Neutralization of KIT Oncogenic Signaling in Leukemia with Antibodies Targeting KIT Membrane Proximal Domain 5

Marianne Le Gall1,2,3,4,5, Ronan Crépin5, Madeline Neiveyans2,3,4, Christian Auclair5, Yongfeng Fan6, Yu Zhou6, James D. Marks6, André Pèlegri1,2,3,4, and Marie-Alix Poul1,2,3,4

Abstract

KIT is a cell surface tyrosine kinase receptor whose ligand stem cell factor (SCF) triggers homodimerization and activation of downstream effector pathways involved in cell survival, proliferation, homing, or differentiation. KIT-activating mutations are major oncogenic drivers in subsets of acute myeloid leukemia (AML), in mast cell leukemia, and in gastrointestinal stromal tumors (GIST). The overexpression of SCF and/or wild-type (WT) KIT is also observed in a number of cancers, including 50% of AML and small cell lung cancer. The use of tyrosine kinase inhibitors (TKI) in these pathologies is, however, hampered by initial or acquired resistance following treatment. Using antibody phage display, we obtained two antibodies (2D1 and 3G1) specific for the most membrane proximal extracellular immunoglobulin domain (D5) of KIT, which is implicated in KIT homodimerization. Produced as single chain variable antibody fragments fused to the Fc fragment of a human IgG1, bivalent 2D1-Fc and 3G1-Fc inhibited KIT-dependent growth of leukemic cell lines expressing WT KIT (UT7/Epo) or constitutively active KIT mutants, including the TKI imatinib-resistant KIT D816V mutant (HMC1.2 cell line). In all models, either expressing WT KIT or mutated KIT, 2D1 and 3G1-Fc induced KIT internalization and sustained surface downregulation. However, interestingly, KIT degradation was only observed in leukemic cell lines with oncogenic KIT, a property likely to limit the toxicity of these antibodies in patients. These fully human antibody formats may represent therapeutic tools to target KIT signaling in leukemia or GIST, and to bypass TKI resistance of certain KIT mutants. Mol Cancer Ther; 14(11): 2595–605. ©2015 AACR.

Introduction

KIT is a 145 kDa type III tyrosine kinase receptor that functions as a growth factor receptor (1). Type III tyrosine kinase receptors are characterized by five N-glycosylated immunoglobulin (Ig) domains (D1 to D5) in the N-terminal extracellular region and by a split tyrosine kinase intracellular domain. D2 and D3 domains are involved in the binding of KIT ligand, SCF, whereas the fourth (D4) and fifth (D5) domains are implicated in receptorimerization following SCF binding (2, 3). KIT intracellular region contains the catalytic domain composed of the ATP-binding site and the phosphotransferase domain. KIT stimulation by SCF induces KIT dimerization and transphosphorylation, which activates downstream effector pathways (1). SCF is a major cytokine for self-renewal, proliferation, and differentiation of hematopoietic lineage, germ cells, melanocytes, gut, and central nervous system in embryo (4). In adult, KIT is expressed in a limited number of tissues and its defects induce impaired hematopoiesis, decreased number of tissue mast cells, decreased fertility and pigmentation, and defective development of the interstitial cells of Cajal responsible for intestinal pacemaker activity, for review see (ref. 5).

Abnormal KIT signaling is observed in cancer either due to overexpression of SCF and/or KIT itself or due to gain-of-function KIT mutations. These mutations of KIT are generally heterozygous (6) and are major oncogenic drivers in gastrointestinal stromal tumors (GIST; ref. 7), that derive from Cajal cells, in subsets of acute myeloid leukemia (core-binding factor acute myeloid leukemia, CBF-AML; refs. 8), in mast cell leukemia (9), in melanoma (10), and less frequently, in other cancers. Consequently, oncogenic KIT inhibition with specific tyrosine kinase inhibitors (TKI) has proven successful in those pathologies (11).

KIT mutations can be classified in two main categories corresponding to distinct structural and functional locations (12). Catalytic mutants display point mutations located in the kinase domain, mainly in the activation loop. For instance, the mutation D816V is keeping the activation loop in extended conformation corresponding to constitutive phosphotransferase activity (13). Catalytic KIT mutants are found in CBF-AML (8), germ cell tumors (14), mast cell tumors, and mastocytosis (9). Regulatory mutants, found in GIST (12), display point mutations or short deletions in the juxtamembrane domain that induce constitutive dimerization therefore permanent activation of the receptor (15).

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Marie-Alix Poul, INSERM, ICM, 208 rue des Apothicaires, 34298 Montpellier Cedex 5, France. Phone: 33-0467612404; Fax: 33-0467613787; E-mail: marie-alix.poul@inserm.fr

doi: 10.1158/1535-7163.MCT-15-0321

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Wild-type KIT is also involved in a number of malignant diseases mainly derived from cell types that express KIT transiently during embryogenesis (16). The SCF/KIT axis works as an autocrine or paracrine loop sustaining proliferation and/or migration. Indeed, KIT is present on 50% of AML (17) and AML blasts frequently respond to SCF stimulation by increased proliferation (18). Consistently, AML patients refractory to chemotherapy can be cured by KIT inhibition by TKI (19). A number of solid tumors also frequently express KIT and/or SCF, including small cell lung cancer (SCLC), melanoma, and seminoma. Seventy percent of SCLC coexpress SCF and KIT (20), and high KIT levels are associated with poor prognosis (21). Other solid tumors have been reported to express KIT, including breast cancers, neuroblastomas, colon cancers, gynecologic tumors, gliomas, and nonsmall cell lung cancer (21).

Pharmacologic inhibition of KIT is an efficient targeted approach to treat malignancies that are partly or completely dependent on KIT signaling. In GIST patients, ATP-competitive type II TKI (as defined by TKI binding to the inactive conformation of the kinase) imatinib (22) has proven successful. However, some KIT catalytic mutants exhibit initial resistance to imatinib (23), and long-term TKI treatment with imatinib induces secondary mutations and resistance to the drug (24). ATP-competitive type I TKI (as defined by TKI binding to the active conformation of the kinase) has been developed like dasatinib (25), but their use in the clinic is impaired by toxic side effects due to broad specificity (26).

Because of their high specificity combined to their natural ability to recruit immune effectors or their conjugation to toxic drugs or radio-isotopes, mAbs offer an attractive approach to target KIT (27). Independently of effector recruitment to KIT-positive cells, inhibitory effect on KIT signaling could be obtained with mAbs by direct blocking SCF binding to KIT, inhibition of KIT dimerization, and/or induction of KIT degradation. Recent studies reported preclinical data in GIST using naked anti-KIT mAb either competing with SCF (28) or targeting KIT D4 dimerization domain (29). A radiolabeled anti-KIT antibody was also shown to decrease SCLC progression in a mouse model (30).

We report here the generation and characterization of a panel of fully human anti-KIT single-chain variable antibody fragment (scFv) selected by phage display on a recombinant form of KIT. Among the eight scFv obtained, five bound to endogenous KIT and were produced in fusion to the Fc fragment of a human IgG1 as dimeric scFv-Fc (31). Two of the scFv-Fc, namely 2D1 and 3G1, were found to target KIT D5 membrane most proximal domain and to efficiently inhibit KIT-dependent signaling and cell growth in various cells models of leukemia dependent of KIT signaling for their growth and survival. In all the models, either expressing WT KIT or various KIT mutants, mAbs induce KIT internalization. However, interestingly, KIT degradation is only observed in leukemic cell lines with mutated KIT, a property likely to limit the toxicity of these mAbs in patients.

### Materials and Methods

#### Cells and cell culture conditions

None of the cell lines were authenticated. Ba/F3, Ba/F3-KIT, and TF-1 cell lines (a gift from Patrice Dubreuil, Institut Paoli-Calmettes, Marseille, France, in 2009) were grown in complete IMDM supplemented either with EPO (2 U/mL, Abnova) or SCF. Recombinant human SCF (Cell Signaling Technology) or CHO-K1 (a recombinant CHO cell line expressing mouse SCF) culture supernatant (gift from Michel Arock, ENS Cachan) was used as a source of SCF. HMC1.1 and HMC1.2 cell lines that harbor a V560G KIT or a V560G CD816V KIT allele, respectively (gift from Michel Arock, in 2010), were grown in complete IMDM. The HEK-T cell line was grown in complete DMEM. All these cells were cultured at 37°C in a humidified atmosphere enriched in CO2 (5%), were checked for absence of mycoplasma infection, dependency to cytokines sensitivity to TKI dasatinib and imatinib. Insect Sf9 and High Five cells (Invitrogen) were grown at 27°C in, respectively, Grace medium, 10% FBS, or Express Five medium (both from Fisher Scientific) supplemented with 20 mmol/L of l-Glutamine.

#### Antibodies and pharmacologic reagents

For Western blot analysis, anti-KIT-extracellular domain (ECD; H300) and anti-Erk 2 antibodies were obtained from Santa Cruz Biotechnology, anti-phospho-Akt (Ser 473) and anti-phospho-Akt (Thr 202/Tyr 204) from Cell Signaling Technology, anti-Akt and anti-GAPDH from Millipore, and anti-phospho-KIT (Y823 or Y568-570) from Invitrogen. HRP-conjugated anti-mouse or rabbit IgG antibodies were from Sigma. PE/Cy5 or PE/Cy7-conjugated anti-human KIT 104D2 mAb (that does not compete with SCF for binding to KIT (information provided by the manufacturer: Biologend)) anti-SV5 mAb (Invitrogen), conjugated to Alexa-647 (Labeling Kit Molecular Probes), and anti-c-myc peptide mAb 9E10 (Sigma) were used for FACS analysis. PE-anti-mouse Ig and anti-human Fc F(ab’2) fragment were from Beckton Dickinson and Rockland, respectively. Imatinib was a gift from Serge Roche (CRBM) and dasatinib was obtained from Santa Cruz Biotechnology.

#### Recombinant soluble KIT extracellular domain expression and purification

Soluble recombinant KIT-ECD (a.a 26 to 509, P10721 UniprotKB/Swiss-Prot) was produced using baculovirus/insect cell system. C-terminal His-tagged KIT-ECD cDNA was generated by PCR amplification from plasmid pSKasiAP1-K (a gift from Frédéric Subra, ENS Cachan) and cloned into transfer vector pVL 1393 (Beckton Dickinson). Recombinant KIT-ECD viruses were produced in Sf9 cells and recombinant protein KIT-ECD in High Five cells following the BD Baculogold protocol. The culture medium, containing the secreted 6His-tagged KIT-ECD, was collected and dialyzed in PBS before purification with Ni-NTA affinity chromatography (Qiagen). The eluted fraction (elution buffer PBS-Imidazole 250 mmol/L) was concentrated and buffeted into PBS using Amicon Ultra-4 30K centrifugal Filter Units (Millipore). Total protein concentration was determined by spectrophotometry (1 uA360 nm = 0.83 g/L) and purified products analyzed by SDS-PAGE.

#### Selection of KIT-ECD–specific phage antibodies

General steps for the selection have been described elsewhere (32, 33). Briefly, 100 μL of KIT-ECD at 50 μg/mL were used to coat a well of a maxisorp 96-well plate (Nunc) at 4°C overnight. The well was saturated with PBS-2% milk for 2 hours at RT before
adding 10^{11} cfu of the phage antibody library (34) diluted in PBS-2% milk. After a 2-hour incubation at RT, the plate was washed 20 times with PBS-Tween 0.1% and 10 times with PBS. Bound phages (output phages) were eluted with triethylamine 100 mmol/L, neutralized with a solution of Tris 1M pH 7, and amplified for the next round of selection. Selection was monitored by titration of input/output phages by infection of Escherichia coli TG1 for each round of selection. Screening for KIT binders was performed by soluble scFv-ELISA. Monoclonal scFvs were expressed from single colonies grown in 96-well plates and induced with IPTG 1 mmol/L for 12 to 16 hours. Crude culture supernatant containing soluble scFv was tested by ELISA for binding to recombinant KIT-ECD or BSA (Sigma). The diversity of ELISA-positive clones was determined by sequencing. For FACS staining, 5 mL induced supernatants were concentrated 10 times and buffered in PBS.

**Human scFv-Fc expression and purification**

To express scFv in fusion with human Fcγ1, scFv cDNA was NcoI/NolI subcloned from the library phagemid into pFUSE-hFc2 (IL2s) vector (31), a gift from Frank Perez, Institut Curie, Paris, France. Soluble 110 kDa scFv-Fc was produced by transient transfection of HEK-T cells (Supplementary Materials and Methods). scFv-Fc–binding Bolton Hunter neurotoxin (Bot-Fc) and TR1 (transferrine receptor-1; H7-Fc; ref. 35) were used as controls.

**Quantification of scFv-Fc binding to recombinant KIT-ECD by ELISA**

A maxisorp 96-well plate was coated with KIT-ECD (or BSA) 5 μg/mL in PBS overnight at 4°C. Wells were saturated with PBS-1% BSA for 2 hours at RT. scFv-Fc was diluted in PBS-1% BSA and incubated 2 hours at RT. Bound scFv-Fc were detected by HRP-conjugated anti-human IgG (Fc specific) secondary antibody (Sigma) followed by ABTS enzymatic substrate solution incubation (Roche). Fits and EC_{50} were determined with GraphPad software.

**Flow cytometry**

Staining was performed at 4°C. Two hundred thousand mammalian cells were resuspended in 100 μL of FACS buffer (PBS-0.5%BSA) and incubated with crude concentrated bacteria supernatant containing myc-tagged scFv or purified scFv-Fc then with secondary antibodies. For competition experiments, cells were preincubated with SCF (1 μg/mL) during 40 minutes before adding the scFv-Fc (10 μg/mL). For internalization experiments, cells were seeded at 5 × 10^{3} in 700 μL complete growth medium per well and incubated with antibodies (2D1-Fc, 3G1-Fc, or Bot-Fc, 10 μg/mL) at 37°C. KIT cell surface expression was detected on cells returned at 4°C with anti-KIT 104D2 mAb. Yeast cells (1 million) displaying SV5 epitope-tagged KIT truncated forms (Supplementary Materials and Methods) were stained with anti-KIT scFv-Fc followed by secondary antibodies or with PE-conjugated 104D2 mAb directly. Cells were analyzed with a FACS Quanta (Beckman Coulter).

**Western blot analysis**

Cells were washed with stop buffer [150 mmol/L NaCl, 50 mmol/L Tris-Cl (pH 6.7), 1 mmol/L EDTA, 2 mmol/L Na_{2}VO_{4}, 7.5 mmol/L Na_{3}P_{2}O_{7}, 100 mmol/L NaF] and lysed in stop buffer complemented with 1 mmol/L PMSF, 0.25% DOC, 1% NP40, and protease inhibitor cocktail (Roche Diagnostic). Lysates were cleared by centrifugation at 13000 × g for 10 minutes at 4°C, quantified with BCA assay (Interchim), and 30 μg of proteins were separated by SDS-PAGE (10% polyacrylamide) for analysis by Western blot analysis.

**Proliferation assay**

UT-7/Epo was seeded at 10^{4} per well and treated for 4 days with scFv-Fc at 5 or 50 μg/mL in presence of EPO or CHO-KL supernatant diluted 500 times. HMC1.1 and 1.2 were seeded at 2.10^{3} cells per well and treated the same but for 7 days and with 2% PBS and without cytokines. Cell viability was estimated with MTS assay (Promega) as recommended by the manufacturer.

**Results**

Selection and initial characterization of KIT-ECD–specific single chain Fv antibodies

The goal of this work was to isolate fully human KIT-specific antibodies that could inhibit KIT signaling to interfere with the growth of KIT dependent tumors. We proceeded by antibody phage-display selection on recombinant human KIT-ECD produced in insect cells. The purified C-terminal 6His-tagged recombinant KIT-ECD analyzed on a reducing SDS-PAGE gel showed a major protein of 60 kDa also detected by Western blot analysis with an antibody directed against KIT-ECD that validated the production (Supplementary Fig. S1). For selections, phages were prepared from a human scFv phage naïve antibody library (34). After three rounds of selection for enrichment of KIT ECD binders (Supplementary Table S1), eight different scFv anti-KIT ECD were obtained, 2D1 being the most frequent (Supplementary Fig. S1). Because these scFv had been selected based on their binding to recombinant human KIT produced in insect cells, that have distinct N-glycosylation enzymatic machineries compared with mammalian cells (36), we tested whether they could also bind to endogenous KIT. Only the antibodies scFv that detected endogenous KIT on HMC1.2 cells (2D1, 2A3, 3G1, 2B12, 2A6; Supplementary Fig. S1) were kept for further analysis.

**Conversion into a bivalent scFv-Fc format**

Five anti-KIT monovalent scFv were converted into a bivalent scFv-Fc format (31) consisting of a fusion at the C-terminal end of the scFv of the hinge domain and the two constant domains of the human γ1 heavy chain, and produced in HEK-T cells. SDS-PAGE analysis of protein A affinity-purified products showed proteins with the expected 110 kDa molecular weight corresponding to covalently disulfide-linked scFv-Fc homodimers (55 kDa each; Fig. 1A). The reformattting conserved antibody specificity since all scFv-Fc bound to the receptor, as shown by ELISA on recombinant KIT-ECD, while an irrelevant antibody fragment Bot-Fc in the same format produced in the same conditions, did not (Fig. 1B; Table 1). Concentrations to reach half of the saturation ELISA signal (EC_{50}) were in the nanomolar range, except for 2A6-Fc, for which signal saturation was not reached suggesting a lower affinity. Within the antibodies tested, bivalent 2D1-Fc displayed the lowest EC_{50} (0.66 nmol/L or 72 ng/mL) therefore had the better apparent affinity for recombinant KIT. We then tested the binding of the 5 anti-KIT scFv-Fc by FACS to cell lines expressing different levels of surface KIT, as assessed by using the commercial 104D2 mouse mAb (ref. 37; Fig. 1C), including human HMC1.2 (high levels of KIT) and erythroleukemic TF-1 (medium levels of KIT) cell lines and the murine pro-B Ba/F3 cell line.
line modified to express human KIT (low levels of KIT). The parental Ba/F3 cell line, which does not express endogenous mouse KIT, was used as a negative control. KIT was detected on HMC1.2 and TF-1 cells by all five anti-KIT scFv-Fc (used at 10 μg/mL), 2A6-Fc showing lower staining, consistent with lower signal observed (Fig. 1B) on ELISA on recombinant KIT-ECD. 2B12, 2D1, and 3G1-Fc, but not 2A6-Fc or 2A3-Fc, detected low level of KIT on the Ba/F3-KIT cell line (Fig. 1D).

Antibody interference with WT KIT

**Signaling interference.** Anti-KIT antibodies interference with WT KIT signaling was first tested using the TF-1 cell line. Starved TF-1 cells were incubated with scFv-Fc before stimulation with recombinant SCF. Western blot analysis showed that 2A6, 2D1, and 3G1-Fc inhibited SCF-induced KIT phosphorylation on tyrosine 823 (Y823). 2D1 and 3G1-Fc being the most effective while irrelevant antibody Bot-Fc did not (Fig. 2A). Downstream AKT phosphorylation was also reduced by the same antibodies, with stronger effect with 2D1 and 3G1-Fc. Preincubation with SCF at 4°C reduced the binding of 2A6, 2D1, and 3G1-Fc to HMC1.2 cells, while 2B12 and 2A3-Fc binding was not affected (Fig. 2B). To narrow the binding site on KIT, truncated forms of KIT were displayed on yeast (Fig. 2C and Supplementary Table S2) and tested for their detection by the anti-KIT antibodies (Fig. 2D). 2D1, 3G1, and 2A6-Fc bound to KIT D4-D5 and to KIT D5 but not to KIT D4 and KIT-D1-D3 constructs, whereas 2A3 only detected KIT-D1-D3, similarly to the 104D2 commercial antibody. 2B12-binding site on KIT was not found using this method. Moreover, 3G1 but not 2A6 competed with 2D1 for binding to KIT (Supplementary Fig. S2), suggesting an overlapping epitope for 2D1 and 3G1 on KIT D5. In the rest of the study, we focused on the strongest KIT signaling inhibitory anti-KIT-D5 2D1 and 3G1 antibodies.

Table 1. EC50 of the scFv-Fc binding to KIT-ECD by ELISA

<table>
<thead>
<tr>
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<th>2A3-Fc</th>
<th>2A6-Fc</th>
<th>2B12-Fc</th>
<th>2D1-Fc</th>
<th>3G1-Fc</th>
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<tr>
<td>nmol/L</td>
<td>1.40</td>
<td>NA</td>
<td>4.56</td>
<td>0.66</td>
<td>2.04</td>
</tr>
<tr>
<td>SD</td>
<td>0.11</td>
<td>NA</td>
<td>0.25</td>
<td>0.06</td>
<td>0.28</td>
</tr>
</tbody>
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NOTE: Concentrations (nmol/L) of scFv-Fc antibody format giving 50% of maximal OD at 405 nm are given. Fits and EC50 were determined with GraphPad software.

Abbreviation: NA, not appropriate.

To investigate whether the antagonistic effect of 2D1 and 3G1 scFv-Fc antibody formats on SCF-dependent KIT signaling translated into functional effects, we used the megakaryoblastic leukemia cell line UT-7/Epo, which is dependent on either Epo or SCF.
cytokines for its proliferation in vitro (ref. 38 and Supplementary Fig. S3). UT-7/Epo cells were incubated 4 days with antibodies and the effect on cell viability was measured. 2D1-Fc and 3G1-Fc alone had no effect on cells grown with Epo (Fig. 3A). 2D1-Fc and 3G1-Fc decreased cell viability in a dose-dependent manner when cells were cultivated with SCF (Fig. 3B), while H7-Fc, an anti-TfR1 antibody (35) in the same format, decreased cell viability both in Epo and SCF cultivated cells and irrelevant antibody Bot-Fc had no effect. Like in TF-1 cells, the anti-KIT antibodies inhibited downstream KIT signaling pathways, with inhibition of phosphorylation of KIT on Y823, of phosphorylation of AKT and ERK2, and when used alone did not stimulate KIT signaling (Fig. 3C).

**Antibody modulation of KIT levels.** In a therapeutic orientated strategy, one way to reduce KIT signaling in cancer cells would be to decrease KIT levels at the cell surface in order to reduce its access to SCF. SCF binding to KIT at the cell surface has been shown to rapidly induce internalization and degradation of KIT (39), while reexpression of KIT at the cell surface after internalization requires neosynthesis (40). We analyzed the effect of 2D1 and 3G1 anti-KIT antibody treatment on KIT surface level by FACS using the 104D2 anti-KIT mAb, that does not interfere with 2D1 and 3G1-Fc for KIT binding on cells (Supplementary Fig. S4). Treatment of UT-7/Epo cells for 1 to 24 hours with 2D1 or 3G1-Fc induced KIT cell surface downregulation with a 50% decrease but did not affect total KIT levels (Fig. 4A and B). Kinetics of KIT surface level decrease were similar when cells were treated with SCF from 1 to 24 hours, but after 72 hours, KIT surface levels stayed low with antibodies while going back to initial levels with SCF (Fig. 4C). Because in the same cell model, TKI imatinib was also recently shown to decrease KIT surface levels (41), we tested the effect of a combination treatment of anti-KIT antibody and imatinib on UT-7/Epo cells cultivated in the presence of EPO as a source of cytokine. Interestingly, the combination with TKI had an additive effect with 2D1 or 3G1 antibody treatment on KIT surface level reduction (Fig. 4E).

To sum up, these data show that bivalent 2D1 and 3G1 scFv-Fc antibodies, that target KIT D5 membrane proximal region, do not induce KIT phosphorylation and downstream signaling but inhibit SCF-induced KIT signaling resulting in reduced growth of KIT-dependent cell lines. The antibodies induce the internalization but limited downregulation of KIT and combination treatment with imatinib increases this effect.

**Antibody interference with oncogenic KIT**

To test the effect of anti-KIT D5 antibodies on oncogenic KIT activity, we used the mast cell leukemic cell lines HMC1.1 and HMC1.2 that express constitutively active KIT V560G regulatory or KIT D816V catalytic oncogenic KIT mutants, respectively, and depend on KIT signaling (but not on SCF) for their growth and...
survival (42). As expected, HMC1.1 cells were sensitive to TKI imatinib and dasatinib, but HMC1.2 cells were only sensitive to dasatinib due to the resistance of the KIT D816V mutant to imatinib (ref. 23; Supplementary Fig. S3). When HMC1.1 and HMC1.2 cell lines were treated with 2D1 and 3G1-Fc at doses of 5 and 50 μg/mL for 7 days, we found that both anti-KIT antibodies decreased the viability of both cell lines (Fig. 5A). Antibody 3G1-Fc was less active than 2D1 on HMC1.2 cells and irrelevant antibody Bot-Fc had no effect.

To understand the mechanism of inhibition of anti-KIT antibodies, surface KIT level modulation kinetics were analyzed like on the UT-7/Epo cell line. In both mast cell leukemic cell lines, after 1 hour, both 2D1 and 3G1-Fc induced a strong decrease of KIT levels similar to SCF treatment (Fig. 5B). Upon a 72-hour treatment, the downregulation of surface KIT was maintained (Fig. 5C). Unlike in UT-7/Epo cells, surface downregulation of KIT was associated with a strong decrease of total KIT levels after 4 hours (Fig. 5D), which was conserved after 72 hours in both cell lines (Fig. 5E). Notably this decrease was accompanied by a decrease of phosphorylated KIT levels, stronger in HMC1.1 than in the HMC1.2 cell line (Fig. 5E). In addition, when tested on the HMC1.2 cell line, the inhibition of kinase activity by TKI dasatinib reversed partially the antibody-induced KIT degradation and surface downregulation, suggesting that degradation is dependent on KIT phosphorylation (Fig. 5F and G). In contrast with antibody treatment, SCF treatment induced only a transient decrease of KIT surface levels on both cell line (Fig. 5C) and total KIT levels were not affected (Fig. 5D and E).

Altogether, these data show that the targeting of KIT D5 membrane proximal region reduces the viability of HMC1 mast cell leukemic cell lines. On these cells, that are dependent on oncogenic KIT for their growth and survival, anti-KIT D5 antibodies induce a strong KIT degradation and reduction of phosphorylated KIT levels contributing to reduce KIT downstream signaling. Interestingly, HMC1.2 cells that are resistant to TKI imatinib due to the D816V mutation are sensitive to 2D1-Fc and, to a lesser extent to 3G1-Fc.

**Discussion**

In this work, we have generated anti-KIT antibodies using antibody phage display to select for binders to recombinant KIT-ECD (Supplementary Fig. S1 and Supplementary Table S1). Eight distinct KIT-ECD phage binders were isolated, but when expressed as soluble monovalent scFv, only five detected endogenous KIT on HMC1.2 cells, maybe due to a low affinity for KIT or to the recognition N-glycosylation motifs specific to insect cells (36) for the three negative scFvs. When reformatted into bivalent scFv-Fc formats and produced in mammalian cells, all five endogenous KIT binders still bound to high KIT-expressing HMC1.2 cells. Nevertheless, two of them (2A3 and 2A6) did not detect KIT on mouse Ba/F3 cells transfected to express WT human KIT (Fig. 1D). This could be due to a low affinity for KIT in the case of 2A6 (Table 1; Fig. 1B), but the reason is unclear for 2A3 because its affinity for KIT and staining intensity of TF-1 and HMC1.2 cells by FACS were similar to that of 2B12, 2D1, and 3G1 that indeed also stained KIT on Ba/F3-KIT cells (Table 1; Fig. 1D). This approach therefore allowed the rapid selection of a panel of five distinct human antibodies, isolated from a natural naïve antibody phage library (34) that bind endogenous KIT.

![Figure 3](image-url)

Figure 3. Effect of anti-KIT D5 domain scFv-Fc 2D1 and 3G1 on the UT-7/Epo cell line. A and B, UT-7/Epo cells were incubated 4 days in Epo (A) or CHO-KL supernatant (B) in the presence of anti-KIT antibodies or control antibody as indicated at 5 μg/mL (dashed) or 50 μg/mL (gray). Cell viability was measured by MTS assay and is expressed in percentage compared with nontreated cells (black). Data were analyzed with a Kruskal-Walls test. Differences were considered significant when \( P < 0.05 \). C, UT-7/Epo cells were starved overnight in serum-free medium and incubated with scFv-Fc (10 μg/mL) before 5 min stimulation with SCF (100 ng/mL). KIT, AKT, and ERK1/2 phosphorylation were analyzed by Western blot with phospho-specific antibodies. Total KIT and ERK2 levels were visualized after stripping the membranes and reprobing with anti-KIT, anti-AKT and anti-ERK2 antibodies.
KIT-ECD is structured in five Ig domains. From the crystal structure, it is known that the binding of dimeric SCF to domain 2 and 3 of KIT favors KIT homodimerization at the cell surface. This results in homotypic interactions between domain 4 and domain 5 of each monomer that induce a twist in the intracellular domain, leading to the intracellular kinase domain activation (3). We used yeast display to identify the domain bound by the various antibodies. KIT truncated forms were successfully displayed on yeast (Fig. 2C and D). Lower molecular weight truncated forms were better displayed than larger constructs, as previously observed (43). Anti-KIT 2B12 scFv-Fc did not detect any KIT-truncated form despite strong detection of KIT on all mammalian cell lines tested but, using this technique, we determined that 2A3 binds to KIT D1-D3 ligand-binding region and that 2D1, 3G1, and 2A6 bind to KIT D5 membrane most proximal domain. 2D1 and 3G1, but not 2A6, binding to KIT was mutually exclusive, suggesting a shared epitope for the formers and a distinct one for the latter.

Because of their dimeric format, we expected a possible agonistic activity of the anti-KIT scFv-Fc suggested by their potential induction of KIT dimerization, but none of the five anti-KIT scFv-Fc induced activation of KIT downstream signaling nor were able to maintain the growth of SCF-dependent cell lines in cytokine-free medium. On the contrary, interestingly, all three anti-KIT D5 antibodies (2A6-Fc, 2D1-Fc, and 3G1-Fc) inhibited SCF-dependent KIT activation (Figs. 2A and 3C) and the most potent, 2D1 and 3G1, also reduced cell viability of leukemic cell lines dependent on SCF and KIT signaling for their survival (Fig. 3B).

One major effect of targeting KIT D5 domain with 2D1 and 3G1 antibodies was the rapid induction of KIT internalization and the long-term decrease of KIT cell surface levels, both in leukemic cells expressing only WT KIT (UT-7/Epo) and in cells expressing a WT and a constitutively mutated KIT allele (HMC1.1 and HMC1.2). KIT is described as a rapidly renewed receptor that is not recycled at the cell surface after ligand-induced internalization (40, 44). Intrinsic kinase activity and phosphorylation of KIT are required for efficient SCF-induced degradation of KIT and involve the recruitment to KIT of the Cbl E3-ubiquitin ligase, ubiquitinylation of KIT, and degradation by both lysosomal and proteasomal pathways (39). As previously observed by others (45), we found that KIT turnover rate was in the same range whether KIT was

![Figure 4.](image-url)
Figure 5. Oncogenic KIT modulation by anti-KIT D5 scFv 2D1 and 3G1 in HMC1 cell lines. A, HMC1 cell lines were incubated for 7 days in IMDM 1% FCS in presence of anti-KIT (2D1 and 3G1-Fc) or control scFv-Fc antibody (Bot-Fc) at 5 μg/mL (dashed) or 50 μg/mL (gray). Cell viability is expressed in percentage compared with nontreated cells (black). Data were analyzed with a Kruskal–Wallis test. Differences were considered significant when P < 0.05; *, P < 0.01, versus nontreated cells. B–G, HMC1 cells were incubated in IMDM 10% FCS in presence of scFv-Fc antibodies (10 μg/mL), SCF, and dasatinib (1 μmol/L) as indicated for 1, 2, and 24 hours (B and D), 72 hours (C and E), or 4 hours (F and G). Treated cells were analyzed by FACS using 104D2 anti-KIT antibody (B, C, and G) or by Western blot analysis using anti-KIT and GAPDH antibodies (D, E, and F). Representative data of three independent experiments each time.
constitutively active or not (Supplementary Fig. S3). We also showed that despite early KIT internalization induction similar to anti-KIT D5 2D1 or 3G1 antibody treatment, treatment with SCF did not change KIT surface level in the long term (Figs. 4C and 5C). This difference is likely due to increased KIT neosynthesis upon SCF treatment, but not upon antibody treatment.

Although anti-KIT D5 2D1 and 3G1 scFv-Fc–induced internalization of KIT was observed independently of the presence of KIT-activating mutations, KIT degradation was only observed in cells harboring a constitutively active mutated KIT allele (Figs. 4B and D and 5D and E). Indeed, antibody-induced KIT degradation was dependent on effective mutant KIT kinase activity since it was reversed by treatment with TKI dasatinib (Fig. 5F). Dasatinib also reversed partially the decrease of KIT surface levels induced by 2D1 and 3G1 in HMC1.2 cells (Fig. 5G). Notably, an opposite effect was obtained on UT-7/Epo leukemic cells that express exclusively WT KIT. In those cells, imatinib and anti-KIT D5 antibody combination enhanced KIT cell surface reduction compared with treatment with either reagent alone (Fig. 4E). This observation is in line with a recent study in the same cell model that reports that the occupancy of the KIT intracellular kinase ATP-binding site by the TKI imatinib induces KIT internalization and degradation, without the requirement of KIT phosphorylation (41). Therefore, in a context where KIT is not mutated, the association of TKI and anti-D5 antibody could durably decrease KIT from the cell surface of cancer cells, avoiding its activation by SCF. To summarize, anti-KIT D5 scFv-Fc inhibits KIT signaling in leukemic cells by distinct mechanism depending on KIT status. Inhibition of cell growth is obtained by induction of the degradation of constitutively activated KIT or by intracellular sequestration of WT KIT.

Recently, two studies presenting naked anti-KIT antibodies for cancer therapy have been reported. Edris and colleagues (28) studied the effect of SCF competing anti-KIT antibody SR1 (46, 47) on GIST cell lines presenting various constitutively active KIT-mutant alleles. In a mouse model, SR1 was reported to limit GIST progression essentially by a macrophage-dependent mechanism, while KIT degradation upon SR1 treatment was not observed suggesting a different mechanism of inhibition than 2D1 and 3G1 antibodies. Reshetnyak and colleagues presented an in vitro study with an anti-KIT antibody binding to an epitope implicated in homotypic D4-D4 KIT interactions required for KIT activation after SCF binding. Similarly to the anti-D5 antibodies of this study, the anti-KIT D4 antibody inhibited SCF-induced WT KIT activation (29). It also inhibited the growth of BaF/3 cells modified to express a constitutively active KIT mutant found in 10% of GIST patients that harbors a 2 amino acid AY502-503 insertion in KIT Domain 5. No details were provided on mutant KIT degradation or effector functions with this particular antibody.

Compared with small inhibitors, antibody-based immunotherapy of tumors has the advantage to recruit immune effectors through the Fc part of the antibody and to potentially medium ADCC (antibody-dependent cell cytotoxicity) or cell lysis through the complement cascade (CDC; ref. 27). To our knowledge, within anti-KIT antibodies published or commercially available, of mouse or human KIT specificity, none was described to mediate ADCC or CDC (30, 48), which probably explains the mild toxicity observed in animal models where anti-KIT antibodies were injected (48, 49). Together with the fact that in GIST patients, long-term treatment with imatinib does not induce profound hematologic toxicity, these observations let anticipate that treatment with KIT specific inhibitory antibodies will not elicit strong toxic secondary effects. Because 2D1 and 3G1 are cross-reactive with mouse KIT, they will be helpful to explore further toxicity issues of KIT D5 targeting on mouse models.

To conclude, we have obtained two anti-KIT D5 antibodies that inhibit mast cell leukemic cell line growth in vitro, including cells harboring a D816V mutation on KIT that renders them resistant to imatinib. These fully human antibodies, that do not elicit immune effector function targeted to KIT expressing cells, therefore anticipating limited toxicity in vivo, represent promising molecules for killing cancer cells depending on KIT signaling for their survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Le Gall, R. Crépin, C. Auclair, J.D. Marks, M.-A. Poul
Development of methodology: R. Crépin, M. Neiveyans, Y. Fan, Y. Zhou, M.-A. Poul
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Le Gall, R. Crépin, M. Neiveyans
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Le Gall, R. Crépin, C. Auclair, Y. Zhou, J.D. Marks, A. Pelegrin, M.-A. Poul
Writing, review, and/or revision of the manuscript: M. Le Gall, R. Crépin, C. Auclair, Y. Zhou, J.D. Marks, A. Pelegrin, M.-A. Poul
Study supervision: C. Auclair, M.-A. Poul

Acknowledgments
The authors thank Veronique Fabre-Merssemann for help with the production of recombinant KIT-ECD.

Grant Support
This work was supported by the program “Investissement d’avenir” grant agreement: Label MaBlMprove, ANR-10-LABX-53-01. M. Le Gall and R. Crépin were supported by PhD fellowships from the French Government. M.-A. Poul was supported by the mobility program of Cancéropole Grand Sud Ouest, France.

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Received April 21, 2015; revised August 14, 2015; accepted August 20, 2015; published OnlineFirst September 10, 2015.

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Molecular Cancer Therapeutics

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Marianne Le Gall, Ronan Crépin, Madeline Neiveyans, et al.

Mol Cancer Ther 2015;14:2595-2605. Published OnlineFirst September 10, 2015.

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