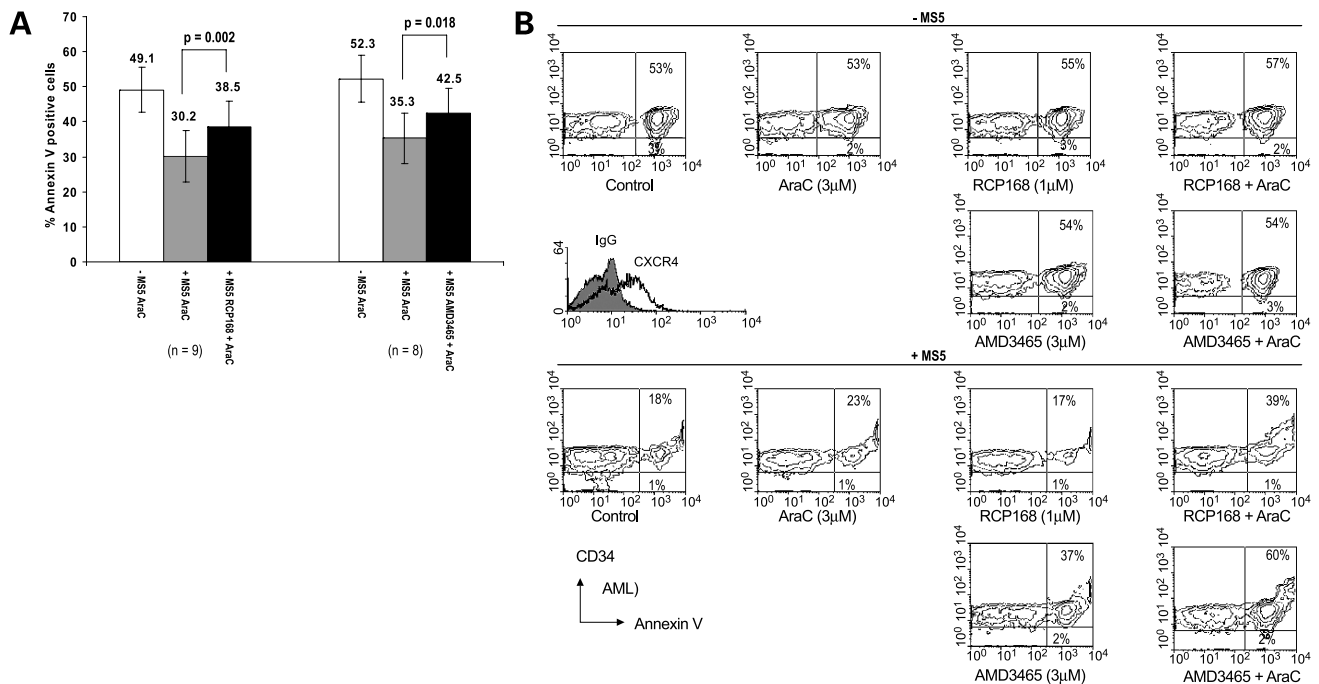


Figure 6. Inhibition of CXCR4 by RCP168 or AMD3465 sensitizes AML cells to chemotherapy. **A**, average percentage of apoptotic cells in primary AML samples induced by cytarabine alone or in combination with either RCP168 (9 of 15 samples shown) or AMD3465 (8 of 15 samples shown). Paired data were analyzed using the Wilcoxon paired matched test. **B**, contour dot plot of flow cytometry results in primary AML sample treated with 3 μ mol/L cytarabine alone, or pretreated with 1 μ mol/L RCP168 (*top*) or AMD3465 (*bottom*) followed by cytarabine in MS-5 cocultures. Apoptotic cells were detected by Annexin V flow cytometry after gating on CD34⁺ leukemic cells.

favorably compare this class of peptides with other peptidic inhibitors of CXCR4, such as T140 (41), thus encouraging further clinical development of these compounds. Several other CXCR4 antagonists have been described, one of which is a small-molecule reversible inhibitor of SDF-1 α /CXCR4 (AMD3100) that has recently shown a remarkable ability to mobilize normal progenitor cells (42, 43). In our AML studies, RCP168 and an analogue of AMD3100 (AMD3465) showed comparable activity. Importantly, the ability of these two structurally different CXCR4 inhibitors to sensitize AML cells to chemotherapy in stromal cocultures was strongly correlated, thus strongly implicating a mechanism-based mode of action.

Our observation that RCP168 inhibits stroma-mediated protection in most CLL samples tested, and only in a subset of AML samples that bear FLT3 mutations suggests that other factors, independent of CXCR4 but associated with FLT3 signaling, play important roles in stroma-mediated AML cell survival. Albeit it would be expected that constitutively activated FLT3 (by mutations or ITD) would perhaps antagonize the antileukemia effects of CXCR4 inhibition, our results suggest a more complex picture whereby constitutively activated FLT3 further increases AML cell dependency on the CXCR4 signaling pathway. The clinical implications of these results are that (a) in principle, CLL is amenable to combination treatment with CXCR4 inhibitors and cytotoxic agents; (b) that modulating FLT3 signaling may be necessary to enhance the efficacy of CXCR4 inhibitors in

combination with cytotoxic chemotherapy; and (c) that there is a potential clinical benefit of treating FLT3 mutant AML, the most common mutation in AML, with CXCR4 inhibitors in combination with cytotoxic agents. These concepts are currently being investigated in our ongoing studies.

Taken together, our findings indicate that SDF-1 α /CXCR4 interactions contribute to the resistance of leukemia cells to chemotherapy-induced apoptosis and identify significant differences in the contributions of this pathway to the chemoprotection of myeloid versus lymphoid malignancies and wild-type versus mutant FLT3 AML. Most importantly, our data provide proof of principle that the inhibition of CXCR4 signaling can help overcome the protection conferred by stromal cells in CLL and FLT3 mutant AML and thereby enhance the efficacy of chemotherapy. These results indicate a potential role for CXCR4 inhibitors as a novel strategy for targeting leukemia cell/bone marrow microenvironment interactions.

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