The novel CXCR4 antagonist POL5551 mobilizes hematopoietic stem and progenitor cells with greater efficiency than Plerixafor

INTRODUCTION

Mobilization of hematopoietic stem and progenitor cells (HSPCs) describes their enforced egress from the bone marrow (BM), their natural place of residence in post-natal mammals, into the peripheral blood (PB). HSPC mobilization occurs in response to a wide variety of physiological or pharmacological stimuli, such as intense physical exercise, infection or inflammation, and administration of cytokines or chemotherapy.1–5 The clinically most relevant mobilizing agent, granulocyte colony-stimulating factor (G-CSF), promotes mobilization by a complex chain of indirect convergent cellular and molecular events including interference with the CXCL12/CXCR4 axis.6,7 The 5-day course of G-CSF stimulation required for optimal HSPC mobilization5,8,9 results in substantial variability in mobilization efficiency.10 Added to the adverse effects of G-CSF,11–15 such as significant BM disruption16–18 and the lingering threat of adverse genetic events induced by G-CSF,19,20 these shortcomings have driven the quest for alternative mobilizing agents devoid of some of these inherent disadvantages. Direct targeting of CXCR4 with small molecule antagonists has been used to mobilize HSPCs, most prominently with the bicyclam antagonist Plerixafor.21–24 However, CXCR4 inhibitors available to date have proven too weak for efficient clinical mobilization when given as a single agent.25,24

CXCR4-deficient hematopoiesis is characterized by a severe HSPC retention defect in the BM that manifests as constitutive mobilization.25 This phenotype suggests that the cellular target of CXCR4 antagonists that results in HSPC egress from marrow is the HSPC proper. Indeed, this mechanism has been assumed by many,22,26–28 however, direct evidence of this hypothesis has been lacking and recently published data potentially challenge this notion.28

We here report on a novel, potent and highly selective CXCR4 antagonist, POL5551, which was developed using the Protein Epitope Mimetics technology.29 Using in vitro and in vivo assays, we explored in mice the potential of POL5551 as an HSPC-mobilizing agent. Using labeled compound, we also sought to identify the cellular target of CXCR4 antagonist-mediated mobilization.

MATERIALS AND METHODS

Mice

C57BL/6 wild-type (CD45.2) mice purchased from Janvier (Le Genest-Saint-Isle, France) or Charles River Laboratories (Sulzfeld, Germany) were used for most experiments. B6.SJL-Ptprc<sup>Pep3</sup>/BoyJ (CD45.1, Charles River Laboratories) and F1-hybrid mice (CD45.1/2) were used for engraftment experiments. B6.SJL-Ptprc<sup>Pep3</sup>/BoyJ and DBA/2 mice (Janvier) were used for some mobilization experiments. Animals were housed at the Johann Wolfgang Goethe-University Medical School vivarium under non-SF conditions, with autoclaved chow and water ad libitum. Following lethal irradiation (1 × 9.5 Gy, except for homing assays, where 1 × 12.5 Gy were used, using a Cesium source with a dose rate of 1 Gy/min) and transplantation, mice were kept on antibiotic medication, 0.025% Baytril

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minipumps (model 2001, Alzet, Palo Alto, CA, USA), which were implanted under general anesthesia into a dorsal subcutaneous pouch. Monocyto- 
biotinylated POL5551 (Polyphor Ltd) was suspended in PBS (Life Technolo-
gies GmbH, Darmstadt, Germany) and injected i.p. rh-CSF (Granocyte, 
Chugai, Frankfurt, Germany) was suspended in dH2O and diluted in saline to a 
final concentration of 0.5 μg/μl for i.p. injection. Mice received G-CSF 
injections every 12 h at a dose of 100 μg/kg for a total of nine doses i.p., 
referred to as ‘standard regimen’ throughout the manuscript. Subsequent 
blood withdrawal and/or administration of POL5551 were performed 
directly after the last G-CSF injection on day 5. Cyclophosphamide (CY) or 
Plerixafor (both from Sigma-Aldrich) were administered as single i.p. 
injections at doses of 200 mg/kg or 5 and 10 mg/kg, respectively.

Mouse model of diabetes
Diabetes was induced in 12-week-old CS7BL/6 mice with a single i.p. 
injection of 200 mg/kg Streptozotocin (Calbiochem, Merck Millipore, 
Darmstadt, Germany) dissolved in citrate buffer (pH 4.7–5.3). Blood glucose 
levels were measured with a portable glucose meter (Accu-check Aviva, 
Roche Diagnostics, Mannheim, Germany). Only animals with glucose 
values higher than 300 mg/dl were used for mobilization experiments 2–3 
weeks post Streptozotocin injection.

Hematopoietic colony assay
For enumeration of CFU-C, aliquots of cells were incubated in duplicate 
in commercially available growth-factor-supplemented methyl cellulose 
medium for mouse CFU-C (Stem Cell Technologies, Vancouver, BC, USA 
or Cell Systems, Kirkland, WA, USA) towards CXCL12 (100 ng/ml, Peprotech, Rocky 
Hill, NJ, USA or Cell Systems, Kirkland, WA, USA), or control medium 
(Ao.o1_hCXCR4 cells (see above) were used to study occupation of different 
receptor domains by the natural ligand of CXCR4, CXCL12, in comparison 
to the antagonists Plerixafor and POL5551. A total of 1–2 × 10⁶ cells were 
concurrently incubated with CXCL12, Plerixafor or POL5551 (1 μM 
in phosphate-buffered saline (PBS)/bovine serum albumin, 0.5%, for all) and 
allo and one of the two different CXCR4 antibody clones 12G5 (binding to 
extracellular loops) or 1D9 (binding to the N-terminus). Controls were 
icubated with the antibodies alone or stained with appropriate 
immunoglobulin G isotype controls. Incubation was performed at 4°C 
(to prevent internalization) in the dark for 30 min followed by a wash step 
and fluorescence-activated cell sorting analysis of the samples.

Migration
Migration of BM or PB cells through 5-μm pore-size transwells (Corning- 
Costar, Tewksbury, MA, USA) towards CXCL12 (100 ng/ml, Peprotech, Rocky 
Hill, NJ, USA or Cell Systems, Kirkland, WA, USA), or control medium 
(spontaneous migration), performed as described,23 was assessed after 4 h. 
Input cells and cells from the lower chamber were plated into a colony 
assay; colony-forming unit culture (CFU-C) migration is expressed as the 
percent of migrated CFU-C of total CFU-C contained in the inoculum 
(input).

Actin polymerization assays
BM cells preincubated either with medium or POL5551 (1 μM) were 
stimulated with 100 ng/ml CXCL12 at 37 °C for the indicated time, fixed 
in 5% formaldehyde (Carl Roth GmbH, Karlsruhe, Germany) and permeabi-
lized with 0.1% saponin (Carl Roth GmbH), as described.31 F-actin was then 
stained with AlexaFlour568-conjugated phallodin (Molecular Probes, 
Eugene, OR, USA) followed by flow cytometric analysis of the relative 
staining intensity.

Ca²⁺ flux assay
Ca²⁺ flux assay was performed with CXCR4-transfected 30–19 murine pre-B 
cells as described in Supplementary Methods.

HSPC mobilization
POL5551 (Polyphor Ltd, Allschwil, Switzerland) was suspended in saline 
and either injected as bolus intraperitoneally (i.p.) or intravenously (i.v.) 
(0.5–100 μg/g body weight) or filled into continuous-release osmotic 
minipumps (model 2001, Alzet, Palo Alto, CA, USA), which were implanted 
under general anesthesia into a dorsal subcutaneous pouch. Mono-

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Tissue processing and immunohistochemistry
Tissue processing and immunohistochemistry were performed as described in Supplementary Methods.

Detection of biotinylated POL5551
For the detection of biotinylated POL5551, blood collected in heparin-coated tubes (Sarstedt AG & Co, Nümbrecht, Germany) was treated directly with the crosslinking reagent Bis(sulfosuccinimidyl) suberate (BS³, Thermo Fisher Scientific Inc, Rockford, IL, USA) at a final concentration of 5–10 mM (at first resuspended in PBS, Life Technologies GmbH). BM was flushed in PBS and resuspended in fresh 5 mM BS³ solution. Crosslinking was performed at room temperature for 30 min followed by quenching of the reaction with 15 mM Tris-HCl (pH 7.5, Carl Roth GmbH). Subsequent fixation of the samples was carried out with 5% formaldehyde (Carl Roth GmbH), followed by staining with streptavidin and anti-CD45 antibody performed simultaneously in fresh PBS/bovine serum albumin.

Human subjects’ protection
Human cells, which served as the source for CXCR4 mRNA, were from anonymized leftover materials from quality control samples, used with permission of the local internal review board (IRB, no. 329/10) in agreement with the WMA Declaration of Helsinki. Written donor approval was obtained.

Statistics
Descriptive statistics and Student’s t-tests, with Bonferroni correction where indicated, were calculated using Excel (Microsoft, Redmond, WA, USA).

RESULTS
POL5551 is a potent CXCR4 antagonist
We compared binding properties of POL5551 to those of the natural ligand, the chemokine CXCL12, and the well-characterized CXCR4 antagonist Plerixafor. Binding of two CXCR4 antibodies (Abs), clone 12G5 (which binds extracellular loops 1 and 2) and clone 1D9 (which recognizes an epitope within the N-terminus), was tested using flow cytometry after concurrent incubation of Ao.o1_hCXCR4 cells with Abs and compounds (Figure 1a and Supplementary Figure S1). In agreement with previous reports, Plerixafor interfered with 12G5 binding without affecting the binding of 1D9. By contrast, CXCL12 blocked the binding of both clones, indicative of its interaction with both the extracellular loops and the N-terminus, again in agreement with published data. Similar to Plerixafor, POL5551 bound to the extracellular loops but not to the N-terminal moiety recognized by 1D9. This was also confirmed by the molecular model of a POL5551 analog bound to CXCR4 (Figure 1b).

We next sought to confirm antagonistic properties of POL5551 in functional in vitro assays. Responsiveness of cells pretreated with either Plerixafor or POL5551 (both at 1 μM) to CXCL12 was assessed by standard chemotaxis and F-actin polymerization assays. CXCL12-induced transwell migration of sBM CFU-C (~6%) was completely blocked by pre-incubation with either of the CXCR4 inhibitors (Figure 1c). By contrast, whereas POL5551 pretreatment completely abrogated polymerization of F-actin filaments following CXCL12 stimulation, Plerixafor did not show an inhibitory effect in this assay (Figure 1d). A quantitative comparison of POL5551- and Plerixafor-mediated inhibition of cellular Ca²⁺-Flux was performed (Figure 1e). The resulting value of 2–3 nM for POL5551 was ~200-fold lower than the IC₅₀ concentration determined for Plerixafor (400–600 nM). Thus, except for the chemotaxis assay, which favors slowly acting antagonists because of the long incubation time (4 h) and where the activity of the antagonists was the same, in vitro performance of POL5551 as the CXCR4 inhibitor was superior to that of Plerixafor.

Rapid and potent mobilization of hematopoietic progenitor cells by POL5551
Time and dose responsiveness of HSPC egress after POL5551 injection were evaluated next in C57BL/6 mice. CFU-C mobilization after a single bolus injection of POL5551 (5 mg/kg, i.p., Figure 2a) occurred rapidly with a significant increase observed at 1 h (1500 CFU-C/ml) and a peak reached after 4 h (2200 CFU-C/ml), representing a 10- and 14-fold increase, respectively, compared with baseline circulating CFU-C levels (~160 CFU-C/ml). The majority of mobilized progenitors disappeared from the circulation quickly thereafter. Peak plasma concentration of the compound was reached 1 h after injection; after 4 h, >90% of POL5551 had been cleared from the circulation (Supplementary Figure S2A). After i.v. administration of POL5551, mobilization kinetics were similar to the i.p. treatment, whereas the efficiency was increased by >50%. (Supplementary Figure S2B).

Whole blood count analysis showed a peak of white blood cell mobilization at 2 h after POL5551 injection (Supplementary Figure S3A). Compared with control groups receiving G-CSF (standard regimen) or bolus injection of Plerixafor (5 mg/kg, i.p.), no significant differences were found in the relative composition of mature leukocyte subsets in POL5551-mobilized blood. The frequency of neutrophils was increased in mobilized (most prominently in G-CSF-mobilized) versus non mobilized blood (Supplementary Figure S3B). Further analysis of mobilized subsets confirmed the relative increase in the myeloid fraction (Gr1⁺, Mac1+) in mobilized blood (Supplementary Figure S3C). No differences in the ratio of cytotoxic T cells and T-Helper cells were observed (Supplementary Figure S3D).

Injection of escalating doses of POL5551 (0.5–100 mg/kg, Figure 2b and Supplementary Figure S4) resulted in a positive, non-linear dose response of mobilized CFU-C for the doses tested. Mobilization achieved with Plerixafor (5 mg/kg, i.p.) or the standard regimen of G-CSF was in the range of what has been reported previously by us and others. At doses >5 mg/kg, POL5551 induced HSPC mobilization (2200 CFU-C/ml) to significantly higher levels than Plerixafor (1300 CFU-C/ml). Moreover, at doses of 20–30 mg/kg, mobilization levels (2800–4000 CFU-C/ml) were comparable to and at higher doses even exceeded mobilization with G-CSF (3800 CFU-C/ml, Figure 2b, and data not shown).

To assess the magnitude of the difference in mobilization of C57BL/6 and DBA/2 mice in response to POL5551, we next evaluated responsiveness of DBA/2 mice to POL5551 (5 and 50 mg/kg, i.p.), with G-CSF- and Plerixafor-treated mice as controls. Indeed, at both doses mobilization with POL5551 was increased by almost threefold in DBA/2 relative to C57BL/6 mice (Figure 2c) similar to the relative increase found with Plerixafor between the two strains. G-CSF mobilized at least six times more CFU-C in DBA/2 than in C57BL/6 mice.

Streptozotocin-induced diabetic mice (type 1 diabetes) were used as a disease model of G-CSF refractoriness. POL5551 (30 mg/kg)-treated mice were compared with mice treated with G-CSF (standard regimen) and Plerixafor (10 mg/kg, i.p.). Both CXCR4 antagonists were therefore tested at equimolar doses. Treatment with all three agents resulted in markedly decreased (to approximately one-fourth) mobilization in diabetic as compared with healthy mice (Figures 2b and d). Addition of either of the CXCR4 inhibitors (30 mg/kg for POL5551 and 10 mg/kg for Plerixafor) after the ninth G-CSF dose could rescue diabetes-induced hyporesponsiveness to G-CSF (Figure 2e).

POL5551 synergizes with G-CSF and CY
Synergistic mobilization by Plerixafor and G-CSF has been reported for various treatment schedules of both agents. We therefore tested whether a POL5551 bolus injection...
(5 or 30 mg/kg, i.p.) given at the end of a standard regimen of G-CSF could similarly enhance mobilization. Mice mobilized with the combination of G-CSF and Plerixafor (5 or 10 mg/kg) served as controls, with 10 mg/kg of Plerixafor and 30 mg/kg of POL5551 representing equimolar doses of the inhibitors. In the combined treatment regimens, mobilization was noticeably enhanced (Figure 3a).

The combination of POL5551 or Plerixafor with the cytotoxic agent CY was investigated next. On day 8 after CY injection, when peak mobilization occurs,42 addition of a single dose of POL5551 or Plerixafor (both at 5 mg/kg, i.p.) mobilized \(450,000\) or \(440,000\) CFU-C per ml PB, respectively (Figure 3b). The synergism between CY and POL5551 or Plerixafor was more pronounced than the combination of CY plus G-CSF (Figure 3c).

Properties of POL5551-mobilized stem and -progenitor cells
If POL5551 mobilizes HSPCs by directly targeting the CXCR4 receptor, this raises the question whether as a consequence POL5551-mobilized cells found in circulation can still sense CXCL12. We therefore performed migration assays with POL5551-mobilized blood HSPCs (Figure 4a). At both doses tested (5 and 30 mg/kg), POL5551-mobilized CFU-Cs were highly responsive towards the chemokine signal, more so than untreated BM and to a similar degree as was also observed for G-CSF-mobilized blood. All three mobilized specimen had lower expression of cell adhesion receptors when compared with ssBM progenitors (Supplementary Table S2). Interestingly, CXCR4 surface expression on POL5551-mobilized progenitors was significantly higher relative to ssPB (Supplementary Figure S5).
There is controversy regarding the role of the CXCR4/CXCL12 pathway for efficient homing of HSPCs.\textsuperscript{25,43–45} Given the unprecedented potency of POL5551 as CXCR4 antagonist, we tested how efficiently POL5551 mobilized CFU-C to the BM of lethally irradiated recipients by determining the recovery of donor cells from hematopoietic organs 20 h after transplantation. As shown in Figure 4b, mobilization of POL5551-mobilized CFU-C was as efficient as that of ssBM. Our next experiment consequently addressed whether POL5551-mobilized progenitors can also provide timely early engraftment. As determined by serial blood count analyses of all three examined transplant sources, ssBM, as well as G-CSF- or POL5551-mobilized blood, showed similar kinetics of engraftment in lethally irradiated hosts (Figure 4c).

POL5551 mobilizes CRU

The frequency of long-term RU in POL5551 bolus-mobilized blood was quantified and compared with G-CSF-mobilized blood using a standard limiting dilution CRU assay.\textsuperscript{34} Based on the dose response studies depicted in Figure 2b, we selected a dose of POL5551 (30 mg/kg, i.p.) that induced CFU-C mobilization in the range of G-CSF. The proportion of engrafted mice increased with the volume of transplanted blood (Figure 5a). At the doses used, POL5551 and G-CSF mobilized CRU into blood at similar frequencies (47 and 34 CRU/ml blood, respectively, Figure 5b).

In addition, a RU assay was performed to directly compare the RU concentration in blood from mice mobilized with G-CSF, Plerixafor (10 mg/kg, i.p.) or POL5551 (30 mg/kg, i.p.), as well as with G-CSF in combination with Plerixafor or POL5551. The relative concentration of RU in each of the mobilized specimen replicated the agents’ (agent combinations’) efficiency at CFU-C mobilization (Supplementary Figure S6).

POL5551 treatment has minimal effects on macrophage and osteoblast distribution within the BM and endosteal microenvironments.

We previously confirmed that mobilization of HSPC using either G-CSF or CY, but not Plerixafor, occurs through a mechanism that initiates collapse of HSC niche cellular components.\textsuperscript{17} Using a similar immunohistochemistry approach, we examined whether the distribution of mature osteoblasts and macrophages within the BM and endosteal environment was disturbed by treatment with POL5551. Observations are reported relative to saline-treated control samples that exhibited expected cellular distributions for skeletally mature mice (Figures 6a and b). In contrast to G-CSF (Figures 6c and d), treatment with POL5551 had no apparent effect on macrophage or osteoblast distribution within the BM and endosteum. The F4/80+ osteomac canopy (Figure 6e, arrows) covering osteocalcin+ osteoblasts (Figure 6f, arrows) within the endosteal region was clearly maintained. In contrast to G-CSF (Figures 6c and d), treatment with POL5551 initiates collapse of HSC niche cellular components.\textsuperscript{17}
POL5551 mobilizes hematopoietic and progenitor cells by targeting them directly.

By injecting biotin-labeled POL5551 (Bio-POL) into mice and analyzing BM and blood for Bio-POL binding at time points preceding mobilization (30 min after injection), we sought to determine whether it directly targets HSPCs. Indeed, Bio-POL was detected on hematopoietic cells (CD45-positive) in the BM within 30 min of i.p. injection (Figure 7a), as well as on circulating cells (Figure 7b). In addition, POL5551 was detected in BM fluid samples prepared from treated animals (Supplementary Figure S7).

**DISCUSSION**

In this study, in vitro and in vivo properties of the novel CXCR4 antagonist POL5551 as a mobilizing agent were evaluated. The markedly improved potency of POL5551 compared with Plerixafor was shown by its superior ability to block CXCL12-induced responses in vitro and reflected in its in vivo efficacy. Dose escalation of POL5551 as a single mobilizing agent resulted in mobilization in excess of G-CSF-induced mobilization, which to our knowledge was not previously achieved by CXCR4 antagonists mobilization in excess of G-CSF-induced mobilization, which to our knowledge was not previously achieved by CXCR4 antagonists. Indeed, Bio-POL was detected on hematopoietic cells (CD45-positive) in the BM within 30 min of i.p. injection (Figure 7a), as well as on circulating cells (Figure 7b). In addition, POL5551 was detected in BM fluid samples prepared from treated animals (Supplementary Figure S7).

For comparison, the circulating CFU-C numbers from mice treated with POL5551 on day 8 as well as from mice that received POL5551 (5 mg/kg, 2 h) or Plerixafor (5 mg/kg, 1 h) on day 8 from b are shown. ***P < 0.001, *P < 0.05, ND: not determined.

The RU assay confirmed the relative potency of single agents as well as the combination of G-CSF with CXCR4 antagonists, reproducing the CFU-C mobilization pattern associated with these modalities. Limiting dilution competitive transplantation assays with POL5551-mobilized blood demonstrated the presence of CRUs, as experimental evidence of mobilization of true stem cells. The evidence provided is critically important if clinical transplantation of POL5551-mobilized stem cell grafts is planned.

The cellular integrity of the endosteal region was maintained after treatment with POL5551. These data demonstrating differential effects on marrow architecture by G-CSF and POL5551 are not unexpected. The rapid kinetics of CXCR4 antagonists would likely not allow for significant architectural changes in the BM, and indeed similar data were previously reported with the CXCR4 antagonist Plerixafor. Nevertheless, given the several-fold weaker action of Plerixafor, the absence of BM remodeling in response to these CXCR4 inhibitors therefore suggest strain-specific differences in mice (presumably modelling individual-specific differences in humans) in the firmness of CXCL12-mediated stem cell retention or in the relative microanatomical distribution of the cells within the BM.

Synergistic mobilization by G-CSF and CXCR4 antagonists has generally been attributed to only partial targeting of the CXCR4/CXCL12 pathway as well as expansion/relocation of CXCR4 antagonist-mobilizable pools over the course of G-CSF treatment. In agreement with previous reports, G-CSF-mediated mobilization was markedly enhanced by the addition of POL5551. Moreover, the combination of POL5551 or Plerixafor with the cytotoxic agent CY resulted in synergistic mobilization in excess of that observed with G-CSF plus CY, which can be explained by the significant overlap in pathways targeted by G-CSF and CY.

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Brisk responsiveness of POL5551-mobilized HSPCs to CXCL12 in vitro is in agreement with reported data on efficient CXCL12-directed transwell migration as a common property of all mobilized specimen. The observed efficient homing of POL5551-mobilized progenitors is consistent with publications about the homing of CXCR4-deficient or of Plerixafor-mobilized cells. It has been assumed that the molecular mechanism of mobilization of HSPCs by CXCR4 antagonists in vivo is disruption of the CXCL12/CXCR4 axis at the level of the HSPC, and
migration assay and compared with migration of steady-state BM (mean POL5551 at the indicated dose or standard regimen of G-CSF. Migration of PB-mobilized CFU-C towards CXCL12 was assessed by a transwell
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mobilized HSPCs do respond to CXCL12
derived from the marrow stroma. However, CXCR4 antagonist-
consequently, egress of HSPCs deprived of CXCL12 signaling input
Figure 4. Properties of POL5551-mobilized HSPCs. (a) CXCL12 responsiveness of POL5551-mobilized HSPCs: mice received a single injection of
in vitro
Figure 5. Mobilization of CRU by POL5551. CRU frequency in POL5551 (30 mg/kg)-mobilized blood was determined using a CRU assay and compared with the CRU frequency in G-CSF (standard regimen)-mobilized blood. Lethally irradiated recipients (n = 5–10 per group) received transplants of 250 000 BM competitor cells (CD45.2) together with indicated limiting volumes of mobilized blood (CD45.1, 3 pooled donors per experimental group). CRU engraftment, defined as multilineage engraftment of >0.5% per lineage was quantified 16 weeks after transplantation. (a) Percentages of negative mice were plotted against blood volume; f(x) = −3.4709x + 100 (R² = 0.86) for G-CSF and f(x) = −3.7672 x + 100 for POL5551 (R² = 0.75). The mean CRU (LTRC) frequency (b) was calculated using Poisson's statistic (LCALC software, Stem Cell Technologies) (mean ± s.e.m., n = 5–10 per group). **P < 0.01.

CONCLUDING REMARKS

In summary, we demonstrate that POL5551 is a fast-acting, efficient and safe mobilizing agent for immature hematopoietic cells, including long-term repopulating stem cells. At higher doses, its potency exceeds that of G-CSF in C57BL/6 mice, which sheds new light on the size of CXCR4 antagonist-mobilizable pools. With respect to mechanisms of mobilization by CXCR4 inhibitors, we provide evidence supporting the notion that mobilization with POL5551 is the result of direct targeting of CXCR4 on HSPCs in the BM. Provided that the data can be corroborated in humans, POL5551 possesses promising therapeutic potential alone or in combination with the standard mobilizing agents.

As a result, it has been recently hypothesized that CXCR4 antagonists also target the stroma cells, causing an alteration of the CXCL12 gradient and eliciting HSPC egress by this indirect mechanism. Proof of either of the hypotheses hinges on the demonstration of the antagonist binding to CXCR4 on HSPCs in the BM at early time points after injection of the compound—that is, prior to mobilization. Detection of biotin-labeled POL5551 on the surface of hematopoietic cells in BM of Bio-POL-mobilized animals demonstrated here is the first direct evidence of binding of CXCR4 antagonists to HSPCs in vivo supporting direct targeting of HSPCs by CXCR4 antagonists as the mechanism underlying their mobilization. Whether attenuation of the CXCL12 gradient between the BM and plasma also contributes to cell egress cannot be excluded.
Figure 6. Macrophage and osteoblast distribution within the BM in response to mobilizing agents. Immunohistochemical staining of bone and BM collected from mice treated with saline (a, b), G-CSF (c, d), bolus POL5551 delivery (e, f). Specific antibody staining (brown) was performed using antibodies for F4/80 (left panel) or osteocalcin (right panel) and confirmed by comparison to isotype-matched control staining within the same experiment (data not shown). All sections were counterstained with hematoxylin (blue nuclei). Images within treatment groups are from serial sections. Bone matrix is demarked as ‘Bone’ and this text is placed in a similar location in paired images, providing a landmark reference point. Arrows indicate canopy F4/80⁺ osteomacs in the left panel or mature osteocalcin⁺ osteoblasts in the right panel. Arrowhead in a indicates a resting bone surface F4/80⁺ osteomac. Original magnification of all images is × 40.

Figure 7. Targeting of hematopoietic cells by POL5551. POL5551 labeled with a single biotin molecule (Bio-POL) was injected i.p. (30 mg/kg). Control mice received PBS. Thirty minutes after the injection, blood and BM CD45⁺ cells were analyzed for the presence of Bio-POL on their surface using fluorescence-coupled streptavidin. a and b show representative stainings of blood and BM samples, respectively (mean ± s.e.m., n = 3, 3 independent experiments).
CONFLICT OF INTEREST
BR, KP, EC and KD are employees of Polyphor that provided the compound and partially funded the studies described. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
DK, KD, GS, DC, EW, MS and AP performed experiments. DK, JPL, BR, KP, EC, KD and HB planned and analyzed experiments. DK and HB wrote the paper. HB et al. Leukemia (2013) 2322 – 2331

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