Anti-KIT monoclonal antibody inhibits imatinib-resistant gastrointestinal stromal tumor growth

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Contributed by Irving L. Weissman, January 3, 2013 (sent for review August 2, 2012)

Gastrointestinal stromal tumor (GIST) is the most common sarcoma of the gastrointestinal tract and arises from the interstitial cells of Cajal. It is characterized by expression of the receptor tyrosine kinase CD117 (KIT). In 70–80% of GIST cases, oncopgenic mutations in KIT are present, leading to constitutive activation of the receptor, which drives the proliferation of these tumors. Treatment of GIST with imatinib, a small-molecule tyrosine kinase inhibitor, inhibits KIT-mediated signaling and initially results in disease control in 70–85% of patients with KIT-positive GIST. However, the vast majority of patients eventually develop resistance to imatinib treatment, leading to disease progression and posing a significant challenge in the clinical management of these tumors. Here, we show that an anti-KIT monoclonal antibody (mAb), SR1, is able to slow the growth of three human GIST cell lines in vitro. Importantly, these reductions in cell growth were equivalent between imatinib-resistant and imatinib-sensitive GIST cell lines. Treatment of GIST cell lines with SR1 reduces cell-surface KIT expression, suggesting that mAb-induced KIT down-regulation may be a mechanism by which SR1 inhibits GIST growth. Furthermore, we also show that SR1 treatment enhances phagocytosis of GIST cells by macrophages, indicating that treatment with SR1 may enhance immune cell-mediated tumor clearance. Finally, using two xenotransplantation models of imatinib-sensitive and imatinib-resistant GIST, we demonstrate that SR1 is able to strongly inhibit tumor growth in vivo. These results suggest that treatment with mAbs targeting KIT may represent an alternative, or complementary, approach for treating GIST.

Results

SR1 Inhibits GIST Cell-Line Growth in Vitro and Reduces KIT Cell-Surface Expression. KIT protein expression was determined by immunohistochemistry (IHC) on the human GIST cell lines GIST48, GIST430, and GIST882 and as a negative control on a human leiomyosarcoma (LMS) cell line, LMS05 (Fig. 1A). GIST48 and GIST430 were derived from clinical specimens that had developed resistance to imatinib treatment; GIST48 harbors a primary KIT exon 11 missense mutation and a secondary heterozygous KIT exon 17 missense mutation, and GIST430 harbors
a primary heterozygous KIT exon 11 in-frame deletion and a secondary heterozygous KIT exon 13 missense mutation (11). In contrast, GIST882 was derived from a patient before treatment with imatinib and is sensitive to treatment with imatinib in vitro; GIST882 harbors a homozygous missense mutation in KIT exon 13 (6). Consistent with their clinical origins, GIST882 cells treated with increasing doses of imatinib strongly inhibited cell viability, whereas GIST48 and GIST430 cells were significantly less sensitive to treatment in vitro; KIT-negative LMS05 cells showed no response to increasing doses of imatinib (Fig. 1B).

To determine whether KIT-induced cell growth could be affected using an anti-KIT mAb, GIST48, GIST430, GIST882, and LMS05 cells were plated and allowed to adhere overnight before grown in the presence of anti-KIT mAb (clone SR1) or IgG control antibody for 9 d. SR1 was equally effective in slowing the growth rate of both imatinib-resistant cell lines (GIST48 and GIST430) and an imatinib-sensitive cell line (GIST882), whereas the KIT-negative LMS05 cells showed no significant alteration in cell viability as a result of long-term culture with SR1 (Fig. 1C). Together, these results demonstrate that anti-KIT mAb possesses the potential to inhibit KIT-mediated cell growth in GIST cells independently of their resistance or sensitivity to imatinib treatment.

We observed no obvious decreases by Western blot in levels of phosphorylated or total KIT upon treatment with SR1 (Fig. S1) and hypothesized that SR1 treatment of GIST cells could down-regulate levels of KIT expression specifically on the cell surface. To determine levels of KIT cell-surface expression, we used a second anti-KIT mAb (104D2) that binds a KIT epitope different from SR1 (12). We found no decrease in the ability of 104D2 to bind KIT in the presence of SR1 at 4 °C, a condition where receptor internalization or shedding does not occur (Fig. S2). Bortezomib, a small-molecule proteasome inhibitor that has been shown to induce down-regulation, internalization, and degradation of cell-surface KIT, was used as a positive control (13). GIST48, GIST430, GIST882, and LMS05 cells were treated with IgG, SR1, DMSO, or bortezomib for 12 h, and cell-surface KIT expression was analyzed by flow cytometry. We found that, like bortezomib, SR1 was able to induce a significant decrease in cell-surface KIT expression (Fig. 1D).

**Fig. 1.** SR1 treatment slows in vitro GIST cell growth and reduces cell-surface KIT expression. KIT protein expression was analyzed by IHC on paraffin-embedded pellets of GIST48, GIST430, GIST882, and LMS05 cell lines (A). Cell viability assays were carried out to ascertain sensitivity to imatinib treatment for 72 h with GIST and LMS cell lines (B). Viable cell number, as measured by WST-1 absorbance, after 9 d in the presence of 10 μg/mL IgG control or SR1 was evaluated in GIST and LMS cells (C). Cell-surface KIT expression was evaluated in GIST cells by flow cytometry after 12 h of incubation with 10 μg/mL IgG, 10 μg/mL SR1, DMSO (1:1,000), or 100 nM bortezomib (D). All experiments were performed in triplicate. *P < 0.05 and **P < 0.01, as calculated by Student t test.

**SR1 Allows for Phagocytosis of GIST Cells by Macrophages.** As mAbs can also mediate immune cell-mediated tumor clearance, we next performed coculture assays with macrophages to ascertain whether SR1 treatment could induce phagocytosis of GIST tumors. GIST48, GIST430, GIST882, and LMS05 cells were grown fluorescently labeled and then incubated for 2 h with bone marrow-derived macrophages from red fluorescent protein (RFP)-positive mice in the presence of PBS, control IgG, or SR1. The cells were then analyzed by flow cytometry to determine the level of tumor cell phagocytosis by the macrophages. We found that in all three GIST cell lines, SR1 treatment led to a statistically significant increase in macrophage phagocytosis (Fig. 2 A–C and E and Fig. S3). In contrast, KIT-negative LMS05 cells showed no difference in their potential to be phagocytosed regardless of PBS, IgG, or SR1 treatment (Fig. 2 D and F and Fig. S3).

**SR1 Inhibits Growth of Both Imatinib-Sensitive and Imatinib-Resistant GIST Xenografts in Mice.** We next evaluated whether anti-KIT mAbs could inhibit the growth of xenotransplanted GIST tumors in mice. Imatinib-resistant GIST48 and GIST430 cells, and imatinib-sensitive GIST882 cells, were first transduced in vitro with a lentivirus designed to express GFP and luciferase, enabling the use of bioluminescent imaging to monitor tumor engraftment and growth in vivo. For all cell lines, 100,000 cells were injected into the peritoneal cavity of 4- to 8-wk-old NOD.Cg-Prkdcscid Il2rgtm1Wjl, Rag2<sup>−/−</sup>/SzJ (NSG) immunodeficient mice, which lack functional T-cells and B-cells but retain macrophages capable of phagocytosis (14). Two weeks after transplantation, engraftment was evaluated using bioluminescent imaging. GIST48 tumors failed to engraft, but tumor cell engraftment was confirmed in GIST430 and GIST882 mice. The animals were then randomized according to baseline tumor luminescence and treatment commenced (full treatment protocols are available in Table S1).

In imatinib-resistant GIST430 xenografts, treatment with SR1 potently inhibited tumor growth, as evidenced by an ~10-fold decrease in average bioluminescent signal compared with the
IgG-treated controls after 8 wk of treatment (Fig. 3A and B and Table S2). Mice were killed after 8 wk of treatment and autopsies were performed under fluorescent light to aid in identification of tumor masses (Fig. 3C). All mice treated with control IgG showed gross tumor masses whereas only one SR1-treated animal showed a gross tumor. The identity of the tumor masses found in control- and SR1-treated mice was confirmed by histologic examination and IHC. GIST430 xenografts showed a proliferation of a monotonous spindle-cell neoplasm in various locations in the peritoneal cavity. No preference of these tumor masses was found for a specific location although one lesion grew in the wall of the intestine, reminiscent of the site of origin for GIST in human disease. Most tumor masses were large and contained no residual indication of lymph node architecture. The identity of the tumor cells was confirmed by IHC for KIT and DOG1, a marker for GIST (Fig. 3D). The single tumor that was grossly identified in an SR1-treated GIST430-bearing mouse expressed KIT at a level that did not appear different from the control treated group, but the immunoperoxidase technology is likely not sensitive enough to determine the decrease in surface expression of KIT seen on flow cytometry studies, especially because intracellular KIT may still react by immunodetection in formalin-fixed, paraffin-embedded (FFPE) sections.

In contrast to the mice injected with GIST430, the mice that carried GIST882 xenografts showed significantly smaller lesions. Despite this inability of GIST882 cells to generate large tumor masses, we saw a strong inhibition of in vivo tumor growth in mice treated with SR1 compared with those treated with control IgG, with the SR1-treated mice showing a fivefold decrease in average bioluminescent signal compared with IgG-treated controls after 6 wk of treatment (Fig. 4A and B and Table S3). GIST882-bearing animals were euthanized, and no grossly apparent tumor masses were evident. However, under fluorescent light, small tumor foci were seen in many areas in control mice (Fig. 4C, Left) whereas only rare and smaller foci were seen in mice treated with SR1 (Fig. 4C, Right).
Fluorescent microscopy was used to evaluate the presence of GFP-positive growths after animal sacrifice (19). Representative images of SR1 and IgG control-treated mice are shown (B). The identity of GIST430 xenotransplanted tumors was confirmed by H&E staining and by IHC for GIST markers KIT and DOG1 (D).

In conclusion, we show that treatment of human GIST cell lines with the anti-KIT mAb SR1 can inhibit tumor growth in vitro and in vivo and that decreasing cell-surface KIT expression and immune cell-mediated tumor clearance are two mechanisms that likely contribute to SR1’s efficacy. Importantly, SR1-mediated inhibition in tumor growth was independent of imatinib sensitivity or resistance in human GIST cell lines, suggesting that anti-KIT mAb therapy may effectively address the significant clinical problem of imatinib resistance in GIST and thereby form the rationale for evaluating the clinical efficacy of mAb therapy in GIST patients.

Materials and Methods

Cell Culture. GIST cell lines were developed in the Fletcher laboratory at Brigham and Women’s Hospital, Boston, using a protocol approved by the Brigham and Women’s Hospital institutional research board (6, 11). Their derivations and culture conditions have been described previously (6, 11, 24).

Imatinib sensitivity assays, cells were seeded at a density of 4,000 cells per well in 96-well tissue culture plates and allowed to adhere overnight. Imatinib mesylate (Santa Cruz Biotechnology) was dissolved in DMSO to a stock concentration of 10 mM, and cells were then treated with a range of concentrations from 0 to 5 μM; total DMSO was held constant. After 72 h, cell numbers were assessed using WST-1 absorbance. For SR1 sensitivity assays, cells were seeded at a density of 4,000 cells per well in 96-well plates and allowed to adhere overnight. Imatinib mesylate (Santa Cruz Biotechnology) was dissolved in DMSO to a stock concentration of 10 mM, and cells were then treated with a range of concentrations from 0 to 5 μM; total DMSO was held constant. After 72 h, cell numbers were assessed using WST-1 absorbance. For SR1 sensitivity assays, cells were seeded at a density of 4,000 cells per well in 96-well plates and allowed to adhere overnight. Cells were then cultured in the presence of SR1 or mouse IgG control antibody at a concentration of 10 μg/mL. The antibody-containing media was refreshed every 3 d. SR1-mediated inhibition in tumor growth was independent of imatinib sensitivity or resistance in human GIST cell lines, suggesting that anti-KIT mAb therapy may effectively address the significant clinical problem of imatinib resistance in GIST and thereby form the rationale for evaluating the clinical efficacy of mAb therapy in GIST patients.

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Cell Viability Assays. For imatinib sensitivity assays, cells were seeded at a density of 4,000 cells per well in 96-well tissue culture plates and allowed to adhere overnight. Imatinib mesylate (Santa Cruz Biotechnology) was dissolved in DMSO to a stock concentration of 10 mM, and cells were then treated with a range of concentrations from 0 to 5 μM; total DMSO was held constant. After 72 h, cell numbers were assessed using WST-1 reagent (Roche); viable cell numbers are directly related to WST-1 absorbance. For SR1 sensitivity assays, cells were seeded at a density of 4,000 cells per well in 96-well plates and allowed to adhere overnight. Cells were then cultured in the presence of SR1 or mouse IgG control antibody at a concentration of 10 μg/mL. The antibody-containing media was refreshed every 3 d, and cell viability was assessed using WST-1 reagent (Roche) after 9 d of antibody treatment. Purified SR1 mAb was provided by the laboratory of Judith Shizuru (Stanford University School of Medicine). Control mouse IgG antibody was purchased from Innovative Research.

Cell Surface Protein Expression Assays. A total of 500,000 tumor cells were plated in each well of a six-well tissue culture plate (BD Biosciences), allowed to adhere overnight, and treated in triplicate with 10 μg/mL SR1, 10 μg/mL IgG control, DMSO (1:1,000), or 100 nM bortezomib (Selleck Chemicals) for 12 h. The cells were then dissociated using TrypLE (Life Technologies), washed...
with PBS, and stained with anti-KIT mAb (clone 104D2) directly conjugated to phycoerythrin (STEMCELL Technologies), which can bind to KIT in the presence of SR1 (Fig. S2). The cells were then washed twice, stained with DAPI, and analyzed on an LSRFortessa cell analyzer (BD Biosciences).

**Western Blotting.** Protein lysates were prepared from GIST430 and GIST882 cell monolayers using RIPA buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche) and PMSF (Sigma-Aldrich). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Electrophoresis and immunoblotting for KIT, phospho-KIT Y703, phospho-KIT Y719, and \( \beta \)-actin were carried out as previously described (25). Protein expression and phosphorylation changes were visualized by chemiluminescence, captured using a GelDoc system (Bio-Rad), and processed using GIMP and Inkscape software.

**Macrophage Phagocytosis Assays.** C57BL/J6, Rosa26-mRFP1 transgenic mice were used for red-fluorescent macrophage derivation (26), carried out as described previously (27). Tumor cells were green fluorescently labeled with 1 \( \mu \)M carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) for 10 m at 37 °C, washed twice with HBSS (Invitrogen), and incubated for 30 m in Iscove’s modified Dulbecco’s media (IMDM) (Invitrogen) with mouse IgG or SR-1. A total of 50,000 macrophages were then incubated with 100,000 tumor cells for 2 h in Ultra-low Cluster 96-well plates (BD Biosciences), washed twice, stained with DAPI, and analyzed on an LSRFortessa cell analyzer (BD Biosciences). The phagocytic index was defined as the percentage of macrophages that had successfully phagocytosed tumor cells (i.e., RFP-positive and CFSE-high cells) (Fig. 2 E and F and Fig. S3), and differences in phagocytosis between treatment groups were evaluated using Student’s t tests.

**Xenotransplantation Studies.** All animal procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University. Lentiviral production of a pCDH-CMV-EF1-puro construct (Systems Bioscience) containing a ubiquitin promoter driving the expression of a fusion protein containing the Luc2 (pgl4) luciferase gene (Promega) and the eGFP gene (Becton Dickinson) was carried out using standard protocols. NSG mice were used for xenotransplantation studies (14). GIST48, GIST430, and GIST882 cells were transduced with lentivirus, and 100,000 GFP+ cells were injected intraperitoneally into 4- to 8-wk-old NSG mice as described previously (28). Bioluminescent activity was visualized in vivo after a-luciferin injection (Biosynth) on an IVIS Spectrum (Caliper Life Sciences) instrument.

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**Fig. 4.** SR1 treatment inhibits tumor growth in a xenotransplantation model of imatinib-sensitive GIST. Six weeks of treatment with SR1 significantly decreased GIST882 xenograft growth compared with IgG control treatment (A). Representative images of SR1 and IgG control-treated mice are shown (B). Fluorescent microscopy was used to evaluate the presence of GFP-positive growths after animal sacrifice. White arrows denote tumor nodules (C).

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Edris et al. PNAS Early Edition | 5 of 6
and quantified using Image 4.0 software, as described previously (28). Total flux (photons/second) values were obtained from mice. Mice were matched based on total flux 2 wk after cell engraftment and subsequently treated weekly via i.p. injections with SR1 (500 µg) or mouse IgG. Mice were imaged every 2 wk, and differences in tumor growth were assessed using Student t test. At autopsy, tumor masses were visualized using a fluorescent dissecting microscope (Leica). Each symbol in Figs. 3A and 4A (GIST430 and GIST882, respectively) represent an individual mouse. All treatment cohorts consist of five mice, with the exception of the SR1 cohort in the GIST430 treatment (n = 4) due to the death of one animal while performing bioluminescent imaging before initiation of treatment.

**Histology and Immunohistochemistry.** KIT and DOG1 protein expression was evaluated on FFPE cell pellets or on full cross-sections of FFPE xenograft tumors, which were dissected after animal sacrifice, as described previously (24). Samples were then stained with primary antibodies against KIT (Dako; 1:200) and DOG1 (Leica; clone K9; 1:100) on a Benchmark autostainer (Ventana Medical Systems). The IHC reactions were visualized using mouse versions of the EnVision + system (Dako) with diamobenzidine.

**ACKNOWLEDGMENTS.** We thank A. Logan, A. M. Ring, G. Krampitz, J. Oak, R. Li, X. Guo, S. K. Gupta, E. Gilbert, and members of the Stanford Immunodiagnosis Laboratory for technical assistance and helpful discussions. Grant support came from National Institutes of Health Grants CA 112270 and CA 139490, the National Cancer Institute (F30 CA168059 to K.W.), Deutsche Forschungsgemeinschaft (Grant VO 1976/1 to A.K.V.), the Life Raft Group, the GIST Cancer Research Fund, the Jacob Program of Excellence in Gynecologic–Ovarian Cancer Research and Treatment, and the Ludwig Institute for Cancer Research. B. E. is a recipient of the National Science Foundation Graduate Research Fellowship. Dedicated to the memory of our colleague Angela Lee Riegel, who lost her life to GIST.

Fig. S1. Effect of SR1 treatment on phospho-KIT and total KIT expression. GIST430 and GIST882 were treated for 24 h with increasing doses of SR1, IgG (50 μg/mL), or imatinib (1 μM) controls, and phospho-KIT Y703, phospho-KIT Y719, and total KIT levels were analyzed by Western blot (A). GIST882 cells were treated with 10 μg/mL SR1 for 1 h, 6 h, 24 h, 3 d, 6 d, and 9 d or with 10 μg/mL IgG for 9 d, and phospho-KIT Y703, phospho-KIT Y719, total KIT, and actin levels were analyzed by Western blot (B).

Fig. S2. Anti-KIT mAb 104D2 can bind KIT on GIST cells in the presence of SR1. GIST882 cells were suspended in FACS buffer [PBS + 2% (wt/vol) FBS] and incubated on ice (to prevent receptor internalization) for 30 min in the presence of 10 μg/mL IgG or 10 μg/mL SR1. Cells were then stained with 104D2 and analyzed by flow cytometry to evaluate KIT expression (A). No differences were observed in mean fluorescence intensity (MFI) signal (Student t test, P = 0.8704), indicating that SR1 does interfere with 104D2’s ability to stain KIT on GIST cells (B).
Fig. S3. Treatment of GIST cells with SR1 increases phagocytosis by macrophages. A total of 50,000 red fluorescent protein (RFP)-positive mouse macrophages were incubated with 100,000 GFP-positive tumor cells for 2 h after 30 min of pre-incubation of tumor cells with PBS, IgG, or SR1. The cells were then analyzed by flow cytometry. Macrophages that had successfully phagocytosed tumor cells were defined as RFP- and CFSE-double-positive cells. Dot plots are shown for macrophages or tumor cells alone (A), and gates were subsequently drawn to identify double-positive populations for each treatment condition with percentages of double-positive cells labeled in green. Experiments were performed in triplicate, and a representative experiment is shown for GIST48 (B), GIST430 (C), GIST882 (D), and LMS05 (E).
### Table S1. Details of GIST430 and GIST882 xenotransplantation models and SR1 treatment protocols

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*Duration between injection of tumor cells and initiation of antibody treatments.
†Per mouse. Antibody in PBS administered via i.p. injection.

### Table S2. Fold-changes in bioluminescent signal measurements of xenotransplanted GIST430 tumors in mice treated with IgG or SR1

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### Table S3. Fold-changes in bioluminescent signal measurements of xenotransplanted GIST882 tumors in mice treated with IgG or SR1

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*ND (no data) due to technical error during signal measurement.
†ND (no data) due to exclusion by Grubbs statistical outlier test.