Enhanced Unique Pattern of Hematopoietic Cell Mobilization Induced by the CXCR4 Antagonist 4F-Benzoyl-TN14003

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Key Words. CXCR4 • Mobilization • Hematopoietic stem cells • Hematopoietic progenitors

ABSTRACT

An increase in the number of stem cells in blood following mobilization is required to enhance engraftment after high-dose chemotherapy and improve transplantation outcome. Therefore, an approach that improves stem cell mobilization is essential. The interaction between CXCL12 and its receptor, CXCR4, is involved in the retention of stem cells in the bone marrow. Therefore, blocking CXCR4 may result in mobilization of hematopoietic progenitor and stem cells. We have found that the CXCR4 antagonist known as 4F-benzoyl-TN14003 (T-140) can induce mobilization of hematopoietic stem cells and progenitors within a few hours post-treatment in a dose-dependent manner. Furthermore, although T-140 can also increase the number of white blood cells (WBC) in blood, including monocytes, B cells, and T cells, it had no effect on mobilizing natural killer cells. T-140 was found to efficiently synergize with granulocyte colony-stimulating factor (G-CSF) in its ability to mobilize WBC and progenitors, as well as to induce a 660-fold increase in the number of erythroblasts in peripheral blood. Comparison between the CXCR4 antagonists T-140 and AMD3100 showed that T-140 with or without G-CSF was significantly more potent in its ability to mobilize hematopoietic stem cells and progenitors into blood. These results demonstrate that different CXCR4 antagonists may have different therapeutic potentials.

Granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral-blood mononuclear cells are routinely used as a source of HSCs for transplantation. Despite the potency of G-CSF in mobilizing stem cells, it results in broad interindividual variations in circulating progenitor and stem cell numbers [4], requiring repeated dosing, and is frequently associated with side effects. Thus, optimal and improved methods to mobilize and collect peripheral-blood progenitor and stem cells for hematopoietic rescue are warranted.

Over recent years, it has become apparent that the interaction between CXCL12 and its receptor, CXCR4, plays a pivotal role in hematopoietic cell mobilization and engraftment [5–7]. The CXCR4 receptor is widely expressed on many cell types, including HSCs and HPCs, and the interaction with its ligand was found to be involved in chemotaxis, homing, and survival. The CXCL12/CXCR4 axis is also involved in the retention of hematopoietic cells within the BM microenvironment [8]; consequently, the disruption of CXCL12/CXCR4 interactions results in mobilization of hematopoietic cells. Indeed, blocking the CXCR4 receptor with an antagonist, such as AMD3100, results in the mobilization of HPCs. Moreover, combining AMD3100 with G-CSF has produced an additive effect [9]. These approaches suggest that antagonizing the interactions of BM-produced CXCL12 with CXCR4 that is expressed on HSCs could be an effective HSC mobilizing strategy.

Today, there are several known CXCR4 antagonists that have been described as having different levels of efficiency [10, 11].

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

All mature blood cells are derived from hematopoietic stem cells (HSCs) through intermediates that are termed hematopoietic progenitor cells (HPCs) [1]. Hematopoietic cells at various stages of differentiation are localized within the bone marrow (BM), their main site of production. Their trafficking between BM and blood is a physiological process [2, 3], but under steady-state conditions, HPCs and HSCs circulate in peripheral blood at very low frequency. Stem cell frequencies in peripheral blood can be considerably increased both in response to various growth factors and during the recovery phase following myeloablative treatment with high doses of chemotherapy or radiation. The CXCR4 receptor is widely expressed on many cell types, including HSCs and HPCs, and the interaction with its ligand was found to be involved in chemotaxis, homing, and survival. The CXCL12/CXCR4 axis is also involved in the retention of hematopoietic cells within the BM microenvironment [8]; consequently, the disruption of CXCL12/CXCR4 interactions results in mobilization of hematopoietic cells. Indeed, blocking the CXCR4 receptor with an antagonist, such as AMD3100, results in the mobilization of HPCs. Moreover, combining AMD3100 with G-CSF has produced an additive effect [9]. These approaches suggest that antagonizing the interactions of BM-produced CXCL12 with CXCR4 that is expressed on HSCs could be an effective HSC mobilizing strategy.

Today, there are several known CXCR4 antagonists that have been described as having different levels of efficiency [10, 11].
MATERIALS AND METHODS

Reagents

AMD3100 was purchased from Sigma-Aldrich (Rehovot, Israel, http://www.sigmaaldrich.com), G-CSF (Neupogen [filgrastim], Amgen, Thousand Oaks, CA, http://www.amgen.com) was kindly provided by Prof. Arnon Nagler. T-140 was kindly provided by Biokine Therapeutics, Ltd. The activity of T-140 was neutralized by incubating with Proteinase K (DAKO, Glostrup, Denmark, http://www.dako.com) for 20 minutes at 37°C followed by 10 minutes of incubation at 95°C.

Mice and Experimental Protocol

Female C57BL/6 mice (7–8 weeks old) were purchased from Harlan Israel (Rehovot, Israel, http://www.harlanisrael.com) and maintained under specific pathogen-free conditions at the Hebrew University Animal Facility (Jerusalem, Israel). All experiments were approved by the Animal Care and Use Committee of the Hebrew University (Jerusalem, Israel). Mice were injected subcutaneously with various doses of T-140 or AMD3100 (1, 2.5, 5, and 10 mg/kg) in a total volume of 200 μl, 2 hours before sacrifice. In some experiments, mice were sacrificed at different time points: ½, 1, 2, 4, and 24 hours postinjection. G-CSF was subcutaneously injected at a dose of 2.5 μg per mouse twice a day for 4 days. In the combination experiments, 18 hours after the last injection of G-CSF, mice were injected with either T-140 or AMD3100. Control mice were injected with phosphate-buffered saline (PBS) at the appropriate volume.

Cell Isolation and Differential Counts

Peripheral blood cells were collected from mice by cardiac puncture into tubes with heparin followed by lysis of erythrocyte population using a red blood cell lysis solution (0.155 M NH₄Cl, 0.01 M KHCO₃, 0.01 mM EDTA; pH 7.4). Cells were counted using a hemocytometer, and total number of cells per 1 ml of blood was calculated. In some experiments, blood samples were resuspended and processed to cytospin slides, centrifuged, air-dried, and stained with Giemsa. The percentage and the morphology of mobilized cells were determined by differential counts of Giemsa-stained cytospin slides.

Flow Cytometry

Flow cytometry was used to assess the number of cells in blood and distinguish between the different populations. Cells were gated according to forward scatter and side scatter to exclude dead cells and to determine granulocytes, monocytic cells, and mature macrophage populations (Fig. 2E). The number of each cell population was counted. Cells were also stained in 0.1 ml of fluorescence-proliferation and migration [12]. The number of each cell population and to determine granulocytes, mononuclear cells, and mature macrophages (CFU-GEMM) were assayed by plating the cells in Iscove’s modified Dulbecco’s medium containing 1% methylcellulose, 15% fetal bovine serum, 1% bovine serum albumin, 3 U/ml recombinant human (rh) EPO, 10⁻¹⁰ M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml recombinant mouse (rm)SCF, 10 ng/ml rmIL-3, 10 ng/ml rh interleukin-6 (IL-6), and 200 μg/ml human transferrin (Methocult GF M3434; Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Seven days later, typical colonies were visually scored by morphologic criteria using a light microscope, and the frequency of CFU was calculated. Staining colonies with benzidine dihydrochloride (Sigma-Aldrich) was used to localize hemoglobin-containing cells.

HSC Assay

C57BL/6 mice, serving as donors, were injected with 5 mg/kg of either T-140 or AMD3100. Two hours later, peripheral blood cells were collected, followed by erythrocyte lysis (as described above), and i.v. transferred into C57BL/6 recipient mice that had been pretreated with a lethal dose of irradiation (900 cGy) 24 hours before. Total cells obtained from 900 or 225 μl of blood were inoculated into a single recipient mouse in a total volume of 200 μl of PBS. Recipient mice that were inoculated with cells obtained from blood of untreated mice or with normal BM cells (5 × 10⁶ cells per mouse) served as controls. The survival of mice was monitored for 4 months. Four months after the first transplantation, BM cells recovered from the first recipient mice repopulated by donor cells were injected i.v. into lethally irradiated secondary mice.

Statistical Analysis

Results are expressed as average ± SD. Statistical differences were determined by an analysis of two-tailed Student’s t test. Values of p < .05 were considered to be statistically significant.

To evaluate whether the effect we observed for the combined treatments was synergistic, we used Student’s t-test and compared the sum of values obtained for each different treatment (estimated additive effect) and the values obtained for the combined treatment. Synergistic effect was defined as an effect that was significantly greater (p < .05) than the sum of the individual effects.

RESULTS

T-140 Administration Induces White Blood Cell Mobilization

We evaluated the effect of T-140 on mobilization of cells to the blood of intact mice and analyzed the kinetics and the dose-response of this effect. First, mice were injected with 5 mg/kg T-140, and the mobilization of white blood cells (WBC), at various time points postinjection, was examined. At ½, 1, 2, 4, and 24 hours after injection of T-140, mice were sacrificed and
cells were collected from blood. A single-dose administration of 5 mg/kg T-140 resulted in a rapid elevation in the total number of WBC in blood, a threefold change from the control after 30 minutes, slowly decreasing until full return to the baseline level after 24 hours (Fig. 1A). The characterization of the mobilized WBC, using a flow cytometer, showed alteration in the absolute number of granulocytes (stained positive with Gr-1), mononuclear cells (MNCs), and mature macrophages (stained positive with both mac-1 and F4/80 [13]). Further characterization using cytospin assay and Giemsa staining defined the granulocyte population as neutrophils. When we looked at the number of neutrophils in blood, there was an elevation starting 30 minutes postinjection, reaching a peak after 1–2 hours (more than a 12-fold change from control), and slowly decreasing to the baseline level after 24 hours (Fig. 1B). The same pattern was observed for mature macrophages; these cells reached a peak 1 hours postinjection of T-140 and returned to the baseline level after 24 hours (Fig. 1D). When we tested the effect of T-140 on the mobilization of MNCs, we observed a twofold rapid elevation change from control after 30 minutes, which rapidly decreased until full return to the baseline level after 4 hours (Fig. 1C).

To determine the effect of different doses of T-140, concentrations of 1, 2.5, 5, and 10 mg/kg were subcutaneously injected into mice. Two hours postinjection, mice were sacrificed, and the total number of WBC in 1 ml of blood was measured. T-140 induced the mobilization of WBC in a dose-dependent manner. Peak mobilization of WBC occurred at a dose of 5 mg/kg of T-140 (Fig. 2A). When we looked at the different populations, we found a similar dose-response elevation in the absolute number of granulocytes (8-fold increase), mature macrophages (6-fold increase), and MNCs (3.5-fold increase) in the blood following treatment with 10 mg/kg of T-140 (Fig. 2B–2D, respectively).

To further identify the different populations of MNCs that were mobilized by T-140, we stained the cells with specific antibodies for T cells (CD3), B cells (B220), Mac-1 $^+$ cells, and NK cells (NK1.1) (Fig. 2E). As shown in Figure 2F, T-140 had an effect on the mobilization of T cells, B cells, and Mac-1 $^+$ cells but had no effect on the mobilization of NK cells. We did not observe any significant alteration in the number of platelets or other coagulation factor in blood following treatment with T-140 (data not shown).

T-140 Synergizes with G-CSF to Mobilize WBC

Recently, the CXCR4 antagonist AMD3100 was reported to promote mobilization of stem cells and was found to synergistically augment G-CSF-induced mobilization of HPCs [9, 14]. Thus, we evaluated the mobilizing capability of T-140 in comparison with G-CSF, AMD3100, and the combination of both CXCR4 antagonists with G-CSF. Similarly to T-140, injection of AMD3100 (s.c.) induced the mobilization of WBC after 2 hours at the optimal concentration of 5 mg/kg. Both T-140 and AMD3100 induced a 2.5-fold increase in the number of WBC in blood compared with control. When G-CSF was injected subcutaneously at a dosage of 2.5 μg per mouse twice a day for 4 days, there was a 1.9-fold increase in the number of WBC in blood. To study the combination of G-CSF and either T-140 or AMD3100, the CXCR4 antagonists were injected at a concentration of 5 mg/ml, 18 hours after the fourth injection of G-CSF; 2 hours later, mice were sacrificed, and the number and type of cells mobilized in blood were tested.

The combination of T-140 with G-CSF induced a 5.1-fold increase in the number of WBC in blood (Fig. 3A). This induction was significantly higher than the increase seen in WBC stimulated by a combination of AMD3100 and G-CSF (3.7-fold increase over control; $p = .006$; Fig. 3A).

Next, we analyzed the relative number of neutrophils, MNCs, and mature macrophages in blood of treated mice. We found that T-140 and AMD3100 increased the mobilization of neutrophils by 6.1-fold and 8-fold over control, respectively ($p < .05$), whereas G-CSF alone stimulated no more than a 4.4-fold increase in the number of neutrophils over control. Combination of T-140 or AMD3100 with G-CSF synergistically increased the number of neutrophils in blood (13.3- and 11.7-fold increase over control; $p = .005$; Fig. 3C).

T-140 and AMD3100 induced a significant and similar fold change in the number of MNCs in blood compared with control (2.3- and 3.4-fold increase over control, respectively; $p < .005$). Comparison with both CXCR4 antagonists showed that G-CSF, in our experimental model, is an inferior mobilizer of MNCs (Fig. 3B, 3D). The combination of G-CSF with T-140 induced a synergistic increase in the number of MNCs (4-fold increase over control), whereas the combination of G-CSF with AMD3100 did not induce such an effect (Fig. 3D). T-140 and AMD3100 also induced a significant change in the number of mature macrophages in blood compared with control (4.2-
6.1-fold increase over control, respectively; \( p < .05 \). Comparison with both CXCR4 antagonists showed that G-CSF is also a poor mobilizer of mature macrophages (Fig. 3B, 3E). Similarly to the effect on MNCs, the combination of G-CSF with T-140 induced a synergistic increase in the number of MNCs (8.3-fold increase over control; \( p < .005 \)), whereas the combination of G-CSF with AMD3100 did not induce such an effect (Fig. 3E). These results show that the CXCR4 antagonists are capable of mobilizing different subtypes of cells compared with G-CSF and that T-140, but not AMD3100, synergized with G-CSF to further stimulate the mobilization of MNCs and mature macrophages.

**Effects of T-140 Administration on Progenitor Cell Mobilization**

To further study the effect of T-140 on mobilization of HPCs, we collected cells from the blood of treated and untreated mice and tested for the presence of colony-forming cells. Injection of T-140 at a concentration between 1 and 10 mg/kg induced a dose-response increase (up to 10-fold over control) in the number of progenitor cells in blood (Fig. 4A; \( p < .05 \)). These results show that the CXCR4 antagonists are capable of mobilizing different subtypes of cells compared with G-CSF and that T-140, but not AMD3100, synergized with G-CSF to further stimulate the mobilization of MNCs and mature macrophages.

**Figure 2.** Dose-dependent mobilization of white blood cells (WBC) induced by administration of T-140. C57BL/6 mice were s.c. injected with 1, 2.5, 5, or 10 mg/kg of T-140. Two hours postinjection, mice were sacrificed and peripheral blood cells were obtained. (A): Total WBC was counted using a hemocytometer. Numbers of neutrophils (B), mature macrophages (C), and MNCs (D) were evaluated by fluorescence-activated cell sorting (FACS) according to FSC and SSC. (E): A representative FACS analysis of blood cells gated according to FSC and SSC to define mature macrophages (R1), neutrophils (R2), and MNCs (R3) is shown. Mature macrophages were further identified by staining for MAC-1 and F4/80 expression; neutrophils were also identified by staining for Gr-1. To analyze the MNC subpopulations, T cells were stained for CD3. NK cells were stained for NK1.1, but no CD3 or B cells were identified by staining for B220. (F): T-140 administration induced the mobilization of monocytes, T cells, and B cells but not NK cells. The data are the average ± SD of six mice per group from a total of two separate experiments performed (*, \( p < .05 \)). Abbreviations: FSC, forward scatter; MNC, mononuclear cell; NK, natural killer; SSC, side scatter; T-140, 4F-benzoyl-TN14003.

T-140 Synergizes with G-CSF to Mobilize HPCs

Blocking the CXCR4 receptor with AMD3100 resulted in the mobilization of HPCs [9, 15, 16]. When we compared the effect of T-140 (5 mg/kg) on the mobilization of progenitor cells to that of AMD3100 (5 mg/kg), we found that T-140 was signif-
icantly more potent in its ability to mobilize progenitor cells into blood. T-140 increased the number of progenitor cells in blood by 7.1-fold over control \((p = .05)\), whereas AMD3100 induced the mobilization of progenitor cells by 4.2-fold over control \((p = .001)\). G-CSF induced a 13.6-fold increase in the number of progenitors in blood over control, whereas in combination with T-140, it induced a 76.8-fold elevation in the number of progenitors over control. This progenitor increase induced by a combination of G-CSF and T-140 was significantly higher than the synergistic induction in the number of progenitors by the combination of G-CSF with AMD3100 (76.8-compared with 46.4-fold over control; \(p = .001\); Fig. 5A).

The effect of CXCR4 antagonists alone and in combination with G-CSF on the mobilization of BFU-E and GEMM progenitor cells was further characterized. T-140 and AMD3100 alone induced a similar increase in the number of BFU-E in blood (nearly 30-fold change from control; \(p = .05\)), whereas G-CSF alone induced a 142-fold increase in BFU-E in blood over control (Fig. 5B). Coinjection of AMD3100 with G-CSF was neither additive nor synergistic (142-fold increase with G-CSF vs. 98-fold increase with combination treatment). However, G-CSF, in combination with T-140, induced a significant elevation of BFU-E (142-fold increase with G-CSF vs. 660-fold increase with combination treatment; \(p < .01\); Fig. 5B). Similar results were observed when we analyzed the number of CFU-GEMM colonies mobilized with G-CSF alone or in combination with the CXCR4 antagonists (Fig. 5C). T-140 was significantly more potent than AMD3100 in its ability to mobilize CFU-GEMM (44- vs. 15.5-fold increase, respectively; \(p = .05\)). G-CSF alone induced a 151-fold increase over control \((p = .01)\), whereas its combination with T-140 induced a 712-fold increase over control \((p < .01)\). This elevation was significantly higher than that induced by the combination of G-CSF with AMD3100 (a 266-fold increase over control; \(p = .01\); Fig. 5C). These results suggest that T-140 synergized more efficiently with G-CSF to induce the mobilization of early progenitors.

To determine whether the effects we observed were specifically related to the activity of T-140, we used the neutralized T-140 treated with proteinase K and compared its effect with the natural T-140. We found that treatment with proteinase K digests the peptide into smaller fragments, as demonstrated in supplemental online Figure 3A and 3B. These fragments did not inhibit the migration of Jurkat T cells toward CXCL12, the ligand for CXCR4, and could not induce the mobilization of WBC or progenitor cells (supplemental online Fig. 3C–3E). These results demonstrate that the mobilizing effect we observed was specifically related to presence of the intact T-140 peptide capable of interacting with CXCR4.

Figure 3. T-140 synergizes with GCSF to mobilize WBC. C57BL/6 mice were s.c. injected with GCSF 2.5 \(\mu g\) twice a day for 4 days. Eighteen hours after the last injection, mice were injected with 5 mg/kg of either T-140 or AMD3100; 2 hours later, mice were sacrificed and peripheral blood cells were obtained. (A): Total number of WBC in blood was counted by fluorescence-activated cell sorting (FACS). (B): A representative FACS analysis of blood cells is shown. The cells were gated according to FSC and side scatter, and subpopulations of mature macrophages (R1), neutrophils (R2), and MNCs (R3) were defined. The numbers of neutrophils (C), MNCs (D), and mature macrophages (E) following the different treatments were analyzed. \(\ast\), \(p < .05\) compared with control; \(\ast\ast\), \(p < .05\) of GCSF+T-140 compared with GCSF+AMD3100; \(\Delta\), \(p < .05\) of T-140 compared with AMD3100; FSC, side scatter; GCSF, granulocyte colony-stimulating factor; MNC, mononuclear cell; T140, 4F-benzoyl-TN14003; WBC, white blood cells.
T-140 Mobilizes HSCs with Long-Term Repopulating Capacity

Although mobilization of HPCs may be of use for short-term repopulation in a transplant setting, HSCs are required for long-term repopulation. To assess the T-140 effect on mobilization of murine HSCs, we used a transplantation model in which donor C57Bl/6 mice were treated with T-140 or AMD3100. After 2 hours, blood was collected, and cells that were isolated from an equal blood volume (900 or 225 μl) were transferred into lethally irradiated recipients C57Bl/6 mice. Survival of recipient mice, as indicated by long-term repopulating activity and presence of HSCs, was monitored. As shown in Figure 6A, 100% of mice transplanted with cells mobilized by T-140 (900 μl of blood) survived the treatment. In contrast, only 73% of mice transplanted with cells that were mobilized by AMD3100 survived the treatment (p < .05; Fig. 6A). Transplantation of cells mobilized by T-140 using a blood volume of 225 μl resulted in the survival of 70.6% of the treated mice, compared with the survival of only 37.5% of the treated mice when mobilization was performed by AMD3100 (p < .05). Although all untreated mice died after irradiation, 100% of mice survived irradiation when normal BM cells had been transplanted (Fig. 6A, 6B). To test for self-renewal and long-term repopulating activity, the transplanted mice were maintained for more than 4 months, and then their BM cells were injected into a second recipient. Stem cells mobilized by T-140 maintained 100% of the transplanted mice for longer than 4 months and were capable of producing long-term rescue in lethally irradiated secondary transplanted mice. These results indicate that T-140 is a powerful mobilizer of long-term repopulating stem cells.

DISCUSSION

Autologous and allogeneic stem cell transplantation has emerged as a preferred strategy in the treatment of a variety of hematological malignancies [17, 18]. In recent years, the use of peripheral blood as a source of HSCs for transplantation followed by a high dose of chemotherapy has emerged into common clinical practice [19]. Successful blood and marrow transplants require the infusion of a sufficient number of HSCs capable of homing to the marrow cavity and regenerating a full array of hematopoietic cell lineages. Thus, an increase in the number of hematopoietic cells in blood will improve the yield of cell collection for transplantation and will also have the potential to shorten recovery from cytopenia and reduce morbidity and mortality.

G-CSF was first identified as a colony-stimulating factor for granulocyte precursors and as a stimulator of granulocyte differentiation and activation [20]. Due to its activity, G-CSF is used to reduce neutropenia and increase the production of neutrophils [21, 22]. In addition to inducing the production and mobilization of neutrophils, G-CSF is also a powerful mobilizer of HSCs. Although several cytokines, such as GM-CSF, IL-3, stem cell factor, and flt-3 ligand, and chemokines, such as IL-8, were also shown to mobilize HPCs and HSCs [23–25], the cytokine G-CSF remains the major growth factor used for mobilization of stem cells for transplantation. The current protocols for stem cell mobilization necessitate 4–5 days of 1–2 s.c.
injections of G-CSF; moreover, one or more pheresis procedures are required because of failure to collect the number of CD34+ cells necessary for successful transplantation. It is suggested that G-CSF induces the mobilization of HSCs through an indirect mechanism by activating neutrophils within the BM. Indeed, it was recently demonstrated that G-CSF stimulates neutrophils to secrete a variety of proteolytic enzymes, including elastase, cathepsin G, matrix metalloproteinase (MMP)-2, and MMP-9, that are capable of degrading CXCL12, the ligand for the chemokine receptor CXCR4, a key molecule that is expressed by the BM stroma and plays an important role in the retention of stem cells expressing CXCR4 within the BM [7, 26].

An important role for the interaction between CXCL12 and its receptor CXCR4 has been described for the mobilization of hematopoietic progenitors and stem cells [5–7]. More specifically, AMD3100, a selective antagonist of CXCR4, was recently shown to rapidly mobilize CD34+ HPCs from the human marrow into the peripheral blood. Furthermore, AMD3100 significantly increased both G-CSF-stimulated (10 μg/kg per day) mobilization of CD34+ cells (3.8-fold) and leukapheresis yield of CD34+ cells. Moreover, AMD3100-mobilized leukapheresis products contained significantly greater numbers of T and B cells compared with G-CSF-stimulated leukapheresis products [27]. AMD3100 was also reported to mobilize HSCs with long-term repopulating capacity in humans [28] and nonhuman primates [29]. In addition, AMD3100 was reported to be a safe and effective agent for the mobilization of CD34+ cells in patients with multiple myeloma or lymphoma receiving prior chemotherapy [30, 31]. On the other hand, a clinical trial with AMD3100 in HIV-infected individuals produced premature ventricular contraction side effects, resulting in the discontinuation of this trial [27].

On the basis of this knowledge, we evaluated the ability of a novel specific CXCR4 antagonist, known as T-140, to mobilize hematopoietic cells. T-140 and its analogues were first developed initially as anti-HIV agents that inhibit HIV-1 infection through their specific binding to CXCR4 [32, 33]. Unlike

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Figure 5. T-140 synergizes with GCSF to mobilize HPCs. C57BL/6 mice were s.c. injected with 2.5 μg of GCSF twice a day for 4 days. Eighteen hours after the last injection, mice were injected with 5 mg/kg of either T-140 or AMD3100; 2 hours later, mice were sacrificed and peripheral blood cells were collected. (A): Number of total CFU cells in the blood was evaluated by colony assay. (B, C): The number of BFU-E and CFU-GEMM was tested by staining the colonies with benzidine dihydrochloride to detect hemoglobin-containing cells. *, p < .05 compared with control; **, p < .05 of GCSF+T-140 compared with GCSF+AMD3100; Δ, p < .05 of T-140 compared with AMD3100. The data are the average ± SD of four mice per group from a total of four separate experiments performed. Abbreviations: AMD, AMD3100; BFU-E, burst-forming units-erythroblasts; CFU-GEMM, colony-forming units granulocyte-erythrocyte-monocyte-macrophage; GCSF, granulocyte colony-stimulating factor; T-140, 4F-benzoyl-TN14003.

Figure 6. T-140 mobilizes hematopoietic stem cells with long-term repopulating capacity that can rescue mice following lethal irradiation. C57BL/6 mice serving as donors were injected with 5 mg/kg of either T-140 or AMD3100. Two hours later, peripheral blood cells were collected and transplanted into C57BL/6 recipient mice that were lethally irradiated (900 cGy) 24 hours before i.v. injection of the cells. Cells obtained from 900 (A) or 225 (B) μl of blood were transplanted into single recipient mice. As a control, recipient mice were also transplanted with cells obtained from blood of untreated mice or with normal BM cells. *, p < .06 of T-140 compared with AMD3100 when 900 μl was transplanted; **, p < .05 of T-140 compared with AMD3100 when 225 μl was transplanted. Data are the average of 17 mice per group from a total of four separate experiments performed. Abbreviations: AMD, AMD3100; BM, bone marrow; T-140, 4F-benzoyl-TN14003.
AMD3100, T-140 is a short, modified peptide. Comparative studies between T-140 and AMD3100 found that each of these agents inhibited CXCR4 via different mechanisms [34, 35]. Whereas T-140 binds residues in extracellular domains of the CXCR4 receptor and regions of the hydrophobic core proximal to the cell surface, amino acids in the central hydrophobic core are critical to binding of AMD3100 [34]. Analysis of antagonists revealed that exposure to AMD3100 induced G-protein activation by CXCR4, whereas T-140 decreased autonomous signaling [35]. Therefore, AMD3100 was defined to have a weak partial agonist activity, whereas T-140 functions as an inverse agonist.

Our results indicate that T-140 can efficiently induce mobilization of mature white blood cells, progenitors, and stem cells into blood within a few hours after treatment in a dose-dependent manner and can efficiently synergize with G-CSF for this effect. A variety of cell types express the CXCR4 receptor, including monocytes/macrophages, B and T lymphocytes, progenitors, and stem cells. Indeed, we found that T-140 can induce mobilization of all of these cell subsets. Although CXCR4 is also expressed by NK cells [36] and platelets [37], we could not demonstrate the effect of T-140 on their mobilization. Similarly, CXCR4/CXCL12 interaction was not found to be involved in platelet activation, as demonstrated by the inability of CXCL12 to augment aggregation or stimulate calcium mobilization of platelets [37].

Administration of T-140, AMD3100, and G-CSF can induce an effective mobilization of WBC into blood. However, whereas G-CSF induces mainly the exit of neutrophils, both CXCR4 antagonists induced a robust mobilization of MNCs and mature macrophages.

T-140 or AMD3100 alone induced the mobilization of a similar number of MNCs and activated macrophages. However, the combination of T-140 with G-CSF induced a significant increase in the mobilization of MNCs and mature macrophages into blood compared with AMD3100.

Even though mobilization of WBC is an important factor for transplantation, the major factor contributing to transplantation efficiency is the number of HPCs and HSCs in blood. When we looked at the effect of T-140 on mobilization of progenitor cells, we observed a significant elevation in the number of both myeloid pluripotent and erythroid progenitors, that is, CFU-GM, CFU-M, CFU-GEMM, and BFU-E. A comparative study demonstrated that administration of a single dose of T-140 resulted in a significant increase of progenitor cell mobilization compared with AMD3100. Moreover, the combination of T-140 with G-CSF resulted in a dramatic elevation in the number of progenitor cells in blood that was superior to G-CSF alone or to a combination of G-CSF with AMD3100. This superiority was highly significant when we witnessed the number of CFU-GEMM and BFU-E in the blood of treated mice.

Although improved mobilization of HPCs in response to T-140 is of interest, proof that T-140 also mobilizes HSCs is of paramount importance. A comparison of the abilities of T-140 and AMD3100 to mobilize HSCs into blood was made using a transplantation model. We found that T-140 is more potent in its ability to mobilize HSCs with long-term repopulating activity compared with AMD3100.

Mobilization of WBC and progenitor cells was synergistically enhanced when T-140 was used in combination with G-CSF, and this effect was significantly superior to that observed for the combination of G-CSF with AMD3100. Future studies should address the questions about the mechanism(s) by which T-140 induces cell mobilization, including studies regarding the blocking of the CXCR4 receptor and the immunogenicity properties of T-140.

In summary, our results indicate that the ability of the CXCR4 antagonist T-140 to mobilize WBC, as well as progenitor and stem cells, differs both qualitatively and quantitatively from that of the well-characterized CXCR4 antagonist AMD3100. Furthermore, T-140 differs from AMD3100 in its ability to efficiently synergize with G-CSF. It is therefore possible that an improved CXCR4 antagonist will provide better methods of collecting mobilized stem cells for transplantation. Future studies should address these issues and define the potential clinical advantage and usefulness of T-140 in clinical stem cell mobilization and transplantation.

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ACKNOWLEDGMENTS

We thank Nobutaka Fujii from Kyoto University (Sakyo-ku, Kyoto, Japan) and Mary Clausen from the Gene Therapy Institute, Hadassah University Hospital (Jerusalem, Israel), for technical assistance. This work supported by a grant from the Lady Davis Fellowship Trust.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

A.P. owns stock in, has acted as a consultant to, has performed contract work for, is a Board member for, and has a financial interest in Biokine Therapeutics.


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Enhanced Unique Pattern of Hematopoietic Cell Mobilization Induced by the CXCR4 Antagonist 4F-Benzoyl-TN14003
Michal Abraham, Katia Biyder, Michal Begin, Hanna Wald, Ido D. Weiss, Eithan Galun, Arnon Nagler and Amnon Peled
Stem Cells 2007;25;2158-2166; originally published online May 24, 2007;
DOI: 10.1634/stemcells.2007-0161

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