Combination of drug therapy in acute lymphoblastic leukemia with a CXCR4 antagonist

Reshmi Parameswaran¹, Min Yu¹, Min Lim¹, John Groffen¹,²,³, and Nora Heisterkamp¹,²,³

¹Section of Molecular Carcinogenesis, Division of Hematology/Oncology and The Saban Research Institute of Childrens Hospital Los Angeles, CA 90027, USA
²Leukemia Research Program, Childrens Hospital Los Angeles
³Leukemia and Lymphoma Program, Norris Comprehensive Cancer Center, University of Southern California, CA, USA

Abstract

The bone marrow (BM) stromal niche can protect acute lymphoblastic leukemia (ALL) cells against the cytotoxicity of chemotherapeutic agents and is a possible source of relapse. The SDF-1/CXCR4 axis is a major determinant in the crosstalk between leukemic cells and BM stroma. In the current study, we investigated the use of AMD11070, an orally available, small molecule antagonist of CXCR4, as an ALL-sensitizing agent. This compound effectively blocked stromal-induced migration of human ALL cells in culture and disrupted pre-established adhesion to stroma. To examine how to optimally use this compound in vivo, several combinations with cytotoxic drugs were tested in a stromal co-culture system. The best treatment regimen was then tested in vivo. Mice transplanted with murine Bcr/Abl ALL cells survived significantly longer when treated with a combination of nilotinib and AMD11070. Similarly, immunocompromised mice transplanted with human ALL cells and treated with vincristine and AMD11070 had few circulating leukemic cells, normal spleens and reduced human CD19⁺ cells in the bone marrow at the termination of the experiment. These results show that combined treatment with AMD11070 may be of significant benefit in eradicating residual leukemia cells at locations where they would otherwise be protected by stroma.

Keywords

AMD11070; AMD3100; SDF-1α; stromal co-culture; Ph-positive ALL; leukemia cell mobilization

Users may view, print, copy, download and text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

Correspondence: Dr N Heisterkamp, Division of Hematology/Oncology, Ms#54, Childrens Hospital Los Angeles, 4650 Sunset Boulevard, Los Angeles CA 90027, U.S.A.; tel 323-361-4595, fax: 323-671-3613. heisterk@hsc.usc.edu.

Conflict of interest

The authors declare no conflict of interest.
Introduction

Acute lymphoblastic leukemia (ALL) accounts for about 80% of childhood leukemias, but comprises only 20% of adult leukemias. Advances in therapy have led to survival rates exceeding 80% in children, but only 30-40% of adults achieve long-term disease-free survival. The complete eradication of residual leukemia-initiating cells and progenitors, which present a persistent risk for disease relapse, forms one of the major challenges in ALL therapy. Thus, the development of new drugs and approaches for the treatment of relapse remains an important goal for improved cure rates.

The lack of efficacy of treatment can be partly attributed to the fact that leukemia cells are protected by their microenvironment. Leukemic cells residing in bone marrow niches are provided with favorable conditions for their growth and survival and thereby escape from chemotherapy-induced death. The binding of stromal-derived factor-1 (SDF-1; also named CXCL12) with its receptor CXCR4 is one of the key interactions that take place between human ALL cells and bone marrow stroma. SDF-1 is constitutively produced by many cell types, including immature osteoblasts and endothelial cells within the bone marrow as well as by epithelial cells in many organs, including the central nervous system and is known to stimulate the growth of normal pre-B cells.

SDF-1 stimulation has multiple effects on cells. Intracellular events induced by it include elevation of cytoplasmic Ca\(^{2+}\) levels, activation of phosphoinositide 3-kinase (PI-3 kinase), and phosphorylation of mitogen-activated protein kinase/extracellular signal regulated kinase (MEK/ERK) in several cell types. In pre-B ALL cells, p38 MAPK signaling is required for SDF-1 induced chemotaxis, but not for proliferation of the cells. Spiegel et al. studied the migration and in vivo homing of pre-B ALL cells to the bone marrow of nonobese diabetic, severe combined immunodeficient (NOD/SCID) mice transplanted with ALL cells in comparison to normal CD34+ progenitors. They found that Toxin-B and pertussis toxin inhibited the homing of the leukemic cells, but not that of normal CD34+ progenitors or normal CD10+/CD19+ precursor-B cells, revealing differences in CXCR4 signaling pathways that are based on changes that were acquired by the leukemic cells. It has also been shown that CXCR4 desensitization, by pretreatment of human ALL cells with high levels of SDF-1 in vitro prior to their transplantation, decreases their homing and engraftment levels in NOD/SCID mice that receive transplants.

Because of the importance of CXCR4-SDF-1 in ALL as well as in other hematological malignancies, there is considerable interest in exploring the possible beneficial therapeutic effects of blocking the activity of this receptor/ligand combination. One of the most widely studied inhibitors is plerixafor (AMD3100). Using an in vitro system, Juarez et al. previously demonstrated a sensitizing role toward treatment with chemotherapeutics for AMD3100 in the pre B-ALL cell line NALM-6 and in several ALL patient samples. Inhibition of SDF-1-induced migration of cultured cells from adult T-cell leukemia/lymphoma patients by AMD3100 has also been reported. Nervi et al. reported that treatment with chemotherapy and AMD3100 decreased the tumor burden in a mouse model of acute promyelocytic leukemia. In multiple clinical studies, AMD3100 was found to rapidly and effectively mobilize hematopoietic stem cells into the circulation and it is
currently under development as a stem cell mobilization agent prior to high-dose chemotherapy for multiple myeloma, non-Hodgkin lymphoma, and other hematologic malignancies.\textsuperscript{25,28} AMD3465, a different CXCR4 antagonist, inhibited migration of AML cells by repressing SDF-1\textalpha/CXCR4 signaling.\textsuperscript{29}

Philadelphia chromosome (Ph)-positive leukemias include chronic myelogenous leukemia (CML) and Ph-positive ALL. The latter represents the most common cytogenetic abnormality in adult ALL, in which a constitutively active Bcr/Abl tyrosine kinase is present.\textsuperscript{30} It is found in 15\% to 30\% of patients, and its incidence increases with age. As in children, prognosis in Ph-positive adult ALL is poor. Both Dillmann and Vianello et al\textsuperscript{31,32} showed that the combination of AMD3100 treatment with the targeted tyrosine kinase inhibitors imatinib or nilotinib in the presence of stroma increased the ability of the therapeutic drug to cause apoptosis in the CML cells.

Limitations of AMD3100 include a relatively short (3.5 -4.9 hrs) half-life and the need to administer it via injections.\textsuperscript{33} We therefore were interested in testing alternative CXCR4 antagonists for activity in the treatment of ALL including poor-prognosis subtypes such as Ph-positive ALL. AMD11070 is a noncyclam, orally available CXCR4 antagonist which was shown to inhibit replication of X4-tropic HIV in peripheral blood mononuclear cells and in cell lines \textit{in vitro}\textsuperscript{34} with a half life of 11.2-15.9 hours.\textsuperscript{35} Its possible use for stem cell mobilization or for the treatment of malignancies has not been reported. We here present evidence that AMD11070 is very effective in inhibiting CXCR4/SDF-1-mediated events in ALL cells and its combination with a chemotherapeutic drug significantly increased survival in two transplant models of ALL.

\textbf{Materials and methods}

\textbf{Mice and cells}

All animal experiments were carried out in concordance with Institutional IACUC and NIH guidelines. We used NOD.Cg-Prkdc\textsuperscript{scid}Il2rg\textsuperscript{tm1Wjl}/SzJ mice (abbreviated here as NSG; Jackson Labs) transplanted with human ALL cells for \textit{in vivo} drug testing.\textsuperscript{36} The human ALL cells used here and the stromal co-culture system have been described previously.\textsuperscript{37,38}

\textbf{Reagents and antibodies}

AMD11070 was obtained from Genzyme, MA, USA. AMD3100 was purchased from Sigma-Aldrich (St.Louis, USA). Anti-human CD184 (CXCR4, clone 12G5) and CD19 as well as anti-mouse AA4.1 antibodies were from BD Pharmingen (San Jose, USA). Nilotinib (AMN107) was obtained from Novartis. Recombinant SDF-1\textalpha was from Peprotech Inc. (New Jersey, USA). Antibodies to total and phosphorylated forms of p38, ERK and AKT were from Cell Signaling Technology (MA, USA). The CXCR4 antibody used for Western blotting was from Abcam (MA, USA).

\textbf{Migration assays}

For migration assays, human ALL cells (5\times10^4) were seeded into the upper well of a 5 \textmu m pore size Transwell. The lower chamber contained either SDF-1\textalpha (200 ng/ml) in medium or...
a layer of irradiated OP9 stromal cells plated 24 hrs earlier. Wells without SDF-1α or stromal cells in the bottom chamber served as controls. ALL cells were pre-treated with AMD3100 or AMD11070 (10 μM) -or left untreated- for 30 minutes at 4°C and then seeded into the upper wells. ALL cells migrated to the bottom wells were counted after 90 minutes (SDF-1α) or after overnight incubation (OP9 stroma), using an automated cell counter.

For adhesion assays, human ALL cells were cultured on OP9 stromal cells. After 2 weeks, culture plates were washed to remove the floating cells and AMD3100 (10 μM), AMD11070 (10 μM), U0126 (10 μM) or SB203590 (10 μM) was added to the new culture media.

**In vitro combination treatments**

US.7 cells were treated with either 2.5 nM vincristine, 1 μM AMD11070 or a combination of both. Medium was changed every alternate day and replenished with fresh drug. Care was taken not to destroy the stromal layer. Different conditions were used for the combination treatment. We added vincristine and AMD11070 together, or treated with vincristine alone for the first 6 days and then started with the combination of AMD11070 and vincristine, or we first treated for 6 days with AMD11070 alone and then used a combination of both. A similar experiment was done with TXL-2 cells, but using AMD11070 (1 μM) and nilotinib (300 nM). US.7 was also tested with 10 μM UO126, 2.5 nM vincristine or a combination of both, and 20 μM SB203590 alone or combined with 2.5 nM vincristine. Cell viability was analyzed by the Trypan blue exclusion method.

**Treatment in NSG model of human leukemia**

US.7 cells (1.5 × 10^6 cells/mouse) were injected i.v. into NSG mice. 5 days after transplantation, groups of 5 mice were treated with saline, vincristine (0.5 mg/kg), AMD11070 (10 mg/kg) or a combination of vincristine (0.5 mg/kg) and AMD11070 (10 mg/kg). For the combination treatment group, mice were treated with vincristine alone (once per week) for the first 2 weeks and then vincristine and AMD11070 were administered on the same day; vincristine was injected (i.p.) 5 hours after oral administration of AMD11070, which was mixed with peanut butter and oil. All vincristine only and control mice were also fed an equivalent amount of peanut butter/oil. All treatment was done once a week (see table Figure 4b). Mice were monitored for condition and weight loss. Once more than 20% of the initial weight was lost, or signs of leukemia became evident, animals were sacrificed. Four of five mice in the combination treatment group were alive and active, without showing any signs of leukemia, at day 56 after transplant. Bone marrow and peripheral blood were collected from all animals. After RBC lysis and blocking with Fc block, cells were incubated with anti human CD19 or anti human CXCR4 antibodies. After 45 minutes incubation, cells were analyzed using an Accuri flow cytometer (MI, USA). Peripheral blood collected 5 hours after treatment was also examined for the expression of human CD19 and human CXCR4 using FACS analysis. Livers were fixed in 10% formalin, dehydrated and embedded in paraffin. 5-μm tissue sections were prepared and stained with hematoxylin and eosin.
Mouse model for Bcr/Abl- positive leukemia in C57Bl/6J mice

C57Bl/6J mice were purchased from Jackson Labs. Mice were intravenously injected with 8093 cells (1×10^5 cells/mouse)\(^39\). Equal leukemia burden was assessed on day 6 after transplant by the presence of AA4.1+ ALL cells in submandibular PB samples (Supplementary Figure 3b), after which mice were randomized for treatment. 5 mice per group were treated with only peanut butter/oil (control). Ten mice were treated with nilotinib (75 mg/kg/d) for 5 days and assessed for leukemia cell load by AA4.1-expressing cells in the PB on day 10. Mice were randomized on d12 to receive continued treatment with nilotinib (75 mg/kg/d) for 7 more days, or with a combination of nilotinib and AMD11070 (10 mg/kg) (Supplementary Figure 3a). For the combination treatment, nilotinib was administered 5 hours after oral administration of AMD11070.

Statistical analysis

All in vitro experiments were done in triplicate wells and student's T-test was performed to assess statistical significance of the results. The significance of survival was analyzed using the log rank test.

Results

AMD11070 binds to human ALL cells

It has been reported that the binding of CXCR4 antagonists such as AMD3100 to CXCR4 blocks the binding of the anti-CXCR4 monoclonal antibody 12G5 to the receptor.\(^40\) We used this method to test the ability of AMD11070 to bind to CXCR4 on the human ALLs US.7, US.7R and TXL-2. US.7 and US.7R are two isolates taken from the same patient before and after that patient developed drug resistance while on therapy. TXL-2 is a Ph-positive ALL from a patient at diagnosis. These cells expressed relatively high levels of cell surface CXCR4 (84%, 81% and 76% respectively; not shown) as detected by the 12G5 anti-CXCR4 antibodies. Five primary Ph-positive ALLs and one Ph-negative ALL before passage in NSG mice also expressed high cell surface levels of CXCR4, varying from 50 to over 99% of the cells, and this was maintained after sequential transplant (Supplementary Table 1). This is in agreement with Crazzolara et al\(^41\) who measured CXCR4 on leukemia blasts from the bone marrow of children with ALL and found that 92% of samples (of the 56 examined) were positive for cell surface CXCR4 using FACS.

Figure 1a shows a comparative analysis between AMD11070 and AMD3100 for their ability to reduce cell surface CXCR4 detection by the anti-CXCR4 12G5 monoclonal antibody on US.7, US.7R and TXL-2. Although the degree of inhibition varied between the individual patient samples, all three showed an increased reduction of CXCR4 cell surface detection after treatment with AMD11070 compared to AMD3100. Concentrations as low as 1 μM AMD11070 inhibited more than 60% of the cell surface CXCR4 detection in comparison to non-treated control cells. Western blot analysis (Figure 1b) was performed to confirm that there was no change in total expression of CXCR4 in these cells and that only the detection of CXCR4 using the monoclonal antibody was affected due to the binding of AMD11070 or AMD3100.
To compare the kinetics of binding, we incubated US.7, US.7R and TXL-2 cells with AMD3100 or AMD11070 for 0-20 minutes. Figure 1c shows that the binding of AMD11070, as judged by reduced binding of the anti-CXCR4 antibody, was rapid and measurable even after 5 minutes of incubation, whereas this could not yet be detected for AMD3100 at this time point.

**AMD11070 inhibits SDF-α induced signaling pathways in ALL cells and blocks the migration of ALL cells towards SDF-1 and stroma**

Since AMD11070 binds to ALL cells and blocks CXCR4 detection, we examined whether it could also block SDF-1/CXCR4 intracellular signaling. Bendall *et al* reported that SDF-1 induces ERK, AKT and p38MAPK pathway activation in pre-B ALL cells recovered from the spleen of xenografted mice and expanded in stroma-supported culture. Therefore, we tested the effect of the presence of AMD11070 on SDF-1α-mediated stimulation of p38, AKT and ERK. As shown in Figure 2a, p38 phosphorylation was induced in the presence of SDF-1α, which could be blocked by pretreatment with AMD11070. SDF-1α also induced ERK phosphorylation that was inhibitable by AMD11070. AKT phosphorylation was visibly not affected by SDF-1α stimulation or by AMD11070 treatment in these cells.

As expected and reported previously for other ALLs, SDF-1α was a strong chemoattractant. As shown in Figure 2b, pretreatment of the cells with AMD11070 inhibited the SDF-1α-induced migration of US.7, US.7R and TXL-2 cells by 79%, 77% and 61% respectively, compared to control cells without AMD11070 treatment. AMD11070 also inhibited the migration of US.7, US.7R and TXL-2 cells towards stroma by 62%, 57% and 51% respectively (Figure 2c). Migration of human ALL cells towards SDF-1α and stroma was inhibited more strongly by AMD11070 than by AMD3100 (Figure 2b, c).

**Combination treatment of AMD11070 with chemotherapeutic drugs reduces recovery from treatment**

If drugs such as AMD11070 are to be used to dislodge leukemic cells from the bone marrow in combination with chemotherapy, they should be able to break existing interactions. To determine the efficacy of AMD11070 for dislodging human ALL cells from stroma, we first co-cultured the ALL cells on a stromal feeder layer for 14 days, at which point the stroma is saturated with leukemia cells. Under these circumstances, leukemia cells form a dynamic association with the stroma, with attachment, migration underneath and appearance of cells in the medium. We then added AMD11070 or AMD3100 and measured the number of cells appearing in the medium after 2, 6, and 9 hours. Because of the motile nature of these ALL cells and their dynamic association with stroma, cells appeared in the medium also in the control at the 6 and 9 hour time points. As shown in Figure 3a, a 6-hour incubation with AMD11070 roughly doubled the number of cells in the medium compared to non-drug treated cells.

Since stimulation of CXCR4 with SDF-1 activates p38 and Erk, we considered the possibility that one or both of these pathways is responsible for the SDF-1α-mediated adhesion of the ALL cells. To investigate this, we also examined if the p38 inhibitor SB203590 or the Erk inhibitor U0126 was able to mobilize ALL cells. However, neither
inhibitor caused the appearance of cells in the supernatant after 8 hours of treatment, as compared to AMD11070 (Supplementary Figure 1a). Since both drugs did affect the viability of the ALL cells alone or in combination with vincristine, a drug that is part of remission induction therapy for pediatric ALL (Supplementary Figure 1b, c), these pathways do appear to be important for survival of ALL cells in the presence of stroma.

We next used US.7 and TXL-2 cells for combination treatments with AMD11070. We treated US.7 cells with 2.5 nM vincristine. TXL-2 can become resistant to 300 nM of the tyrosine kinase inhibitor nilotinib after 2-3 weeks of treatment, when provided with stromal support. In such cultures, the percentage of living cells typically decreases over a period of 14 days, followed by an increase in cell numbers (Supplementary Figure 2). When we added AMD11070 and vincristine together at day t =1, emergence of vincristine-resistant US.7 cells was marginally delayed compared to vincristine-only treated cells (Figure 3b, compare black and open triangles). Not surprisingly, treatment of the cells with AMD11070 alone for 6 days followed by addition of vincristine initially resulted in a higher percentage of viable cells, because AMD11070 alone affected neither the proliferation rate nor the viability of these cells. When vincristine was added as monotherapy for the first 6 days and then AMD11070 was combined with it, cells appeared to have difficulties in recovering and the viability, in comparison with vincristine-only treated cells, did not improve by day 21 (Figure 3b, compare open circles with open triangles). Interestingly, although the mode of action and cellular target of nilotinib is very different from that of vincristine, we obtained very similar results with the nilotinib and AMD11070 combination (Figure 3c, open circles): when we first treated the cells with nilotinib alone for 6 days, followed by combined treatment with nilotinib and AMD11070, viability did not increase at day 21 and cells did not resume proliferation (Supplementary Figure 2 bottom panel). Overall, the three ALL samples tested (US.7, US.7R, TXL-2) showed a qualitative similar response in all parameters tested.

**AMD11070 prolongs the survival of NSG mice transplanted with human ALL cells**

The results of the experiments with co-cultured stromal cells suggested that the optimal method of combining a therapeutic drug with AMD11070 would be to first apply the therapeutic drug. To translate our *in vitro* findings into an *in vivo* system, we used $1.5 \times 10^6$ US.7 cells for a transplant to NSG mice, and allowed the cells to migrate to the bone marrow and to form an enlarged leukemia burden. Five days after the transplant, groups of mice were injected with saline or vincristine (0.5 mg/kg) on days 5, 12, 19, 26 and 33. AMD11070 (10 mg/kg) was added to the vincristine on day 19, 26 and 33 in one group. We also treated mice with AMD11070 alone on day 19, 26 and 33 (see Figure 4b, schematic).

Peripheral blood samples collected from AMD11070-monotreated mice 5 hours after administration of the drug showed mobilization of leukemic cells into the circulation, in comparison to the saline injected mice, as detected by human CD19 expression (Figure 4a). As shown in Figure 4a (right panel), there was reduced detection of CXCR4 in the gated leukemic cell population, compared to the saline treated mice (17% CD19+, CXCR4+ cells [MFI ratio 3.4] versus 93% CD19+, CXCR4+ cells [MFI ratio 12.6]) confirming the binding of AMD11070 to these cells.
To ensure that the leukemic cells pushed into the circulation come in contact with the chemotherapeutic drug, we injected vincristine 5 hours after the oral administration of AMD11070. Treatment was terminated once all the mice in the control and AMD11070-alone treated groups showed a significant reduction in their body weight due to disease. At this dose, the vincristine mono-treatment group showed a small and not statistically significant difference in survival compared to control and AMD alone treated groups. As shown in Figure 4b, a significantly prolonged survival (p<0.05) was observed in those mice that received the combination treatment. At the time of termination of the experiment, 56 days after transplant of the leukemic cells, four of five mice in the combination treatment group looked healthy and had no reduction in body weight (Figure 4c).

**Combination treatment using AMD11070 and vincristine reduces leukemic cell numbers at extramedullary sites**

Terminal bone marrow and peripheral blood samples of all mice were analyzed for the presence of leukemic cells. As shown in Figure 5a, 45-70% of the total cells in peripheral blood of the control, vincristine and AMD11070 treatment groups were CD19-positive, with a clearly reduced amount of circulating leukemia cells in the combination treatment group. Also 51-91% of the cells in total bone marrow were positive for human CD19 by FACS, indicating a very high degree of infiltration and proliferation of the ALL cells in the control, vincristine and AMD11070 treatment groups, with a much lower percentage in the combination treatment group (Figure 5b). Moreover, the spleens of the combination treatment group were markedly smaller as compared to the other treatment groups (Figure 5c). Also, no obvious infiltration of leukemic cells in livers of the combination treatment group was visible, compared to marked infiltration in the vincristine treatment group (Figure 5d).

**AMD11070 prolongs the survival of C57bl mice transplanted with mouse Bcr/Abl-positive ALL cells**

We also tested the sensitizing effect of AMD11070 in a non-immunocompromised mouse model for the subtype of ALL caused by Bcr/Abl. Seven days after transplant of mouse Bcr/Abl lymphoblastic leukemia 8093 cells $^{39}$ ($1 \times 10^5$) into syngeneic C57Bl/6J mice, treatment was started. At day 6, randomly selected mice had a comparably high leukemia burden in the peripheral blood (Supplementary Figure 3b). 5 animals were treated with vehicle only (peanut butter/oil). A second group of 10 animals was treated for 5 days with nilotinib to reduce the tumor burden. Treatment of these animals was equally effective as determined by the AA4.1+ ALL cell burden in PB (Supplementary Figure 3b, d10). 5 mice then received continued treatment with nilotinib for 7 additional days, whereas the other group of 5 was treated with nilotinib and AMD11070 for 7 more days (Supplementary Figure 3a). Treatment was terminated on day 18. As shown in Figure 6a, the group that received the combination treatment showed a significantly prolonged survival (p<0.02) compared with the group treated with nilotinib alone. Peripheral blood and bone marrow analysis showed a clear reduction of tumor load in the combination treatment group, compared with control or nilotinib alone treated group (Figure 6b, c).
Discussion

The current study was undertaken to explore the concept of mobilizing leukemic cells out from a protective niche to a location where they are more sensitive to therapeutic drug treatment. To this end, we sought a compound with strong mobilizing activity. We first compared the effectiveness of the widely studied AMD3100 with that of AMD11070, a noncyclam CXCR4 antagonist that is chemically unrelated to AMD3100, using an in vitro co-culture system of human ALL cells with stroma.

In the in vitro assays used, including blocking of the detection of CXCR4 and the chemotaxis of two different ALL cells towards SDF-1α and stroma, equivalent concentrations of AMD11070 produced a stronger response than AMD3100. Wong et al\textsuperscript{42} who performed a detailed analysis on AMD3100 and AMD11070, concluded that these two compounds make contact with overlapping but not identical residues in the binding pocket of the receptor. For example, AMD11070 but not AMD3100 contacts residue D97 of CXCR4 and is unable to bind to a D97N mutant. Therefore, such compounds may have different receptor affinities, binding constants and/or effects on blocking the multiple downstream effects of CXCR4 receptor engagement, which could explain the different potencies, measured here.

Phase I dose escalation studies in humans performed with AMD11070 by Stone et al\textsuperscript{35} showed a C\textsubscript{min} ranging from 17-210 ng/ml and a C\textsubscript{max} of 320-2430 ng/ml in PB, with a daily oral dose ranging from 50 to 400 mg. In the range of concentrations tested by us in the co-culture system, 1 μM (corresponding to 349 ng/ml) still produced a significant effect and is thus within the range that is clinically relevant in humans. We found that this dose was sufficient to inhibit SDF-1α-induced activation of the p38/Erk kinase pathways and migration towards SDF-1α and stroma. A dose of 10 μM also mobilized ALL cells from under a pre-established stromal feeder layer, indicating it was able to displace or replace SDF-1α that was produced by the stroma and move cells away from the protection provided by it.

The use of mobilizing agents in leukemia has the potential to exacerbate the pathology, by spreading the malignant cells to anatomical locations where they previously were not present. A clinical trial using G-CSF in combination with imatinib did not show any added benefit in CML.\textsuperscript{43} Therefore, we reasoned that it is critically important to ensure that mobilization and leukemia cell killing are well-coordinated. We modeled such treatment in vitro and confirmed that AMD11070 alone has no significant toxic effect on two different types of ALL, one Ph-chromosome-positive, the other Ph-chromosome negative. This suggests that SDF-1α does not provide survival signals to ALL cells, although it did activate the p38 and Erk pathways, and inhibition of these pathways caused cell death. However, the ERK and p38 inhibitors failed to act as mobilization agents for leukemic cells, while AMD11070 was successful in breaking the already existing interaction between stromal cells and leukemic cells. This indicates, that the mechanisms by which those inhibitors kill cells differs from that used by AMD11070 in an adjuvant setting.
To date, no in vivo studies have reported the use of CXCR4 antagonists in ALL therapy. We therefore next tested the combination therapy for ALL, using a mouse model of human leukemia. We adopted the timing and pattern from the in vitro experiment for administering the drugs in vivo. Stone et al\(^3\) reported leukocytosis ranging from 1.3 to 2.9-fold, with a peak 2-4 hours after dosing of AMD11070 in humans, dependent on whether the subjects had eaten or not. Taking this consideration, vincristine was injected 5 hours after the oral administration of AMD11070 to animals, which were allowed to feed ad libitum. The results of this treatment, as compared to vincristine alone, were quite remarkable. Although one animal developed overt leukemia, the remaining had a significantly reduced leukemia burden, with normal-sized spleens, very low peripheral blood leukemic cell numbers and relatively small amounts of residual leukemic cells in the bone marrow. Taking into consideration that this result was obtained by adding three injections of 10 mg/kg AMD11070 to a standard chemotherapy drug, this appears to be a remarkably effective method of boosting the cytotoxicity of vincristine. In addition, we were able to prolong the survival of immunocompetent mice that had a high tumor burden of Bcr/Abl-expressing ALL cells, by combining AMD11070 with the targeted small molecule inhibitor nilotinib. A phase I study using AMD11070\(^3\) did not raise any safety concerns, but long-term animal studies revealed histological changes in the liver.\(^4\) In the treatment protocol used here, there are unlikely to be major adverse side-effects because of the short duration (once per week) and dose of drug used. Although human and rodents cannot be directly compared, a dose of 10 mg/kg used here would translate into 600 mg for a moderately heavy patient. The maximum dose tested by Stone et al\(^3\) was 7 doses of 400 mg administered every 12 hours and thus exceeds by far the amount used here.

CXCR4 is expressed by many other malignant cell types including acute myeloid leukemia, multiple myeloma, B-cell lymphomas, breast cancer, lung cancer, neuroblastoma, colorectal cancer, prostate cancer, melanoma, renal cell cancer and ovarian cancer.\(^4,5\) Based on our results, it would appear to be interesting to examine the effect of AMD11070 in combination with cytotoxic therapies for reducing metastatic load of other cancers in organs such as bone marrow and spleen.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

We thank Donna Foster for excellent care of the mice. This work was supported by PHS grants CA090321 (to NH) and by the William Lawrence & Blanche Hughes Foundation (NH, JG).

**References**

1. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, et al. Cancer statistics, 2004. CA Cancer J Clin. 2004; 54:8–29. [PubMed: 14974761]
2. Fullmer A, O’Brien S, Kantarjian H, Jabbour E. Novel therapies for relapsed acute lymphoblastic leukemia. Curr Hematol Malig Rep. 2009; 4:148–156. [PubMed: 20425428]
3. Sadowitz PD, Smith SD, Shuster J, Wharam MD, Buchanan GR, Rivera GK. Treatment of late bone marrow relapse in children with acute lymphoblastic leukemia: a Pediatric Oncology Group study. Blood. 1993; 81:602–609. [PubMed: 8427957]

4. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med. 2006; 354:166–178. [PubMed: 16407512]

5. Gaynon PS. Childhood acute lymphoblastic leukaemia and relapse. Br J Haematol. 2005; 131:579–587. [PubMed: 16351633]

6. Ottmann OG, Druker BJ, Sawyers CL, Goldman JM, Reiffers J, Silver RT, et al. A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. Blood. 2002; 100:1965–1971. [PubMed: 12200353]

7. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001; 344:1038–1042. [PubMed: 11287973]

8. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. Nature reviews. 2006; 2:107–116.

9. Bradstock K, Bianchi A, Makrynikola V, Filshie R, Gottlieb D. Long-term survival and proliferation of precursor-B acute lymphoblastic leukemia cells on human bone marrow stroma. Leukemia. 1996; 10:813–820. [PubMed: 8656676]

10. Bradstock KF, Makrynikola V, Bianchi A, Shen W, Hewson J, Gottlieb DJ. Effects of the chemokine stromal cell-derived factor-1 on the migration and localization of precursor-B acute lymphoblastic leukemia cells within bone marrow stromal layers. Leukemia. 2000; 14:882–888. [PubMed: 10803521]

11. Mishra S, Zhang B, Cunnick JM, Heisterkamp N, Groffen J. Resistance to imatinib of bcr/abl p190 lymphoblastic leukemia cells. Cancer Res. 2006; 66:5387–5393. [PubMed: 16707466]

12. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood. 2000; 96:2655–2663. [PubMed: 11023495]

13. Manabe A, Murti KG, Coustan-Smith E, Kumagai M, Behm FG, Raimondi SC, et al. Adhesion-dependent survival of normal and leukemic human B lymphoblasts on bone marrow stromal cells. Blood. 1994; 83:758–766. [PubMed: 7507732]

14. Ponomaryov T, Peled A, Petit I, Taichman RS, Habler L, Sandbank J, et al. Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. J Clin Invest. 2000; 106:1331–1339. [PubMed: 11104786]

15. Imai K, Kobayashi M, Wang J, Shinobu N, Yoshida H, Hamada J, et al. Selective secretion of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: a possible role in homing of haemopoietic progenitor cells to bone marrow. Br J Haematol. 1999; 106:905–911. [PubMed: 10519991]

16. Nagasawa T, Kikutani H, Kishimoto T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. Proc Natl Acad Sci U S A. 1994; 91:2305–2309. [PubMed: 8134392]

17. Glocbek AM, Le Y, Dykxhoorn DM, Park SY, Mostoslavsky G, Mulligan R, et al. Focal adhesion kinase is required for CXCL12-induced chemotactic and pro-adhesive responses in hematopoietic precursor cells. Leukemia. 2007; 21:1723–1732. [PubMed: 17568820]

18. Ara T, Itoi M, Kawabata K, Egawa T, Tokoyoda K, Sugiyama T, et al. A role of CXC chemokine ligand 12/stromal cell-derived factor-1/pre-B cell growth stimulating factor and its receptor CXCR4 in fetal and adult T cell development in vivo. J Immunol. 2003; 170:4649–4655. [PubMed: 12707343]

19. Juarez JG, Thien M, Dela Pena A, Baraz R, Bradstock KF, Bendall LJ. CXCR4 mediates the homing of B cell progenitor acute lymphoblastic leukaemia cells to the bone marrow via activation of p38MAPK. Br J Haematol. 2009; 145:491–499. [PubMed: 19344405]

20. Bendall LJ, Baraz R, Juarez J, Shen W, Bradstock KF. Defective p38 mitogen-activated protein kinase signaling impairs chemotaxis but not proliferative responses to stromal-derived factor-1alpha in acute lymphoblastic leukemia. Cancer Res. 2005; 65:3290–3298. [PubMed: 15833862]
21. Spiegel A, Kollet O, Peled A, Abel L, Nagler A, Bielorai B, et al. Unique SDF-1-induced activation of human precursor-B ALL cells as a result of altered CXCR4 expression and signaling. Blood. 2004; 103:2900–2907. [PubMed: 15070661]

22. Juarez J, Bradstock KF, Gottlieb DJ, Bendall LJ. Effects of inhibitors of the chemokine receptor CXCR4 on acute lymphoblastic leukemia cells in vitro. Leukemia. 2003; 17:1294–1300. [PubMed: 12835717]

23. Kawaguchi A, Orba Y, Kimura T, Iha H, Ogata M, Tsuji T, et al. Inhibition of the SDF-1alpha-CXCR4 axis by the CXCR4 antagonist AMD3100 suppresses the migration of cultured cells from ATL patients and murine lymphoblastoid cells from HTLV-I Tax transgenic mice. Blood. 2009; 114:2961–2968. [PubMed: 19657116]

24. Nervi B, Ramirez P, Rettig MP, Uy GL, Holt MS, Ritchey JK, et al. Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. Blood. 2009; 113:6206–6214. [PubMed: 19050309]

25. Devine SM, Flomenberg N, Vesole DH, Liesveld J, Weisdorf D, Badel K, et al. Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin’s lymphoma. J Clin Oncol. 2004; 22:1095–102. [PubMed: 15020611]

26. Zeng Z, Shi YX, Samudio IJ, Wang RY, Ling X, Frolova O, et al. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. Blood. 2009; 113:6215–6224. [PubMed: 18955566]

27. Tavor S, Eisenbach M, Jacob-Hirsch J, Golan T, Petit I, Benzion K, et al. The CXCR4 antagonist AMD3100 impairs survival of human AML cells and induces their differentiation. Leukemia. 2008; 22:2151–2158. [PubMed: 18769446]

28. Liles WC, Broxmeyer HE, Rodger E, Wood B, Hübel K, Cooper S, et al. Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. Blood. 2003; 102:2728–2730. [PubMed: 1285591]

29. Zeng Z, Shi YX, Samudio IJ, Wang RY, Ling X, Frolova O, et al. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. Blood. 2009; 113:6215–6224. [PubMed: 18955566]

30. Heisterkamp N, Groffen J, Carella AM, Daley GQ, Eaves CJ, Goldman JM, Hehlman R. BCR/ABL Gene Structure and BCR Function. Chronic Myeloid Leukemia: Biology and Treatment, 2001:3–17.

31. Dillmann F, Veldwijk MR, Laufs S, Sperandio M, Calandra G, Wenz F, et al. Plerixafor inhibits chemotaxis toward SDF-1 and CXCR4-mediated stroma contact in a dose-dependent manner resulting in increased susceptibility of BCR-ABL+ cell to Imatinib and Nilotinib. Leuk Lymphoma. 2009; 50:1676–1686. [PubMed: 19657955]

32. Vianello F, Villanova F, Tisato V, Lympieri S, Ho KK, Gomes AR, et al. Bone marrow mesenchymal stromal cells non-selectively protect chronic myeloid leukemia cells from imatinib-induced apoptosis via the CXCR4/CXCL12 axis. Haematologica. 2010; 95:1081–1089. [PubMed: 20179085]

33. Hendrix CW, Flexner C, MacFarland RT, Giandomenico C, Fuchs EJ, Redpath E, et al. Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers. Antimicrob.Agents Chemother. 2000; 44:1667–1673. [PubMed: 10817726]

34. Moyle G, DeJesus E, Boffito M, Wong RS, Gibney C, Badel K, et al. Proof of activity with AMD11070, an orally bioavailable inhibitor of CXCR4-tropic HIV type 1. Clin Infect Dis. 2009; 48:798–805. [PubMed: 19193109]

35. Stone ND, Dunaway SB, Flexner C, Tierney C, Calandra GB, Becker S, et al. Multiple-dose escalation study of the safety, pharmacokinetics, and biologic activity of oral AMD070, a selective CXCR4 receptor inhibitor, in human subjects. Antimicrob Agents Chemother. 2007; 51:2351–2358. [PubMed: 17452489]

36. Baersch G, Mollers T, Hotte A, Dockhorn-Dworniczak B, Rube C, Ritter J, et al. Good engraftment of B-cell precursor ALL in NOD-SCID mice. Klin Padiatr. 1997; 209:178–85. [PubMed: 9293448]

Leukemia. Author manuscript; available in PMC 2012 February 01.
37. Parameswaran R, Müschen M, Kim YM, Groffen J, Heisterkamp N. A functional receptor for B-cell activating factor is expressed on human acute lymphoblastic leukemias. Cancer Res. 2010; 70:4346–4356. [PubMed: 20460528]
38. Fei F, Stoddart S, Groffen J, Heisterkamp N. Activity of the Aurora kinase inhibitor VX-680 against Bcr/Abl-positive acute lymphoblastic leukemias. Mol Cancer Ther. 2010; 9:1318–1327. [PubMed: 20388735]
39. Kaur P, Feldhahn N, Zhang B, Trageser D, Müschen M, Pertz V, et al. Nilotinib treatment in mouse models of P190 Bcr/Abl lymphoblastic leukemia. Mol Cancer. 2007; 6:67. [PubMed: 17958915]
40. De Clercq E. Inhibition of HIV infection by bicyclams, highly potent and specific CXCR4 antagonists. Mol Pharmacol. 2000; 57:833–839. [PubMed: 10779364]
41. Crazzolara R, Kreczy A, Mann G, Heitger A, Eibl G, Fink FM, et al. High expression of the chemokine receptor CXCR4 predicts extramedullary organ infiltration in childhood acute lymphoblastic leukaemia. Br J Haematol. 2001; 115:545–553. [PubMed: 11736934]
42. Wong RS, Bodart V, Metz M, Labrecque J, Bridger G, Fricker SP. Comparison of the potential multiple binding modes of bicyclam, monocyclam, and noncyclam small-molecule CXC chemokine receptor 4 inhibitors. Mol Pharmacol. 2008; 74:1485–1495. [PubMed: 18768385]
43. Drummond MW, Heaney N, Kaeda J, Nicolini FE, Clark RE, Wilson G, et al. A pilot study of continuous imatinib vs pulsed imatinib with or without G-CSF in CML patients who have achieved a complete cytogenetic response. Leukemia. 2009; 23:1199–1201. [PubMed: 19262595]
44. Orimo A, Gupta PB, Sgroi DC, Arentzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005; 121:335–348. [PubMed: 15882617]
45. Geminder H, Sagi-Assif O, Goldberg L, Meshel T, Rechavi G, Witz IP, et al. A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. J Immunol. 2001; 167:4747–4757. [PubMed: 11591806]
46. Burger JA, Peled A. CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. Leukemia. 2009; 23:43–52. [PubMed: 18987663]
47. Scala S, Ottaiano A, Ascieto PA, Cavalli M, Simeone E, Giuliano P, et al. Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. Clin Cancer Res. 2005; 11:1835–1841. [PubMed: 15756007]
48. Zeelenberg IS, Ruuls-Van Stalle L, Roos E. The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. Cancer Res. 2003; 63:3833–3839. [PubMed: 12839981]
49. Burger JA, Kipps TJ. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood. 2006; 107:1761–1767. [PubMed: 16269611]
50. Wang J, Wang J, Sun Y, Song W, Nor JE, Wang CY, et al. Diverse signaling pathways through the SDF-1/CXCR4 chemokine axis in prostate cancer cell lines leads to altered patterns of cytokine secretion and angiogenesis. Cell Signal. 2005; 17:1578–1592. [PubMed: 16005185]
Figure 1.
AMD11070 binds to CXCR4 expressed on human ALL cells. (a) Dose-response of US.7, US.7R and TXL-2 to a 30-minute incubation on ice with AMD11070 or AMD3100 (*p<0.05, AMD11070 compared with AMD3100). (b) Western blot analysis of total cellular CXCR4 after 30 minutes exposure to different concentrations of AMD11070 or AMD3100. (c) Time course in minutes of US.7, US.7R and TXL-2 ALL cells treated with 1 μM AMD11070 or AMD3100. Cell surface CXCR4 expression in (a) and (c) was measured using FACS and the 12G5 monoclonal antibody. The percentage of cells positive for CXCR4 in untreated samples (US.7 84%; US.7R 81%; TXL-2 76%) is set at 100%.
Figure 2.
Effect of AMD11070 on SDF-1α-induced and stromal-induced migration of ALL cells. (a) Western blot analysis for phosphorylation of p38, ERK and AKT in US.7 cells. Samples include those subjected to no treatment (−), pre-incubation with AMD11070 (1 μM, 30 min) followed by 100 ng/ml SDF-1α stimulation (SDF+AMD) or only SDF-1α stimulation (+SDF). β-2 microglobulin serves as loading control. (b, c) migration towards SDF-1α (200 ng/ml, b) or stroma (c). Untreated or AMD11070/AMD3100-pretreated human ALL cells were seeded in the upper wells of Transwells. Controls, no SDF-1α (b) or stroma (c) in the bottom wells. Bars, cell counts in the supernatant of triplicate wells (*p<0.05, AMD11070 compared with AMD3100).
Figure 3.
*Ex vivo* modeling of combination treatment using AMD11070 with anti-leukemia drugs. (a) ALL cells grown on OP9 feeder layers were incubated with 10 μM AMD11070 or AMD3100 for 2, 6 and 9 hours. The numbers of non-adherent cells in the medium are indicated (*p<0.05, AMD11070 compared with AMD3100). (b) US.7 cells growing on OP9 stroma were treated with 2.5 nM vincristine and 1 μM AMD11070 in three different ways. *AMD+Vin*: vincristine and AMD11070 added together to the medium; *AMD, Vin*: AMD11070 alone added for the first 5 days and then (arrow) vincristine plus AMD11070; *Vin, AMD*: vincristine alone added for the first 5 days and then (arrow) vincristine plus AMD11070 added together. The percentage viability is the viable cell count (Trypan-blue excluding cells) as percentage of the total cell number. *p<0.05, for vincristine followed by AMD11070 + vincristine treatment, compared to vincristine alone. (c) TXL-2 cells treated with AMD11070 (1 μM) and nilotinib (300 nM) in a similar way as described for (b). *p<0.05, for nilotinib followed by nilotinib + vincristine, compared to nilotinib alone.
**Figure 4.**

*In vivo* combination treatment of human ALL with vincristine and AMD11070. NSG mice were intravenously injected with U8.7 cells (1.5 × 10^6 cells/mouse). Five days later, treatment was started with i.p. injection of either saline or vincristine (0.5 mg/kg) once a week. Three treatment cycles were done as indicated in the table in (b). The time point at which the last treatment was done is indicated with an arrow. For the combination treatment, vincristine was injected 5 hours *after* oral administration of AMD11070. (a) Leukemia cells in the peripheral blood of a saline treated mouse (control, left panel) are compared to that of an AMD11070 treated mouse (mid panel), as detected by FACS analysis for CD19. CXCR4 detection on the gated CD19^+^ cells from a saline-treated (grey) and an AMD11070 treated (black) mice 5 hours after AMD11070 administration. (b) Survival of mice treated with PBS (circles), vincristine (0.5 mg/kg) (triangles up), AMD11070 (10 mg/kg) (squares) or vincristine plus AMD11070 (triangles down). AMD11070 + vincristine *versus* vincristine, *p*<0.05. (c) Percent weight change of the mice during treatment. The percentage weight loss or gain with respect to the initial starting weight is indicated.
Figure 5.
Reduced presence of leukemic cells at extramedullary sites in combination-treated mice. Analysis of treated mice at the time of sacrifice. The percentage of CD19 positive cells in the live gated population of (a) total peripheral blood or (b) total bone marrow of individual mice. (c, left), spleens of the mice from the vincristine-alone treated group and from the combination treatment group. (c, right) weight of spleens. (d) H&E stained sections of livers of representative mouse from the vincristine-alone group and from the combination treatment group (20x). *p<0.0001
Figure 6.
In vivo combination treatment of mouse Bcr/Abl-positive ALL with nilotinib and AMD11070. C57Bl/6J mice (n = 5 per group) were intravenously injected with 8093 cells (1×10^5 cells/mouse). (a) Survival of mice treated with vehicle (control, circles), nilotinib for 12 days (squares), or nilotinib for 5 days followed by combination of nilotinib and AMD11070 for 7 additional days (triangles). AMD11070 + nilotinib versus nilotinib, *p<0.02. The time point at which the last treatment was given is indicated with an arrow. (b, c) Percentage of AA4.1-positive cells in the live-gated population of (b) peripheral blood or (c) bone marrow of individual mice. *p<0.004 for (b) and *p<0.018 for (c).