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A New Anti-CXCR4 Antibody That Blocks the CXCR4/SDF-1 Axis and Mobilizes Effector Cells

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Abstract

The type IV C-X-C-motif chemokine receptor (CXCR4) is expressed in a large variety of human cancers, including hematologic malignancies, and this receptor and its ligand, stromal cell–derived factor-1 (SDF-1), play a crucial role in cancer progression. We generated a humanized immunoglobulin G1 mAb, hz515H7, which binds human CXCR4, efficiently competes for SDF-1 binding, and induces a conformational change in CXCR4 homodimers. Furthermore, it inhibits both CXCR4 receptor–mediated G-protein activation and β-arrestin-2 recruitment following CXCR4 activation. The binding of the hz515H7 antibody to CXCR4 inhibits the SDF-1–induced signaling pathway, resulting in reduced phosphorylation of downstream effectors, such as Akt, Erk1/2, p38, and GSK3β. Hz515H7 also strongly inhibits cell migration and proliferation and, while preserving normal blood cells, induces both antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity against neoplastic cells. In mouse xenograft models, hz515H7 displays antitumor activities with multiple hematologic tumor cell lines, with its Fc-mediated effector functions proving essential in this context. Furthermore, hz515H7 binds to primary tumor cells from acute myeloid leukemia and multiple myeloma patients. Collectively, our results demonstrate two major mechanisms of action, making hz515H7 unique in this regard. Its potential as a best-in-class molecule is currently under investigation in a phase I clinical trial. Mol Cancer Ther. 15(8): 1890–9. ©2016 AACR.

Introduction

The type IV C-X-C-motif chemokine receptor (CXCR4) is a G-protein–coupled receptor (GPCR) expressed predominantly in B and T cells, monocytes, natural killer (NK), and CD34+ bone marrow progenitor cells. The only CXCR4 ligand described to date is stromal cell–derived factor-1 (SDF-1), also known as CXCL12 (C-X-C-motif ligand 12). SDF-1 is highly expressed in lymph nodes, bone marrow, the liver and the lungs, and to a lesser extent in the kidneys, brain, and skin. In adults, the CXCR4/SDF-1 axis is involved in lymphocyte trafficking and in the retention and homing of hematopoietic stem cells in the bone marrow. As with other GPCRs, the contact between SDF-1 and the CXCR4 receptor initiates signaling via an induced conformational change in the receptor, which is transmitted through the membrane to promote replacement of GDP by GTP on associated G proteins (1). CXCR4 activates the Gi/o protein family, which in turn leads to the activation of phospholipase C, the PI3K pathway (2), and to the regulation of gene transcription, cell migration, and cell adhesion. β-arrestin-1 and β-arrestin-2 have classically been assumed to shut off signal transduction following receptor activation, a process termed desensitization. In addition, β-arrestin-2 has been reported to enhance CXCR4-mediated p38 activation and cell migration following SDF-1 stimulation (3).

Tumor tissues from at least 23 different types of human cancers of epithelial, mesenchymal, and hematopoietic origin express CXCR4 (4). Indeed, this receptor has been found to be overexpressed in a large number of cancers, including solid tumors (4–16), leukemia (17), multiple myeloma (18), and lymphomas (19). The CXCR4/SDF-1 axis is involved in cancer cell proliferation, in angiogenesis, and in antiapoptotic and metastases processes (20). CXCR4 is also expressed by cancer stem cells that have been associated with cancer progression and treatment resistance, notably those that confer the resistance of residual, postchemotherapy acute myeloid leukemia (AML) blast to additional chemotherapeutic agents, a major problem in the treatment of AML (21). These data constitute the rationale for cancer treatments that target CXCR4. In the last few years, clinical trials have been initiated on several CXCR4 antagonists, in combination with anticancer drugs, in patients with hematologic malignancies. In particular, plerixafor, a small-molecule CXCR4 antagonist registered by the FDA in combination with GCSF to enhance the mobilization of hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with lymphoma and multiple myeloma, is now
undergoing phase I and phase II clinical trials for several indications, including chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma, AML, multiple myeloma, non-Hodgkin lymphoma (NHL), and Hodgkin disease. Similarly, phase I clinical trials are under way for the CXC4R human immunoglobulin IgG4 mAb BMS-936564, for the treatment of multiple myeloma, AML, diffuse large B-cell leukemia, CLL, and follicular lymphoma. Clinical trials in phase I or II are also ongoing for the BL-8040 (BKT140) peptide, as a treatment for chronic myeloid leukemia, multiple myeloma, and AML.

The above-mentioned CXC4R antagonists can all potentially mobilize CXC4R-expressing cancer cells, facilitating their destruction by anticancer drugs. Here, we describe the generation of hz515H7, a new humanized IgG1 mAb and CXC4R antagonist designed to kill cancer cells via antibody- and complement-dependent cellular cytotoxicity (ADCC and CDC), which efficiently competes for SDF-1 binding and alters the conformation of CXC4R homodimers.

Materials and Methods

Cell lines and reagents

Chinese hamster ovary (CHO)-K1 and NIH3T3 cells from the ATCC were stably transfected to express human CXC4R. The CHO-K1 cells were cultured in DMEM-F12 medium (Cambrex) supplemented with 5% FCS, 1% L-glutamine, and 500 μg/mL geneticin (all obtained from Gibco-Invitrogen) and the NIH3T3 cells in DMEM + GlutaMAX medium (Gibco-Invitrogen) supplemented with 10% FCS, 1% L-glutamine, and 600 μg/mL geneticin. The HEK293 cells (from the ATCC) used for bioluminescence resonance energy transfer (BRET) assays were cultured in DMEM + GlutaMAX medium and 10% FCS (Sigma). U937 human monocyte histiocytic lymphoma and Ramos human B lymphoblast Burkitt lymphoma tumor cell lines were obtained from the ATCC and KARPAS-299 anaplastic large cell lymphoma (ALCL) human T-cell lymphoma cell line from the European Collection of Cell Cultures. Cell lines were cultured according to the manufacturer’s instructions and were authenticated using short tandem repeat (STR) DNA profiling (LGC Standards) on the dates indicated [U937, Ramos, and KARPAS-299 (obtained, 2009; STR, 2016)]. Passages were limited to 20 for experimental procedures (<3 months) before replacing with lower passage number stocks. Recombinant human SDF-1 was produced by R&D Systems, [125]I-human-SDF-1 from PerkinElmer, guanosine 5’-triphosphate γ 32P ([32P]-GTPγS) and scintillation proximity assay (SPA) wheat germ agglutinin (WGA) beads from Amersham Biosciences. The anti-CD20 chimeric mAb rituximab was obtained from Euromedex.

Antibodies

Anti-CXC4R mAbs were generated by immunizing BALB/c mice (Charles River Laboratories) subcutaneously with NIH3T3-hCXC4R-transfected cells. Supernatants of hybridomas were evaluated for the secretion of mAbs raised against the human CXC4R receptor by FACS. Selected hybridomas were cloned using a FACS Vantage device. The best candidate mAb, 515H7, was humanized (yielding hz515H7) using a strategy based on CDR grafting. An hz515H7 IgG1 variant with reduced Fc-mediated effector functions was generated by mutating the leucines at positions 234 and 235 in the amino acid sequence to alanines (22). The recombinant chimeric constructs hz515H7–IgG2a and hz515H7–lgG4 were generated by fusing the Fc region of mouse IgG2a and of human IgG4, to the heavy chain and light chain variable domains of hz515H7, respectively.

Flow cytometry

The specific binding of hz515H7 to human CXC4R was investigated by FACS. NIH3T3 and NIH3T3-hCXC4R–transfected cells were incubated with hz515H7 or an irrelevant hlgG1 mAb control. After incubation with a goat anti-human Alexa 488 secondary (Invitrogen) antibody and three washing steps, propidium iodide was added to each well and viable cells were analyzed by FACS.

To determine the expression level of CXC4R on blasts (17 patients for AML and 8 healthy donors, Hospices Civils de Lyon, Lyon, France) and on plasmocytes (13 patients for multiple myeloma and 10 healthy donors, Hôpitaux Universitaires de Genève, Geneva, Switzerland), bone marrow samples from patients obtained after informed consent were incubated with CD45-V500 (clone HI30, BD560777). CD34-APC (clone 8G12, BD435804) for blasts and CD138-PECy5 (clone BA38, CoulterA51911). CD38-V450 (clone HB7, BD646581) for plasmocytes, as per the manufacturer’s recommendations, and with 5 μg/mL PE-labeled hz515H7 or a PE-labeled isotype control. Two milliliters of BD (BD Biosciences) Pharm Lyse was added to lyse red blood cells, followed by washing with BD Cell Wash and resuspended in BD Cell Wash. Samples were analyzed immediately on a BD FACSCanto II Flow Cytometer. Results were expressed as a CXC4R/MFI isotype mean fluorescence intensity ratio.

[125]I-SDF-1 binding assay

A radioligand binding assay was performed using membranes from CXC4R-transfected CHO-K1 cells mixed with [125]I-SDF-1 (0.1 nmol/L) and SPA-WGA beads in the presence of indicated concentrations of hz515H7 in 96-well plates. The plates were incubated for 1 hour at room temperature, centrifuged at 1,000 × g, and then read on a TopCount liquid scintillation counter (PerkinElmer).

BRET assay of CXC4R homodimers

HEK293 cells transfected with plasmid constructs allowing the expression either of CXC4R fused to R. reniformis luciferase (CXC4R-Fluc), or of both CXC4R-Fluc and CXC4R fused to yellow fluorescent protein (CXC4R-YFP), were incubated in the presence of antibodies (hz515H7 or a control mAb) at 20 μg/mL and 37°C prior to the addition of coelenterazine H with or without SDF-1 (100 nmol/L). After 5-minute incubation at 37°C and 15 minutes at room temperature, light emission acquisition at 485 and 530 nm was initiated using a Mithras LB 940 Multimode Plate Reader (Berthold). BRET ratios were calculated as defined previously (23). For each experimental point, a mean BRET ratio was calculated and expressed in milliBRET units (mBUI).

[35]S-GTPγS binding assay

The [35]S-GTPγS binding assay used in this study is a radioactive assay based on scintillation proximity technology (GE Healthcare). Membranes from NIH3T3-hCXC4R–transfected cells and endogenous adaptor heterotrimeric G proteins were mixed with unlabelled GDP, 10 nmol/L SDF-1, SPA-WGA beads, and [35]S-GTPγS in the presence of indicated concentrations of hz515H7.
mAb in 96-well plates. The plates were incubated for 1 hour at room temperature and then read on a TopCount liquid scintillation counter (PerkinElmer) after 10-minute room temperature centrifugation at 1,000 × g.

β-arrestin-2 recruitment BRET assay

The antagonist potency of hz515H7 on β-arrestin-2 recruitment following SDF-1-induced human CXCR4 activation was evaluated using a BRET assay. CXCR4-Rluc and CXCR4-luc/β-arrestin-2-YFP transfected HEK293 cells were incubated for 15 minutes at 37 °C in the presence of different concentrations of hz515H7. The cells were incubated for a further 5 minutes at 37 °C after adding coelenterazine H (5 μmol/L) and SDF-1 (100 nmol/L). Light emission acquisitions at 485 and 530 nm were then performed using a Mithras LB 940 reader.

Western blot analysis

Ramos cells were incubated overnight in serum-free RPMI1640 medium with 1% t-glutamine, then incubated or not with hlgG1 or hz515H7 at 10 μg/mL and stimulated or not with SDF-1 (50 ng/mL). After centrifugation, lysis buffer was added to the cells for 90 minutes at 4 °C. After immunoprecipitation, the immunocomplexes were suspended in sample buffer, loaded on a 4% to 12% SDS-PAGE, and transferred to nitrocellulose blot membranes. Each blot was saturated, incubated with appropriate (Cell Signaling Technology) primary antibodies for 4 hours at room temperature, and incubated with horseradish peroxidase (HRP)-linked secondary antibodies (GE Healthcare). The blots were subsequently assayed using the ECL detection system (Amersham Biosciences). The membranes were then stripped, saturated and incubated with anti-GAPDH antibodies for 1 hour at room temperature, and finally washed and incubated with HRP-linked secondary antibodies. Optical density maps were produced using imagej 1.46 (24) and results expressed as an optical density ratio after normalization with GAPDH.

Cell migration assays

High-throughput screening 96-well 8.0-μm Transwell plates (Corning Life Sciences) were used to perform chemotaxis assays. U937 cells were suspended in RPMI medium (BioWhittaker) and incubated for 40 minutes at 37 °C with PBS, hlgG1 irrelevant antibody or hz515H7 (10 μg/mL). Then, 75 μL of cell suspension was added to the inserts. The inserts were incubated at 800 ng/mL in 235-μL total volume of RPMI ± 2% FCS in the bottom wells. Transwell migration was conducted for 4 hours at 37 °C. The cells that had migrated into the bottom wells were quantified using ATP measurements, expressed as relative luminescence unit.

Proliferation assay

Ramos cells were incubated in RPMI1640 medium with 1% t-glutamine and 10% FBS, with an IgG1 isotype control or hz515H7 at 10 μg/mL for 24 hours at 37 °C with or without a cross-linking antibody (a mouse anti-human IgG1Fc antibody obtained from LSBio) added at 6-fold excess. The cells were then suspended in culture medium and incubated for 3 days at 37°C. Cell viability was evaluated using ATP measurements (CellTiter-Glo, Promega).

Cytotoxicity assays

ADCC and CDC assays on cancer cells were performed as described in refs. 25 and 26, respectively. CFSE-stained Ramos cells were spiked into blood before adding buffer, human IgG1 isotype control, hz515H7, or rituximab at 10 μg/mL and then incubated for 4 hours at 37°C. The percentages of both viable (CFSE -ToPro3−) and dead (CFSE-ToPro3+) Ramos cells were measured. To evaluate the effect of hz515H7 on normal blood cells, 500 μg/mL of hz515H7, human IgG1 isotype control, or rituximab was incubated overnight at 37°C with blood from healthy donors. The viability of B cells, T cells, monocytes, and NK cells was tracked by FACS analysis using the appropriate primary antibodies (CD19, CD3, CD14, or CD56 from Invitrogen) and ToPro3. The results represent the percentage of viable (ToPro3−) cells.

Binding of mAbs to the mouse Fc gamma receptor IV

Real-time surface plasmon resonance interaction assays were carried out using a Biacore X device and reagents supplied by Biacore (GE Healthcare). To study the binding of mAbs on the mouse FcγRIV extracellular domain (ECD; Sino Biological Inc.), 1,033.5 RIU of the m-FcγRIV ECD was chemically grafted on the FC2 of a CM4 sensor chip (GE Healthcare) using the Amine Coupling Kit. The first flow cell (FC1) served as the reference surface to subtract the nonspecific interactions. Kinetic experiments were carried out at 25°C a flow rate of 30 μL per minute. An injection of HBS-EP running buffer analyte was used as a double reference. The binding of mAbs to mouse FcγRIV was evaluated using BIaevaluation 3.1 (GE Healthcare).

Mouse models

Ten million Ramos cells were engrafted subcutaneously to SCID mice (Charles River Laboratories). After 8 days, the mice were randomized into groups with mean tumor sizes of 100 mm³. The mice were treated weekly by intraperitoneal injections with appropriate doses (0.15–20 mg/kg) of hz515H7 with a loading dose. Five million KARPAS-299 cells were engrafted subcutaneously to NOD/SCID mice (Charles River Laboratories). Six days after implantation, the mice were randomized into two groups with mean tumor sizes of 130 mm³. The mice were treated by intraperitoneal injections of hz515H7 at 40 mg/kg (loading dose) and then weekly at 20 mg/kg. In both models, a histidine buffer group was introduced as control. Tumor volumes were measured twice a week with an electronic caliper and calculated with the formula: π/6 × length × width × height. Ten million U937 cells were engrafted intraperitoneally on 7-week-old NOD/SCID or NOD/SCID γ (NSG) female mice. The antibodies were administered subcutaneously, starting two days after cell engraftment, with a loading dose of 20 mg/kg, followed by weekly injections of a 10 mg/kg dose. The efficacy of the treatment was measured by monitoring the mouse survival rate daily and by comparing the treated groups with the control-untreated group (injected with histidine buffer).

Results

hz515H7 recognizes human CXCR4, antagonizes SDF-1 binding, and induces a conformational change in CXCR4 homodimers

The binding specificity of hz515H7 on both NIH3T3 wild-type cells and NIH3T3-hCXCR4–transfected cells was evaluated by
FACS analysis. Neither hz515H7 nor hIgG1 bound wild-type NIH3T3 parent cells (see insert in Fig. 1A). In contrast, concentration-dependent binding of hz515H7 (but not of hIgG1) was observed on the surface of NIH3T3-hCXCR4–transfected cells (Fig. 1A). The $K_d$ value of the mAb measured on NIH3T3-hCXCR4–transfected cells was $0.290 ^{+0.009}$ nmol/L.

**Table 1.** CXCR4 expression on blasts (AML patients) and on plasmocytes (MM patients)

<table>
<thead>
<tr>
<th>AML patient blast cells</th>
<th></th>
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<th>MM patient plasma cells</th>
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</thead>
<tbody>
<tr>
<td><strong>Patient number</strong></td>
<td><strong>MFI isotype</strong></td>
<td><strong>MFI</strong></td>
<td><strong>MFI ratio</strong></td>
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<tr>
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<tr>
<td>3</td>
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<tr>
<td>16</td>
<td>116</td>
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</tr>
</tbody>
</table>

NOTE: Positive cases of the subjects tested are in bold.
Abbreviations: MFI, mean fluorescence intensity; MM, multiple myeloma.
$^a$MFI ratio is $10 ^{± 6}$ for blast cells from healthy donors.
$^b$MFI ratio is $2.6 ^{± 0.9}$ for CD34$^+$ cells from healthy donors.
Furthermore, to confirm CXCR4 specificity, hz515H7-1 was evaluated for its ability to recognize human cell lines by FACS analysis. This profile of cell recognition was compared with the one obtained by qPCR-quantifying mRNA encoding for hCXCR4. hz515H7-1 was able to recognize cells for which mRNA encoding for hCXCR4 was quantified but not cells for which mRNA encoding for hCXCR4 was not detected (data not shown).

To evaluate the ability of hz515H7 to compete with SDF-1 for CXCR4 binding, saturation-binding experiments were conducted using radio-labeled $[^{125}]$I-SDF-1 and membranes of CHO-K1 cells stably expressing human CXCR4. H515H7 inhibited $[^{125}]$I-SDF-1 binding concentration dependently with an IC$_{50}$ of approximately 1.5 nmol/L (Fig. 1B). As reported previously (27, 28), we used a BRET assay to monitor for ligand-induced conformational changes in CXCR4 homodimers (Fig. 1C). The addition of SDF-1 (100 nmol/L) induced an increase in the BRET signal, reflecting a conformational change. In contrast, the binding of hz515H7 (20 μg/mL) to CXCR4 homodimers led to a decrease in the BRET signal both in the absence and presence of SDF-1. These results suggest that the binding of hz515H7 to a CXCR4 homodimer induces a conformational change in the receptor that prevents any further changes induced by SDF-1.

Hz515H7 recognizes CXCR4 on primary blasts and plasmocytes from AML and multiple myeloma patients

To evaluate CXCR4 expression on primary leukemic blasts and malignant plasmocytes, we stained CXCR4 in fresh bone marrow samples from AML and multiple myeloma patients with PE-labeled hz515H7 or PE-isotype control (18 healthy donors). For 6 of 17 (~35%) samples from AML patients and for 9 of 13 (~69%) from multiple myeloma patients, the surface expression levels of CXCR4 of blast cells (CD45$^+$, CD34$^+$) and of plasmocytes (CD38$^+$, CD138$^+$) were higher.

**Figure 2.** Hz515H7 inhibits the G-protein–dependent and β-arrestin-2 signaling pathways induced by SDF-1. A, inhibition of SDF-1–induced $[^{35}]$S-GTP$_{7}$ binding to membranes of NIH3T3-hCXCR4–transfected cells as a function of hz515H7 concentration. Each data point is the mean of three experiments, and measurements at each concentration were performed in duplicate. B, BRET analysis, following the addition of coelenterazine H (5 μmol/L), of CXCR4-Rluc and CXCR4-Rluc/β-arrestin-2-YFP–expressing HEK293 cells incubated in the presence of hz515H7 with or without SDF-1 (100 nmol/L). The data are means of seven experimental values, and the bars represent the associated SEs. C and D, immunoblot analysis of Ramos cells incubated or not with hIgG1 or hz515H7 at 10 μg/mL and stimulated or not with SDF-1 (50 ng/mL) for the time periods indicated in C, D, optical density (OD) ratios, using GAPDH and the corresponding protein to normalize, obtained from OD maps produced using imageJ software. ctl, control.
than those of normal CD34⁺ cells in healthy bone marrow samples (Table 1).

hz515H7 inhibits both G-protein–dependent and β-arrestin-2–dependent SDF-1–mediated signaling pathways

Following SDF-1 binding, the conformation adopted by CXCR4 leads to the activation of heterotrimeric G proteins associated with an exchange between bound GDP and GTP from the Gα subunit (29). In a first assay, the incorporation of radio-labeled [35S]GTPγS was monitored as a proxy for the activation level of CXCR4. The incubation of hz515H7 with SDF-1 (10 nmol/L) activated membranes of NIH3T3-CXCR4 inhibited [35S]GTPγS binding by up to 89% in a concentration-dependent manner (IC₅₀ 3.5 nmol/L; Fig. 2A).

The binding of SDF-1 to CXCR4 induces its internalization through the recruitment of β-arrestin and clathrin-mediated endocytosis (30). The BRET assay in Fig. 2B demonstrates that hz515H7 inhibits β-arrestin-2 recruitment in response to SDF-1 stimulation with an IC₅₀ of 3 nmol/L. Furthermore, Fig. 2C clearly shows that within 5 minutes, hz515H7 dramatically reduces the SDF-1–induced phosphorylation of Akt, Erk1/2, p38, and GSK3β. This inhibition is maintained for at least 60 minutes (Fig. 2D). Altogether, our results indicate that hz515H7 inhibits both G-protein- and β-arrestin-2–dependent SDF-1–mediated intracellular signaling pathways. Finally, Fig. 3 demonstrates that hz515H7 inhibits SDF-1–induced U937 cell migration and proliferation in the presence of a cross-linking antibody by 80.0 ± 12.0% (Fig. 3A) and approximately 50% (Fig. 3B), respectively.

hz515H7 induces both ADCC and CDC of tumor cells but has no impact on normal blood cells in vitro

ADCC and CDC are important mechanisms in mAb cancer therapy (31). The ability of hz515H7 to trigger ADCC was assessed on Ramos cells using purified NK cells from healthy donors. As shown in Fig. 3C, 10 μg/mL of the hz515H7 IgG1 mAb

Figure 3.
Hz515H7 inhibits U937 cancer cell migration and Ramos cell proliferation. A, U937 cell migration after incubation in PBS, 10 μg/mL hIgG1 isotype control, or hz515H7 in the presence or absence of SDF-1 (800 ng/mL). RLU, relative luminescence unit. The data shown are the mean and SDs of three independent measurements, each performed in triplicate. B, percentage inhibition of Ramos cell proliferation after incubation for 72 hours at 37°C either in medium (RPMI1640 with 1% L-glutamine and 10% FBS) by itself or with an IgG1 isotype control or hz515H7 at 10 μg/mL, with or without cross-linking antibodies (viz. anti-human IgG1 Fc antibodies). The data shown are the mean of two independent experiments, each performed in duplicate, and the associated SEs. C–F, Hz515H7-induced ADCC and CDC in tumor cells. C, mean cytotoxicity percentage (±SDs) of Ramos cells preincubated with 10 μg/mL hIgG1 isotype control or hz515H7 IgG1, or hz515H7 IgG4. Purified NK cells from healthy donors were used as effector cells. D, FACS analysis of CXCR4 expression in Ramos cells preincubated with increasing concentrations of hz515H7 IgG1 before human sera were added. E, mean cytotoxicity percentage (±SDs) of Ramos cells preincubated with 10 μg/mL hIgG1 isotype control or hz515H7 IgG1 in heated or nonheated human or baby rabbit sera (10% of the final concentration) to evaluate CDC. F, mean cytotoxicity percentage (±SDs) of Ramos cells preincubated with buffer, 10 μg/mL hIgG1 isotype control, hz515H7 IgG1, or hz515H7 IgG4 before adding human serum.
induced 50 ± 10% cell cytotoxicity, whereas the IgG1 control mAb and the Hz515H7 IgG4 mAb, which binds tumor cells in a manner similar to that of the corresponding IgG1 antibody, did not lead to ADCC.

Next, we investigated CDC at different culture times in Ramos cells expressing different levels of CXCR4. Hz515H7 induced concentration-dependent cell lysis (Fig. 3D). Interestingly, there is a strong correlation between the concentration response curves of CDC and of Hz515H7 binding to Ramos cells. The EC₅₀ for both assays are similar (1.4 × 10⁻¹⁰ and 1.6 × 10⁻¹⁰ mol/L, respectively). The absence of cell lysis in the presence of heated serum (Fig. 3E) confirms the involvement of the complement in the CDC assay. As expected, the Hz515H7 IgG4 mAb did not trigger any CDC on Ramos cells (Fig. 3F).

Regarding the elimination of tumor cells in blood, Fig. 4A shows that more than 90% of the Ramos cells expressing both CXCR4 and CD20 are killed in the presence of either Hz515H7 or rituximab, but not in the presence of a control isotype. In addition, Fig. 4B reveals that whereas rituximab (a positive control) causes B-cell depletion, Hz515H7 has no major impact

![Image of Figure 4](image-url)

**Figure 4.** Hz515H7 does not affect normal blood cells in vitro. A and B, viability (as measured by FACS) of CFSE-stained Ramos cells spiked into blood before adding buffer, a human IgG1 isotype control (Ctl) mAb, Hz515H7, or rituximab (RTX) at 10 μg/mL and then incubated for 4 hours at 37°C; and of B cells, T cells, monocytes, and NK cells (B) in blood from healthy donors (n = 6) incubated overnight at 37°C in the presence of a human IgG1 isotype control mAb, Hz515H7, or rituximab at 500 μg/mL.

![Image of Figure 5](image-url)

**Figure 5.** Efficacy of Hz515H7 in xenograft mouse tumor models. A and B, evolution of tumor volumes in 7-week-old female SCID mice (A) engrafted subcutaneously with 10⁷ Ramos cells and treated intraperitoneally (n = 6) with appropriate doses of Hz515H7 or a histidine buffer after 8 days and then once a week for 5 weeks; and 7-week-old NOD/SCID mice (B) engrafted subcutaneously with 5 × 10⁶ KARPAS-299 cells and treated intraperitoneally (n = 6) with a histidine buffer or Hz515H7 at 40 mg/kg after 6 days and then once a week at 20 mg/kg. A Mann–Whitney statistical test was performed after each measurement. The data from one experiment representative of at least two are shown.
on normal B cells, T cells, monocytes, or NK cells. These results indicate that in vitro, hz515H7 triggers Ramos cancer cell lysis in whole blood while preserving normal cells.

**Efficacy of hz515H7 in xenograft mouse tumor models and the role of effector functions in the activity of this mAb**

The activity of hz515H7 against tumor growth was evaluated in Ramos human B Burkitt lymphoma and KARPAS-299 ALCCL xenograft models. In the Ramos model (Fig. 5A), hz515H7 inhibited tumor growth in a dose-dependent manner, by as much as 98% at 20 mg/kg at day 23. Tumor growth was also significantly inhibited (by as much as 68%) in the KARPAS-299 model (Fig. 5B). It has to be noted that as hz515H7 does not recognize the murine CXCR4, it does not allow in these mouse models the measurement of the activity of mAb on endothelial cells in the process of angiogenesis and the potential toxicity on the different normal tissues expressing CXCR4.

Finally, the importance of effector functions in hz515H7’s mechanism of action was investigated with a U937 AML xenograft tumor model in NOD/SCID mice. The effects of hz515H7 IgG1 were compared on one hand with chimeric mouse/human mlG2a hz515H7, designed to have optimal effector functions in mice, and on the other with an hz515H7 IgG1 Ala/Ala mutant designed to have reduced effector function (32). Figure 6A clearly shows that mlG2a hz515H7 and hz515H7 IgG1 bind very efficiently to mouse FcγRIV, whereas the Ala/Ala mutant does not. The survival rates of mice treated with the two former constructs are much higher than those of mice treated with the Ala/Ala mutant or in the control group (Fig. 6B). Furthermore, as displayed in Fig. 6C, hz515H7 has virtually no antitumor activity in NSG mice lacking the IL2 receptor γ chain. These results therefore demonstrate that the effector functions of hz515H7 are essential for its antitumor activity.

**Discussion**

Despite huge progress in the treatment of cancer patients, residual disease still leads to cancer relapse and mortality. CXCR4 is known to contribute to microenvironment-mediated chemoresistance, an important barrier to the eradication of residual disease that has yet to be overcome, notably when bone marrow niches
offer protection to cancer cells (33, 34). The value of targeting CXCR4 with IgG4 mAbs (35), peptides (36), and small molecules (37) has already been demonstrated in pharmacologic models. Indeed, these agents mobilize cancer cells, thereby facilitating their elimination by chemotherapeutic agents. These CXCR4 antagonists are now under investigation in clinical trials. Nonetheless, it may be possible to further increase CXCR4 antagonist efficacy. For example, Kularatne and colleagues (38) recently described an antibody–drug conjugate strategy to selectively deliver the cytotoxic agent to cancer cells using a CXCR4 mAb coupled to monomethyl auristatin F, which allowed the selective elimination of CXCR4+ metastatic cancer cells both in vitro and in vivo. Importantly, this approach was also shown to spare CXCR4+ hematopoietic cells in vivo. The new humanized IgG1 mAb reported here, hz51SH7, binds the human chemokine receptor CXCR4, competes for SDF-1 binding, and induces a conformational change in CXCR4 homodimers. It inhibits both CXCR4 receptor–mediated G-protein activation and β-arrestin-2 recruitment following CXCR4 activation. The binding of hz51SH7 to CXCR4 inhibits SDF-1–induced signaling pathways and the migration and proliferation of cancer cells. These properties are similar to those of other CXCR4/SDF1 antagonists currently under clinical trial. In addition, however, this study demonstrates that hz51SH7 induces cell lysis in cancer cells in vitro, via ADCC and CDC. Moreover, our findings for multiple hematologic tumor xenograft models suggest that these in vitro properties translate into a strong antitumor activity in vivo. We also evaluated the in vivo antitumor activity of three different isotopes of hz51SH7: the IgG1 wild-type, an Ala/Ala IgG1 mutant with reduced effector functions, and a chimeric mouse/human construct, mlgG2a hz51SH7, designed to have optimal effector functions in mice. The dramatic improvement in survival rates observed here in a L937 survival model for mice treated with hz51SH7 IgG1 and mlgG2a hz51SH7 demonstrates the importance of effector functions for the antitumor activity of hz51SH7, the Ala/Ala mutant losing most of its in vivo activity and hz51SH7 also proving significantly less efficient in NSG mice lacking NK cell activity.

Several other studies have demonstrated the importance of CDC and ADCC for the therapeutic efficacy of mAbs, particularly for rituximab, a CD20 mAb largely used in the treatment of NHL and CLL (39, 40). Daratumumab, a CD38 mAb, kills multiple myeloma tumor cells by inducing CDC and ADCC (41) and is a therapeutic mAb with high potential for the treatment of multiple myeloma patients. Finally, elotuzumab, a CS1 mAb recently approved by the FDA for the treatment of multiple myeloma patients, has been shown to inhibit the binding of multiple myeloma cells to bone marrow stromal cells and to induce ADCC in multiple myeloma cells (42).

Our results also show that the CDC induced by hz51SH7 is correlated with the expression of CXCR4 on the cell membrane of Ramos cells (Fig. 3). Similar to previous reports (18, 43), we found that CXCR4 is overexpressed in cancer cells, in respectively 35% and approximately 69% of samples from AML and multiple myeloma patients, with expression levels of CXCR4 on blast cells and of plasmocytes higher than those of CD34+ cells in healthy bone marrow samples, suggesting that hz51SH7 should preferentially kill the neoplastic cells while preserving normal hematopoietic cells. Indeed, our in vitro results show that hz51SH7 does not decrease the viability of B cells, monocytes, T cells, or NK cells, whereas rituximab, used as a positive control for ADCC (44) and CDC (40), leads to CD20+ B-cell depletion as described previously (44, 45).

The two major mechanisms of action demonstrated here for hz51SH7, namely interference with the CXCR4/SDF-1 axis and the triggering of effector functions (ADCC and CDC), make it unique among mAbs. A phase I clinical trial is under way on this potentially best-in-class molecule for the treatment of hematologic malignancies. Furthermore, as CXCR4 is expressed in solid tumors, hz51SH7 is also being investigated for its antitumor activity in solid xenograft models.

Disclosure of Potential Conflicts of Interest

C. Dumonnet reports receiving other commercial research support from Pierre Fabre. T. Mathies is a consultant/advisory board member for Phi Pharma SA. No potential conflicts were disclosed by the other authors.

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