Clinical Cancer Research

Mechanistic Insights of an Immunological Adverse Event Induced by an Anti-KIT Antibody Drug Conjugate and Mitigation Strategies

Lawrence L'Italien[1], Olivia Orozco[1], Tinya Abrams[1], Lisa Cantagallo[1], Anu Connor[1], Jayesh Desai[4], Hilmar Ebersbach[2], Hans Gelderblom[5], Keith Hoffmaster[1], Emma Lees[1], Heiko Maacke[2], Siew Schleyer[3], Darko Skegro[2], and Si Tuen Lee-Hoeflich[1]

Abstract

Purpose: Hypersensitivity reactions (HSRs) were observed in three patients dosed in a phase I clinical trial treated with LOP628, a KIT targeted antibody drug conjugate. Mast cell degranulation was implicated as the root cause for the HSR. Underlying mechanism of this reported HSR was investigated with an aim to identifying potential mitigation strategies.

Experimental Design: Biomarkers for mast cell degranulation were evaluated in patient samples and in human peripheral blood cell-derived mast cell (PBC-MC) cultures treated with LOP628. Mitigation strategies interrogated include pretreatment of mast cells with small molecule inhibitors that target KIT or signaling pathways downstream of FcεR1, FcγR, and treatment with Fc silencing antibody formats.

Results: Transient elevation of serum tryptase was observed in patients 1-hour posttreatment of LOP628. In agreement with the clinical observation, LOP628 and its parental antibody LMJ729 induced degranulation of human PBC-MCs. Unexpectedly, KIT small molecule inhibitors did not abrogate mast cell degranulation. By contrast, small molecule inhibitors that targeted pathways downstream of Fc receptors blunted degranulation. Furthermore, interference of the KIT antibody to engage Fc receptors by pre-incubation with IgG or using engineered Fc silencing mutations reduced or prevented degranulation. Characterization of Fcγ receptors revealed human PBC-MCs expressed both FcγRII and low levels of FcγRI. Interestingly, increasing the level of FcγRI upon addition of IFNγ, significantly enhanced LOP628-mediated mast cell degranulation.

Conclusions: Our data suggest LOP628-mediated mast cell degranulation is the likely cause of HSR observed in the clinic due to co-engagement of the FcγR and KIT, resulting in mast cell activation. *Clin Cancer Res; 24(14); 3465–74. ©2018 AACR.*

Introduction

The KIT proto-oncogene receptor tyrosine kinase, KIT, plays important functional roles in interstitial cells of Cajal (ICC), spermatogenesis, melanogenesis, and in hematopoiesis. Hematopoietic stem cells and mast cells are dependent upon KIT signaling for their proliferation and survival (1–3). Terminally differentiated mast cells express high levels of KIT and generally reside in tissues that serve as a barrier to the external environment including skin and small intestines, as well as mucosal tissues (4). The cytoplasm of these cells contain dense granules filled with a mixture of proteases and cytokines such as tryptase, β-hexosaminidase, and histamine, which are released upon stimulation. Degranulation of mast cells in a pathogenic response serves as a host defense mechanism but is also a key contributor to some allergic responses and to chronic inflammation including asthma, cancer, and autoimmune diseases. IgE-mediated Fc-epsilon receptor (FcεR) activation triggers key signaling pathways that induce mast cell degranulation. Alternatively, several receptors for

[1]Novartis Institutes for Biomedical Research, Cambridge, Massachusetts. [2]Novartis Institutes for Biomedical Research, Novartis Campus, Basel, Switzerland. [3]Novartis Institutes for Biomedical Research, Shanghai, China. [4]Royal Melbourne Hospital, Parkville VIC, Australia. [5]Leids Universitair Medisch Centrum, Leiden, The Netherlands.

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L. L'Italien and O. Orozco contributed equally to this article.

Current address for L. L'Italien: Celsius Therapeutics, Cambridge, Massachusetts; current address for O. Orozco: Relay Therapeutics, Cambridge, Massachusetts; current address for T. Abrams: Novartis Institutes of Biomedical Research, Cambridge, Massachusetts; current address for L. Cantagallo: H3 Biomedicine, Cambridge, Massachusetts; current address for A. Connor: Novartis Institutes of Biomedical Research, Cambridge; current address for J. Desai: Royal Melbourne Hospital, Parkville, VIC, Australia; current address for H. Ebersbach: Novartis Institutes for Biomedical Research, Novartis Campus, Basel, Switzerland; current address for H. Gelderblom: Leids Universitair Medisch Centrum, Leiden, Netherlands; current address for Keith Hoffmaster: Novartis Institutes of Biomedical Research, Cambridge, Massachusetts; current address for E. Lees: Jounce Therapeutics, Cambridge, Massachusetts; current address for Heiko Maacke: Novartis Institutes for Biomedical Research, Novartis Campus, Basel, Switzerland; current address for S. Schleyer: CytomX, South San Francisco, California; current address for Darko Skegro Novartis Institutes for Biomedical Research, Novartis Campus, Basel, Switzerland; current address for S.T. Lee-Hoeflich Bicycle Therapeutics, 4 Hartwell Place, Lexington, Massachusetts.

Corresponding Authors: Lawrence L'Italien, Celsius Therapeutics, 215 First Street, 4th Floor, Cambridge, MA 02142. Phone: 415-786-1259; E-mail: llitalien@celsiustx.com; and Si Tuen Lee-Hoeflich, Bicycle Therapeutics, 4 Hartwell Place, Lexington, MA 02421. Phone: 617-588-1066; E-mail: si.lee-hoeflich@bicycletherapeutics.com

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Translational Relevance

LOP628, a KIT targeted antibody drug conjugate (ADC), induced tumor regression in preclinical tumor models and had a tolerable safety profile in nonhuman primates thus supporting its clinical utility. Unexpectedly, three patients enrolled in a LOP628 phase I clinical trial experienced rapid hypersensitivity reactions (HSRs). Here we show that LOP628-mediated mast cell degranulation is likely the cause of the observed HSR and that the co-engagement of the Fc gamma receptor and KIT on mast cells is the underlying mechanism. Small molecule inhibitors that block Fc gamma receptor signaling are capable of blunting mast cell degranulation. These data highlight the challenge of using preclinical models to evaluate the in vivo effects of an antibody therapeutic and identify a risk of antibodies co-engaging targets and Fc receptors.

IgG (FcγRs) that are expressed on mast cells positively or negatively regulate degranulation (5–7). In addition, KIT signaling may also augment Fc-epsilon receptor signaling to enhance degranulation (8).

KIT is also a proto-oncogene (9) that has been shown to be constitutively activated through gain-of-function mutations in several cancer types, such as in gastrointestinal stromal tumors (GISTs), acute myeloid leukemia (AML), and subsets of melanoma. In addition, wild-type KIT overexpression and occasional amplification has been reported in additional cancers such as uveal melanoma, lung cancer, sub-type of breast cancer and renal cancer (10–12). However, small molecule inhibitors targeting KIT are most effective in treating cancers with KIT driver mutations. For example, imatinib, a multi-targeted small molecule that inhibits KIT, is the current first-line treatment for GIST, rending durable responses especially in patients with exon 11 mutations. However, the response to imatinib is reduced in cancers with exon 9 mutations or wild-type KIT (13). Additional KIT inhibitors were developed to improve clinical responses, but invariably the development of subsequent mutations is a common mechanism that leads to drug resistance. Because KIT mutations occur predominantly within the intracellular domain, targeting the extracellular domain of KIT with an antibody drug conjugate working through a different mechanism of action would be an opportunity to treat KIT positive cancers regardless of mutational status of the target.

LOP628, a non-cleavable SMCC-DM1 antibody drug conjugate (ADC) co-developed with Immunogen, Inc., binds similarly to wild type and mutant KIT to efficiently deliver the maytansinoid toxin to the cancer. In support of this, T. Abrams et al (submitted for publication), showed that LOP628 was efficacious in multiple preclinical xenograft models including imatinib sensitive and refractory GIST as well as SCLC and AML models. Despite the expression and function of KIT in normal tissues, including mast cells, administration of LOP628 had a tolerable safety profile and did not cause elevations of tryptase, a biomarker used to determine degranulation of mast cells, in nonhuman primates. These data suggested that LOP628 may have the potential for treating patients with KIT overexpressing cancers. Unexpectedly, three GIST patients enrolled in a LOP628 phase I clinical trial experienced rapid hypersensitivity reactions (HSR). Here we show that LOP628-mediated mast cell degranulation is likely the cause of the observed HSR and that the co-engagement of the Fc gamma receptor and KIT is the underlying mechanism.

Materials and Methods

Clinical aspect

Three patients with GISTs, who failed standard of care, were enrolled in a phase I clinical study (Trial registration ID: NCT02221505). Infusions were given of either 0.3 mg/kg LOP628, without premedication, or 0.15 mg/kg LOP628, with premedication of dexamethasone and cetirizine. When necessary, patients were rescued with combinations of the following: clemastine, dexamethasone, hydrocortisone, promethazine, paracetamol, ranitidine, fluids, and oxygen. Serum samples were obtained under patient consent and the tryptase analysis was conducted by a CLIA/CAP-certified laboratory in the United States.

Antibodies and inhibitors

Anti-KIT antibody LMJ729, whole IgG, F(ab)2 and Fc engineered versions were generated at Novartis. The anti-KIT LOP628 (T. Abrams et al; submitted for publication), and anti-P-cadherin ADC conjugated to SMCC-DM1 (manuscript in preparation) were generated at ImmunoGen, Inc. Anti-human IgE, clone LE27, was purchased from NBS-C Bioscience via antibodies-on-line (#0908-1-100).

Dasatinib, imatinib, midostaurin, and nilotinib were generated at Novartis. Ibrutinib and sunitinib were obtained from Selleck chemicals (#S2680 and #S1042).

Mast cell generation

Human peripheral blood cell-derived mast cells (PBC-MCs) were generated from mobilized peripheral blood $CD34^+$ cells isolated from healthy donors (AllCells #mPB017F). Mast cells were generated either by using a semisolid culturing method (14) or suspension culturing method (15). For the semisolid method, the basic culture medium (BCM) comprises Iscove's modified Dulbecco's medium (IMDM, ThermoFisher, #12440-053) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L β-mercaptoethanol. $CD34^+$ progenitors were cultured in one-part BCM to four parts Methocult (Stemcell Technologies, #H4034) and supplemented with recombinant human 5 ng/mL IL3, 50 ng/mL IL6, and 200 ng/mL SCF (Peprotech; #200-03, #200-06, #300-07). After 2 weeks, an equal volume BCM/Methocult, as above except with the IL3 excluded, was overlaid. At 4 weeks, the culture was supplemented with 1 mL BCM supplemented with 200 ng/mL SCF, 100 ng/mL IL6, 1× insulin-transferrin-selenium (ITS; ThermoFisher, #41400045), and 0.1% BSA (Miltenyi, #130-091-376). After an additional 2 weeks, the maturing mast cells were transferred to a suspension culture medium (BCM supplemented with 100 ng/mL SCF, 50 ng/mL IL6, 1X ITS and 0.1% BSA). Briefly for the suspension culturing method, $CD34^+$ progenitors were cultured in SFEM-BCM medium [StemSpan SFEM-II (Stemcell Technologies, #09655) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 100 ng/mL each IL3, IL1β (Peprotech, #200-01B), IL6, and SCF], at a cell concentration of 0.5×10^6/mL. Every few days, the volume of culture was adjusted to maintain 0.5×10^6/mL with SFEM-BCM without IL3 and IL1β. IL3 and IL1β were only used at culture initiation. After 8 weeks, under both conditions,

PBCMCs were >90% FcεR1α+KIT+ (Supplementary Fig. S1A). Upon IgE sensitization, the cells undergo degranulation in response to the addition of an anti-IgE antibody (Supplementary Fig. S2A) and respond in a non-IgE dependent manner to Compound 48/80 (Supplementary Fig. S2B). PBC-MCs were used between 8 and 12 weeks after initiation of culture.

Degranulation assays

PBC-MCs were untreated or sensitized overnight with 1 μg/mL IgE (Abbiotec #250203) and 10 ng/mL IL4 (Peprotech #200-04) or 150 ng/mL IFNγ (Peprotech #300-02). Cells were harvested and washed with Hanks Balanced Salt Solution (ThermoFisher, #14025) + 0.1% BSA and treated with anti-KIT antibodies/ADCs, control antibodies/ADCs, or compound 48/80 (Sigma, #C2313) for 60 minutes at 37°C. Samples were centrifuged at 300 × *g* for 4 minutes before the cell-free supernatants were collected to measure β-hexosaminidase release. For signal blocking experiments, cells were preincubated with inhibitors for 30 minutes prior to adding antibodies. For crosslinking experiments, KIT antibody was preincubated with a goat anti-human IgG F(ab′)2 specific antibody (Jackson ImmunoResearch, #109-006-097) for 30 minutes prior to addition.

β-hexosaminidase activity was measured by mixing 50 μL supernatant and 50 μL of β-hexosaminidase substrate [1 mmol/L 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma, #M2133) in 0.1 M citric acid (Sigma, #251275) buffer pH 4.5] in a 96-well plate and incubating for 90 minutes at 37°C before quenching the reaction with 50 μL of 1 M Tris-base (Sigma, #T1503). The plates were then read with excitation 355 nm and emission 460 nm on the Spectramax M5 (Molecular Devices).

Flow cytometry analysis

Mast cells were washed twice in FACS buffer (PBS, 0.1% BSA, 0.1% sodium azide) and resuspended to a minimum of 100,000 cells per staining in 100-μL buffer that contained Human FcR Binding Inhibitor (eBioscience, #14-9161-73). Cells were incubated for 15 minutes at 4°C and then mixed with 100 μL of 1:100 dilutions of FACs test antibody, incubated 30 minutes at 4°C in the dark, followed by two washes with FACs buffer. Depending on the staining, all FACs antibodies were anti-human and may include: KIT [104D2, eBioscience, #15-1178-42 (PE-Cy5) or #17-1178-42 (APC)], FcεR1α [AER-37, eBioscience, #48-5899-42 (eFluor 450) or # 48-5899-42 (PerCP-eFluor 710)], CD16 [3G8, BD Bioscience, #557744 (PE-Cy7)], CD32 [6C4, BD Bioscience, #17-0329-42 (APC)], CD64 [10.1, eBioscience, #12-0649-42 (PE)]. A fixable viability dye (eFluor-780, eBioscience, #65-0865-18) was used at a 1:5,000 dilution and added with the test antibodies. To confirm binding of Fc engineered antibodies, an indirect FACs was conducted with the KIT positive cell line, OCI-M1 (acute myeloid leukemia, DSMZ #ACC-529), using a F(ab′)2 fragment goat anti-human, F(ab′)2 fragment specific secondary antibody (Jackson ImmunoResearch, #109116097). The identity of OCI-MI was authenticated by single-nucleotide polymorphism (SNP) fingerprinting (Sequenom) as previously described (T. Abrams et al.; submitted for publication). Data collection was performed using a LSR Fortessa (Becton Dickinson) and analyzed with Flowjo software (Flowjo).

Western blot analysis

PBC-MCs were treated as for the degranulation assay and reactions were terminated with denaturing lysis buffer with lysates prepared as described (16). Proteins were separated on 4% to 12% NuPage Tris-Bis gels using NuPage MES-SDS running buffer (ThermoFisher), transferred onto nitrocellulose membranes, and probed with the following antibodies from Cell Signaling Technology: phospho-KIT (Y719) (#3991), KIT (#3308), pan AKT (#2920), phospho-AKT (S473)(#4060), p44/42 MAPK (#4696), and phospho-p44/42 MAPK (#4370). The membranes were then incubated with the appropriate secondary antibodies (Licor #926-68070 or #926-32211). The immunoreactive bands were visualized on the Odyssey imaging system (Licor).

Gene expression assays

RNA was isolated from mast cells or NK3.3 (17) using Purelink RNA mini (ThermoFisher, #12183020) and reverse transcribed using TaqMan Reverse Transcription Reagents (ThermoFisher, #N8080234). FcγR1 and FcγR3 levels were measured using gene expression assays (ThermoFisher, #Hs00417598 and #Hs00275547) and normalized to GAPDH (ThermoFisher, #Hs02758991). Limit of detection calculated as the ΔCt (undetermined Ct value of 38 – GAPDH Ct). Fold change was calculated as $2^{\Delta\Delta CT\ (\Delta Ct\ sample\ -\ \Delta Ct\ LOD)}$.

Statistical significance

Statistical significance was calculated by GraphPad Software using one-way ANOVA with Dunnett's multiple comparisons test.

Results

Mast cell degranulation is implicated as the root cause of HSRs observed in patients treated with LOP628

During the first in human phase I clinical study of LOP628, HSR were observed in three patients with GISTs during LOP628 infusions. As presented in Fig. 1, patient No. 1 received an infusion of 0.3 mg/kg LOP628 and rapidly developed a grade 3 HSR within 33 minutes. The infusion was stopped, and the patient was given rescue medications of 2 mg clemastine and 4 mg dexamethasone. Patient No. 2 was premedicated with 8 mg dexamethasone and 10 mg cetirizine prior to receiving the reduced first dose (cycle 1) of LOP628 at 0.15 mg/kg and no significant adverse events were observed. However, upon receiving the second dose (cycle 2), patient No. 2 experienced a grade 1 HSR that dissipated when the infusion was interrupted. However, a grade 1 HSR returned when the infusion was resumed. The patient was then given rescue medications consisting of 100 mg hydrocortisone, 12.5 mg promethazine, 1 g paracetamol which completely resolved the symptoms, and the entire dose was successfully administered. Subsequently, patient No. 2 received the third dose of LOP628 (cycle 3) where a grade 1 HSR was observed that dissipated when the infusion was stopped and upon resumption then escalated to grade 2 HSR. The patient was rescued with fluids and oxygen and LOP628 administration was not restarted. Patient No. 3 was premedicated with 8 mg dexamethasone and 10 mg cetirizine and a grade 3 HSR was observed within 45 minutes of LOP628 infusion. The patient was rescued with fluids, oxygen, 5 mg albuterol, 12.5 mg promethazine, 100 mg hydrocortisone, and 50 mg ranitidine and LOP628 administration was not resumed.

To investigate the cause of the HSR, the levels of serum tryptase, a major protease in human mast cell that is released into the bloodstream upon activation (18), was assessed as it is reported to

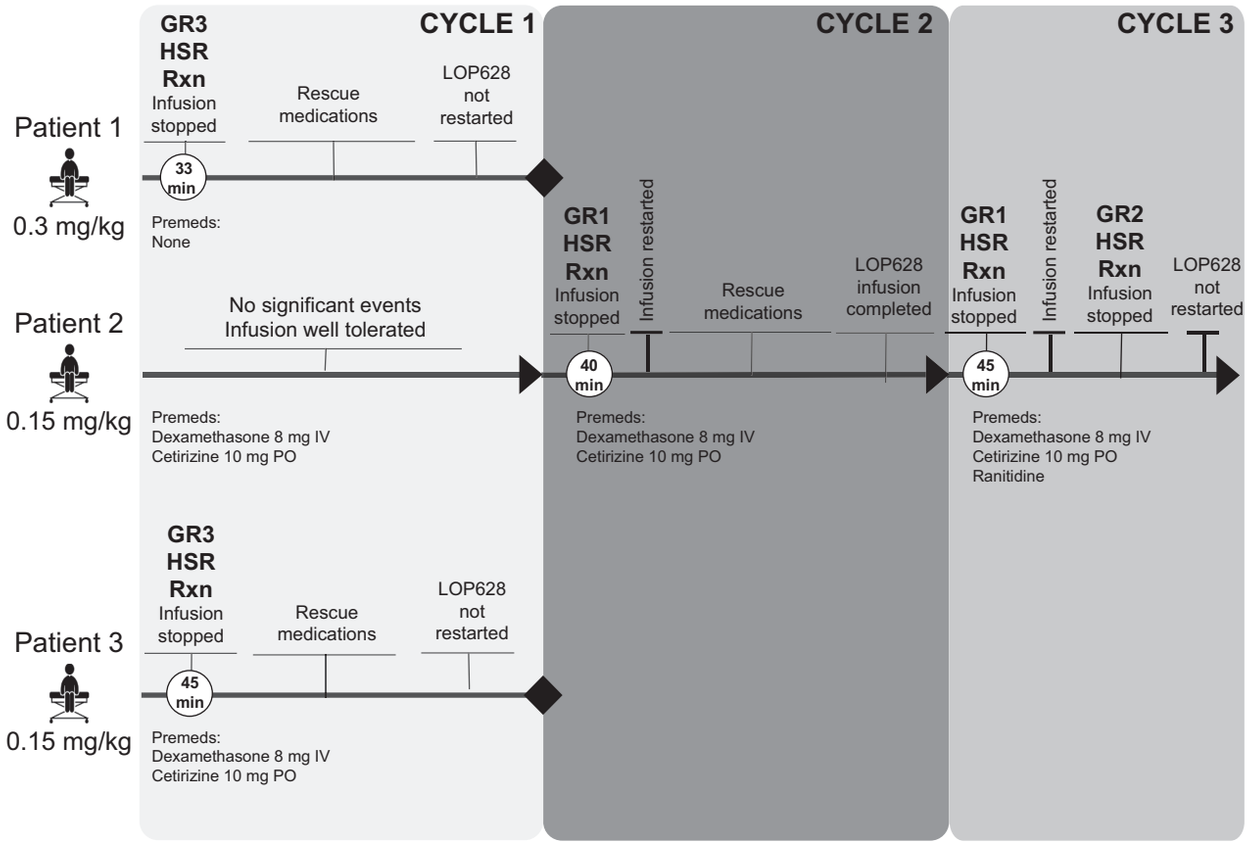

Figure 1.

LOP628 clinical observations of dose-limiting toxicity. Three patients developed hypersensitivity reactions (HSRs) during LOP628 infusions. Patient No. 1 received an infusion of 0.3 mg/kg LOP628 and rapidly developed a grade 3 HSR within 33 minutes. The infusion was stopped, and the patient was given rescue medications of 2 mg clemastine and 4 mg dexamethasone. Patient No. 2 was premedicated with 8 mg dexamethasone and 10 mg cetirizine prior to receiving the reduced first dose (cycle 1) of LOP628 at 0.15 mg/kg, and no significant adverse events were observed. However, upon receiving the second dose (cycle 2), patient No. 2 experienced a grade 1 HSR that dissipated when the infusion was interrupted. However, a grade 1 HSR returned when the infusion was resumed. The patient was then given rescue medications consisting of 100 mg hydrocortisone, 12.5 mg promethazine, 1 g paracetamol which completely resolved the symptoms, and the entire dose was successfully administered. Subsequently, patient No. 2 received the third dose of LOP628 (cycle 3) where a grade 1 HSR was observed that dissipated when the infusion was stopped and then escalated to grade 2 HSR upon resumption. The patient was rescued with fluids and oxygen and LOP628 administration was not restarted. Patient No. 3 was premedicated with 8 mg dexamethasone and 10 mg cetirizine, and a grade 3 HSR was observed within 45 minutes of LOP628 infusion. The patient was rescued with fluids, oxygen, 5 mg albuterol, 12.5 mg promethazine, 100 mg hydrocortisone, and 50 mg ranitidine and LOP628 administration was not restarted.

be elevated in drug-induced patients with HSR (19–21). Blood samples of LOP628-treated patients collected prior to dosing, 1 and 24 hours post-dosing were analyzed. A transient and significant increase in serum tryptase was observed in all three patients 1-hour post-dosing (17–35 ng/mL) relative to basal levels assessed at pretreatment (2 to 6 ng/mL), with levels decreasing by 24 hours posttreatment (2–13 ng/mL; Table 1). This observation suggests that LOP628 treatment might result in mast cell degranulation leading to a HSR.

LOP628 and its parental antibody LMJ729 mediate degranulation in PBC-MCs

Because elevated serum tryptase levels implicate mast cell degranulation as the possible cause of HSR observed in the clinic, we interrogated whether LOP628 would mediate mast cell degranulation *in vitro* using PBC-MCs. PBC-MCs express KIT and FcεR1α (15) and upon IgE sensitization undergo degranulation in response to antigen induced FcεR1α cross-linking (22). We generated PBC-MCs as previously described (14, 15) and confirmed that the addition of an anti-IgE antibody induced degranulation of IgE-sensitized mast cells as measured by the release of β-hexosaminidase, an established marker for mast cell degranulation (Fig. 2). As shown in Fig. 2, LOP628 also induced mast cell degranulation in a dose-dependent manner with and without IgE-sensitization, as did LMJ729, the unconjugated parental antibody of LOP628 (Supplementary Fig. S2A and S2B). LOP628-mediated mast cell activation appears dependent on KIT expression because degranulation was not mediated by an anti-P-cadherin ADC (PCAD-ADC) also conjugated via SMCC to DM1, which targets a protein not expressed on mast cells. Together, these results suggest that the observed mast cell degranulation is not because of the ADC payload, but rather the antibody binding to the KIT receptor expressed on the mast cells.

Table 1. Exploratory evaluation of patient serum tryptase levels from cycle 1

	Serum tryptase (ng/mL)		
Patient	**Pre-dose**	**1 hour**	**24 hours**
No. 1	6	33	13
No. 2	2	17	2
No. 3	3	35	11

Small molecule inhibitors that target downstream signaling of Fc receptors blunt LOP628-mediated degranulation

KIT signaling has been linked to mast cell degranulation. Addition of the KIT ligand, stem cell growth factor (SCF), enhances IgE/FcεR-mediated mast cell degranulation (8). However, KIT signaling may also trigger mast cell degranulation independent of IgE/FcεR and the small molecule KIT inhibitor, imatinib, specifically blocked such KIT-driven degranulation (23). We selected small molecule inhibitors based on the observation that they were either approved as a treatment for mastocytosis, or were currently being evaluated in the clinic (24). Dasatinib, midostaurin, and ibrutinib have also been reported to block IgE-dependent degranulation (25–27). To address whether LMJ729/LOP628-mediated degranulation required signaling through KIT, we tested the blocking ability of imatinib in this context. We postulated that LMJ729 binding to KIT may induce downstream signaling that results in mast cell degranulation and that mitigation of KIT signaling by imatinib would prevent degranulation. As shown in Fig. 3, LMJ729-mediated mast cell degranulation was not inhibited by pretreatment with imatinib. Additional KIT inhibitors, sunitinib and nilotinib, were likewise ineffective (Supplementary Fig. S3A). The lack of inhibition suggests that KIT signaling is not required for LMJ729-mediated degranulation and that an alternative mechanism is involved. Pretreatment of mast cells with dasatinib, ibrutinib, or midostaurin, significantly diminished LMJ729-induced degranulation (Fig. 3). The inhibitory effects of these molecules on mast cell degranulation were also observed with LOP628 treatment (Supplementary Fig. S3B). Although dasatinib and midostaurin inhibit KIT, they also inhibit Src-family kinases (SFK) and Protein Kinase C (PKC), respectively. In contrast, ibrutinib inhibits Bruton's tyrosine kinase (BTK) signaling. SFK, PKC, and BTK are key components of mast cell activation signaling cascades (28, 29). These data suggest that LMJ729-induces mast cell degranulation through mast cell activation signaling pathways that are independent of KIT signaling.

LMJ729-mediated mast cell degranulation requires interaction with the FcR

Because inhibition of key components of mast cell activation signaling cascades was sufficient to prevent LMJ729- and LOP628-mediated degranulation, we postulated that LMJ729 induces mast cell activation signaling by binding to additional receptor(s). All three inhibitors Dasatinib, Ibrutinib, or Midostaurin have been shown to block the key signaling nodes downstream of all Fc receptors comprised of the common Fc gamma chain (30) including FcεRI, FcγRI, and FcγRIIIa, suggesting that Fc receptors may play a role in LMJ729-mediated degranulation (31). To address this, we evaluated the effect of preventing the Fc/Fc receptor interaction on mast cell degranulation. Nonsensitized mast cells were preincubated with either IgG_1 or IgE to occupy the FcγRs or FcεR Fc binding sites prior to the addition of LMJ729. As shown in Fig. 4A, pre-incubation with IgG_1 but not IgE blocked LMJ729-mediated mast cell degranulation in a dose-responsive

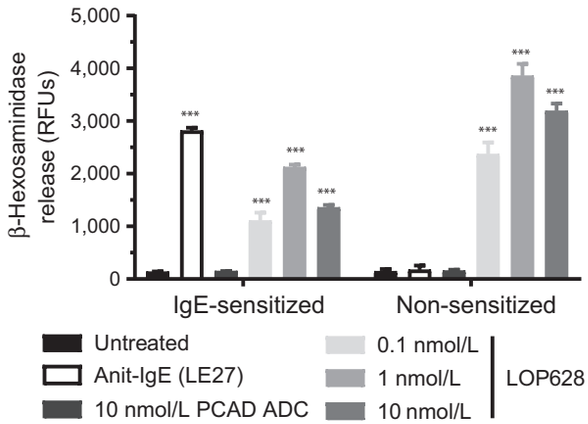

Figure 2.
The KIT antibody drug conjugate, LOP628, mediates mast cell degranulation *in vitro*. Human $CD34^+$ PBC-MCs were cultured with specified treatments in a degranulation assay for 60 minutes prior to β-hexosaminidase release measurement. The relative fluorescence units (RFUs) measurements represent β-hexosaminidase release. Anti-IgE (LE27; 1 μg/mL) served as a positive control for IgE-dependent degranulation. These data are representative of a minimum of three independent experiments conducted in quadruplicate with mast cells derived from at least three individual donors. In some cases, experimental conditions were repeated in more experiments with more donors (example: 1 nmol/L LOP628 or LMJ729 in nonsensitized mast cells). Both IgE-sensitized and non-sensitized human PBC-MCs undergo degranulation only in the presence of LOP628. *P* value calculated by one-way ANOVA comparing to untreated; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

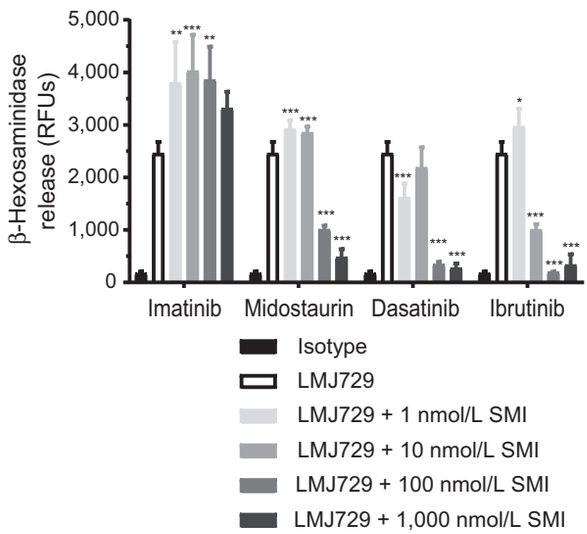

Figure 3.
Small molecule inhibitors that target downstream signaling of Fc receptors blunt LMJ729-mediated degranulation. $CD34^+$ PBC-MCs were pretreated with titrated small molecule inhibitors (SMIs) prior to addition of 1 nmol/L LMJ729 in a degranulation assay. The KIT inhibitor, imatinib, did not block LMJ729-mediated degranulation. Pretreatment of mast cells with dasatinib, ibrutinib, or midostaurin, for 30 minutes blocked LMJ729-mediated mast cell degranulation. These data are representative of three independent experiments conducted in quadruplicate with mast cells derived from two individual donors. *P* value calculated by one-way ANOVA comparing to LMJ729 alone; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

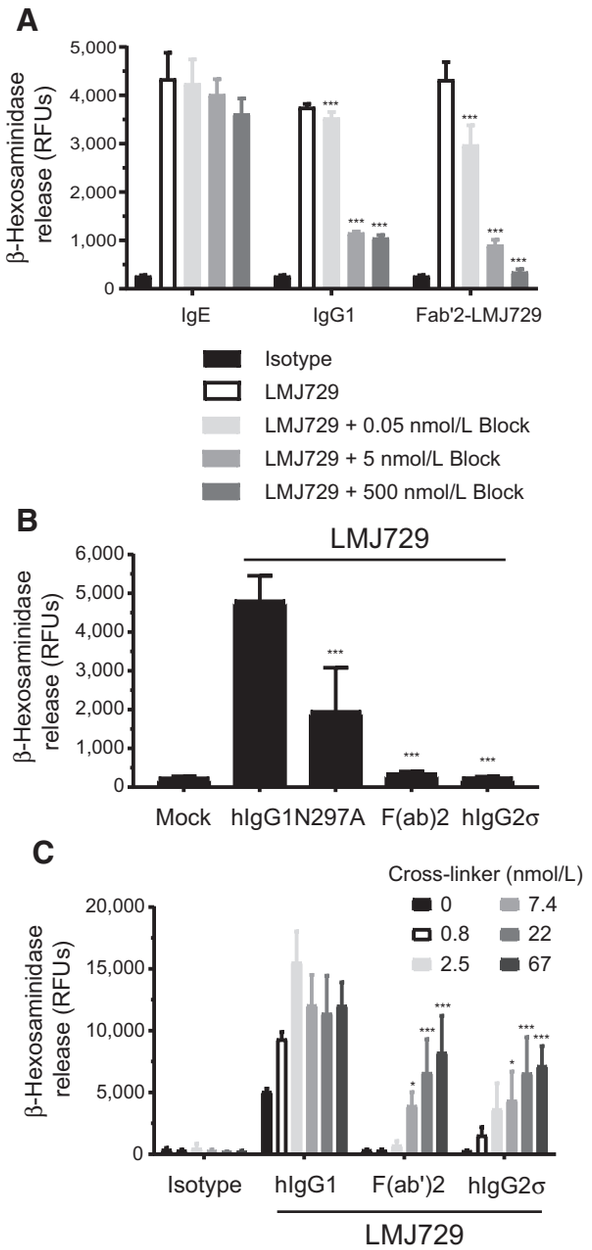

Figure 4.

LMJ729-mediated mast cell degranulation requires the antibody Fc/Fc Receptor interaction. **A,** Pre-incubation with IgG_1 or F(ab′)2-LMJ729 prevented degranulation, whereas IgE did not block LMJ729-mediated mast cell degranulation. $CD34^+$ PBC-MCs were pre-incubated with either titrated IgE, IgG_1, or F(ab′)2-LMJ729 prior to the addition of 1 nmol/L wild-type LMJ729 in degranulation assays. Representative data of three independent experiments conducted in quadruplicate with mast cells derived from three individual donors. *P* value calculated by one-way ANOVA comparing to LMJ729 alone; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B,** Impairing LMJ729 binding to Fc receptors by removing the Fc (F(ab′)2) or engineering Fc-silent mutations into the Fc (N297A or hIgG2σ) reduced or eliminated LMJ729-mediated mast cell degranulation. One nmol/L of either wild type ($hIgG_1$) or Fc modified (F(ab′)2, N297A, or IgG2σ) LMJ729 were used in $CD34^+$ PBC-MCs degranulation assays. Representative data from at least three independent experiments conducted in quadruplicate with mast cells derived from three individual donors. In some cases, experimental conditions were repeated in more experiments with additional donors (example: N297A). *P* value calculated by one-way ANOVA comparing to LMJ729-$hIgG_1$; **, $P < 0.01$; ***, $P < 0.001$. **C,** Pre-incubating titrated cross-linker with Fc modified LMJ729 (F(ab′)2 or IgG2σ) restored the ability of Fc-modified LMJ729 to induce degranulation. Titrated cross-linker pre-incubated with 1 nmol/L wild-type LMJ729 ($hIgG_1$) enhanced degranulation. Titrated cross-linker with isotype control did not induce degranulation. Representative IgG2σ data from two independent experiments conducted in quadruplicate with mast cells derived from one individual donor. Representative data from F(ab′)2, $hIgG_1$, and Isotype, data from three independent experiments conducted in quadruplicate with mast cells derived from three individual donors. *P* value calculated by one-way ANOVA comparing to respective antibody format without cross-linker, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

manner. Because of IgG_1 is known to bind all FcγRs (32) and is the same isotype as LMJ729, these data suggest that the binding to the FcγRs are essential for LMJ729-mediated mast cell degranulation. Consistent with this observation, either removal of the Fc domain of LMJ729 (F(ab′)2), introduction of Fc mutations that have been shown to perturb binding to FcγRs (33) (N297A; ref. 34) or antibody conversion to hIgG2σ that does not bind to any FcγRs (35) blunt mast cell degranulation compared to the parental LMJ729 wild-type IgG_1 antibody (Fig. 4B). All Fc mutants and the F(ab)′2 versions of LMJ729, retained their ability to bind to KIT suggesting that the impaired ability of these constructs to mediate degranulation is not due to interference of binding to KIT (Supplementary Fig. S4). Re-introduction of the Fc-domain via addition of an anti-F(ab′)2–specific IgG crosslinking antibody to LMJ729 was able to restore mast cell degranulation (Fig. 4C). The immune complex formed by cross-linking an isotype control antibody was not sufficient to induce mast cell degranulation (Fig. 4C). These data confirm that the interaction of the Fc domain of LMJ729 to the Fc receptor on mast cells was critical for the observed activity. Conversely, pre-incubation with the LMJ729 F(ab′)2 derivative also blocked degranulation (Fig. 4A), suggesting that both KIT binding and Fc receptor binding on mast cells are together required for LMJ729-mediated degranulation.

Fc Receptors implicated in LMJ729 mediated mast cell degranulation

To further elucidate the nature of the Fc/FcR interaction, non-sensitized mast cells were next profiled for their cell surface expression patterns of the Fc receptors. It has been previously shown that *in vitro* cultured mast cells express FcγRII (CD32) and FcγRI (CD64), but not FcγRIII (CD16) (5). Similarly, significant levels of CD32 and low levels of CD64 but not CD16 were detected by flow cytometry in our PBC-MCs (Fig. 5A). The lack of CD16 expression was further confirmed by TaqMan analysis (Supplementary Fig. S5). These data suggest that LMJ729 might mediate degranulation via CD64 and/or CD32. Because FcγR has been shown to have a role in allergy and auto-immune disorders (reviewed in ref. 36) and CD64 was upregulated upon treatment with IFNγ (5), we further investigated the impact of IFNγ treatment on LMJ729-mediated mast cell degranulation. IFNγ treatment significantly increased CD64 cell surface expression but did not alter CD32 or FcεR1α cell surface expression significantly (Fig. 5A). This increased level of CD64 also correlated to elevated β-hexosaminidase release indicating enhanced degranulation (Fig. 5B). Interestingly, Ibrutinib was able to override degranulation mediated by LMJ729 in the presence of IFNγ (Fig. 5C). The effect of IFNγ on degranulation is LMJ729-specific since the treatment did not result in the mast cells being more reactive to

isotype control (Fig. 5B) nor the Fab′2 version of LMJ729 (Supplementary Fig. S6). This enhanced degranulation of mast cells pretreated with IFNγ was also translated to upregulation of downstream signaling indicated by the increased level of phospho-Akt and phospho-Erk (Fig. 5D).

Discussion

HSRs were observed in all three patients treated with the KIT ADC LOP628 during a phase I clinical trial. Because of these adverse events, the trial has been terminated. High levels of serum tryptase found in all patients after dosing with LOP628 indicated that the reason for the observed HSR was most likely the activation of mast cells. In the present studies, the underlying mechanism of these mast cell activations was investigated with the aim of identifying potential mitigation strategies.

As expected, KIT binding was required because both a non-KIT targeting ADC and an antibody isotype control did not induce mast cell degranulation. Surprisingly, KIT signaling was nonessential because pretreatment with KIT inhibitors was not able to prevent degranulation. This observation implies that blocking KIT signaling with KIT small molecule inhibitors would not be a successful mitigation strategy. To block LOP628-mediated degranulation, we found that inhibiting key Fc receptor signaling

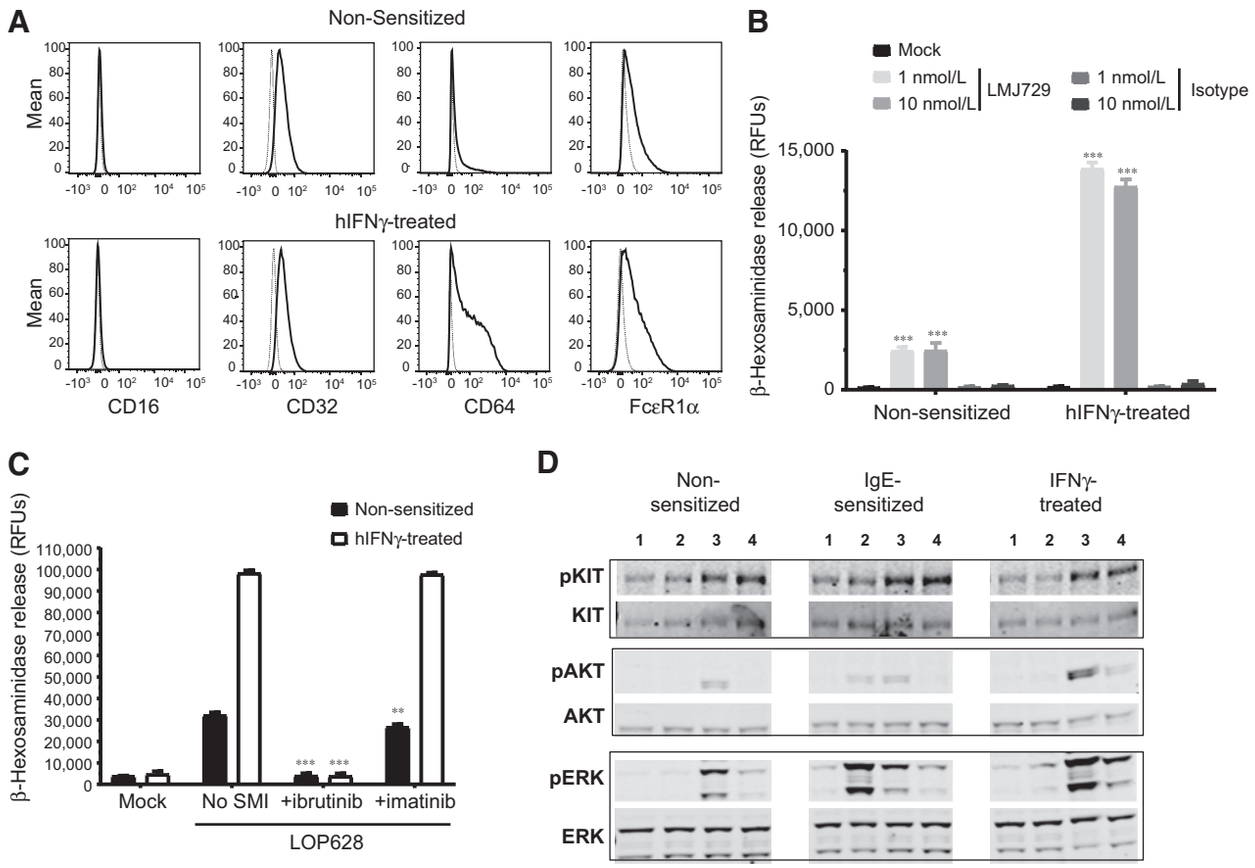

Figure 5.

Fc receptors implicated in LMJ729-mediated mast cell degranulation. **A,** FcγRI (CD64) cell surface expression is enhanced after IFNγ treatment. Mast cells were treated ± IFNγ for 24 hours and then Fc receptors profiled by flow cytometry for FcγRIII (CD16), FcγRII (CD32), CD64, and FcεR1α expression. Solid line: profiled Fc receptor; dotted line: isotype. Representative data of three independent experiments conducted in quadruplicate with mast cells derived from two individual donors. **B,** IFNγ treatment enhanced LMJ729-mediated degranulation. Mast cells were treated ± IFNγ for 24 hours prior to degranulation assay. Representative data of five independent experiments conducted in quadruplicate with mast cells derived from three individual donors. *P* values calculated by one-way ANOVA comparing to isotype control; ***, $P < 0.001$. **C,** Ibrutinib retains ability to block LOP628-mediated degranulation in IFNγ-treated mast cells. Mast cells were pretreated with the small molecule inhibitors (SMI) ibrutinib or imatinib prior to the addition of 1 nmol/L LOP628 in a degranulation assay. One hundred nmol/L Ibrutinib blocked LOP628-mediated mast cell degranulation in either untreated or IFNγ-treated mast cells, whereas 100 nmol/L Imatinib did not block. Representative data of two independent experiments conducted in quadruplicate with mast cells derived from two individual donors. *P* values calculated by one-way ANOVA comparing to LOP628 without inhibitor; *, $P < 0.05$; ***, $P < 0.001$. **D,** IFNγ treatment enhances LOP628-mediated downstream signaling indicated by the increased level of phospho-Akt and phospho-Erk. Nonsensitized, IgE-sensitized, or IFNγ-treated mast cells were used in degranulation assays with the labeled conditions. Western blots of mast cell lysates used in the degranulation assays were probed with antibodies against phospho-KIT, KIT, phospho-Akt, Akt, phospho-Erk, and Erk. Anti-IgE (LE27) served as a positive control in IgE-sensitized mast cells. Mast cell pretreatment with 100 nmol/L Ibrutinib, prior to the addition of 1 nmol/L LOP628 resulted in decreased phospho-Akt and phospho-Erk compared to LOP628 alone; phospho-KIT remained unchanged. Representative nonsensitized and IgE-sensitized data of a two independent experiments done with mast cells derived from two individual donors. IFNγ-treated data of one independent experiment conducted with mast cells derived from one donor. 1, Untreated; 2, anti-IgE (LE27); 3, LOP628; 4, LOP628 + ibrutinib.

molecules was required. Ibrutinib, dasatinib, and midostaurin abrogated or reduced LOP628-mediated mast cell degranulation. Thus, cotreatment of LOP628 with these inhibitors might be a potential strategy. Whether such an approach would be feasible in the clinic requires further investigation. An open question from this study is whether a clinically approved dose of these inhibitors could reach the concentration required to fully prevent HSR.

Our data support that Fc gamma receptors are crucial mediators of mast cell degranulation induced by LOP628. It has been previously reported that Fc receptor activation requires the oligomerization of the receptors into a large immune complex (37). We propose that the binding of LOP628/LMJ729 to KIT may function as a scaffold to promote oligomerization of the Fc receptor. There remains a possibility that co-engagement of KIT and Fc receptor results in another novel mechanism activating mast cells. Perturbing the interactions between this class of receptors and KIT, either by blocking with a nonspecific IgG, or via modification of the KIT antibody, impaired LOP628-mediated mast cell degranulation. Hence, another potential mitigation strategy would be to circumvent antibody engagement of the Fc-receptor through antibody engineering. As expected, an F(ab′)2 antibody format that lacks an Fc domain was incapable of inducing degranulation; however, because of the short half-life of F(ab′)2 *in vivo* further development of a strategy to achieve adequate exposure to drive efficacy in KIT positive tumors would be required. An alternative approach would be to introduce Fc-silencing mutations. The removal of an Asparagine-linked glycosylation site by mutation, N297A, has been previously characterized to reduce $hIgG_1$ binding to Fc receptors (34). Interestingly, a N297A mutated KIT antibody only partially impaired the ability of the antibody to induce degranulation. This result may not be entirely surprising as monomeric IgG_1 binding affinity toward Fc receptors differ from IgG_1 immune complexes (6) and the size of IgG_1 immune complexes may override Fc silencing mutations when certain Fc receptor alleles are present (38). We hypothesize that an Fc silent antibody binding to the KIT receptor might induce sufficient antibody/receptor clustering, mimicking these large IgG immune complexes that allow for simultaneous binding and activation via the Fc receptor. More recent advances have identified an IgG2σ Fc silencing format where the mutations prevent binding to all FcγRs and prevent all residual effector functions (35). In agreement, we generated a KIT Ab with Fc silent mutation analogous to IgG2σ that did not induce degranulation. This demonstrated that engagement of Fc receptor through the Fc domain is critical for LOP628-mediated mast cell degranulation. Yet, the ability of an Fc-silent format antibody to bind Fc receptor might be restored by the presence of an Fc wild-type antibody exemplified by an IgG cross-linking antibody. Indeed, addition of an IgG cross-linker restored the ability of these Fc-silencing antibodies to induce degranulation. We postulate that an antidrug antibody (ADA) has the potential to induce Fc receptor binding similar to an IgG cross-linker and induce degranulation. Because detection of ADA has been reported in 26% of oncology patients treated with a fully humanized antibody (39), an Fc-silent antibodies approach would likely not be feasible in the clinic because of the risk of ADA.

Although not directly investigated in this study, we postulate that soluble KIT cross-linking LOP628/LMJ729 would potentially induce FcγR clustering in a similar fashion as cross-linking an isotype control antibody with an anti-F(ab′)2 cross-linker. Because the immune complex formed by cross-linking an isotype control antibody was not sufficient to induce mast cell degranulation, we speculate soluble KIT will not provide sufficient Fc receptor oligomerization. In addition, if there is a novel mechanism that activates mast cells upon co-engagement of FcγR and KIT, then soluble KIT would not enable such co-engagement. Interestingly, rather than inhibition, LOP628/LMJ729-mediated degranulation was enhanced upon imatinib treatment. Nilotinib, sunitinib, and midostaurin, also showed enhanced degranulation depending on the dose used. Although the exact mechanism is not currently understood, one possibility is that KIT inhibition in mast cells may stabilize KIT on the plasma membrane by inhibiting KIT internalization. Imatinib has been shown to inhibit SCF-mediated KIT internalization (40). A similar mechanism may be occurring where imatinib inhibits the internalization of LOP628. Such a mechanism would likely increase Fc/Fc receptor interaction which potentially supports the hypothesis that KIT may function as a scaffold to promote oligomerization of the Fc receptor. The exact mechanism of enhanced degranulation after KIT inhibition requires further investigation.

HSR reactions in humans were severe and dose limiting. In non-human primates, although infusion reactions were noted, they were tolerable and were not dose limiting (T. Abrams et al; submitted for publication). We postulated that identifying the class of Fc gamma receptor(s) which is responsible for LOP628-mediated mast cell degranulation may provide more insight into the conflicting results. The ability of Fc gamma receptors to induce mast cell degranulation in an IgG-dependent manner has been previously described (5, 41). However, the types and levels of FcγR expressed might vary depending on the mast cell origins. Several studies suggest that human mast cells predominantly express FcγRII, may express FcγRI under certain conditions, but not FcγRIII (reviewed in ref. 36). Interestingly, our data suggest that human PBC-MCs express FcγRII as well as low levels of FcγRI, leading to the possibility that either receptor might function in LOP628-mediated degranulation. Previously, the binding affinities of cynomolgus monkey and human $hIgG_1$ to Fc gamma receptors have been demonstrated to be comparable (42). However, it was noted that the expression level of Fc gamma receptors of specific immune cell types might differ between the two species. Additional studies further characterizing the Fc gamma receptor family of cynomolgus mast cells might provide additional insights. Alternatively, other factors such as in the microenvironment might impact mast cell biology. Previous studies demonstrated that cytokines produced in the microenvironment such as IFNγ modulate expression levels of FcγR1 and as a result might provide sensitization for IgG-induced mast cell degranulation. In agreement, we noted IFNγ treatment increased levels of FcγRI on mast cells and correlated with an increased level of degranulation driven by the KIT antibody. This suggests that an inflammatory microenvironment might heighten the response of mast cells to therapeutic antibody exposure. By contrast, the lack of inflammation might also be the underlying reason that LOP628 mediated-degranulation was not detected preclinically. Finally, it is interesting that mast cells also express CD32b (FcγRIIB), an Fc gamma receptor with suppressive function, as bispecific antibodies that target both FcεRI and CD32b were thought to be able to dampen an overactive immune response (43, 44). Similarly, we speculate that simultaneous engagement of CD32b might overcome LOP628-induced degranulation. Interestingly, an Fc mutation has been shown to specifically engage the inhibitory Fc-

receptor (45); we propose such an antibody may counterbalance the mast cell activation induced by activating Fc-receptors.

In summary, our data show that LOP628-mediated mast cell degranulation is the likely cause of HSR observed in the clinic. We identified co-engagement of Fc gamma receptor and KIT as the underlying mechanism. Furthermore, we propose several potential mitigation strategies that might abrogate degranulation including disruption of Fc receptor engagement via antibody engineering or inhibition of signaling pathways downstream of Fc receptor. These data highlight the challenge of using preclinical models to accurately evaluate the in vivo effects of antibody therapeutics. The inconsistency might arise from the inherent differences of the immune system between species or other contributing factors such as an inflammatory microenvironment. Further investigation would be required before a KIT ADC approach could again be assessed clinically.

Disclosure of Potential Conflicts of Interest

H. Maacke is an employee of Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: L. L'Italien, O. Orozco, T. Abrams, A. Connor, J. Desai, H. Gelderblom, K. Hoffmaster, E. Lees, S. Schleyer, S.T. Lee-Hoeflich
Development of methodology: L. L'Italien, O. Orozco, S.T. Lee-Hoeflich
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. L'Italien, O. Orozco, J. Desai, H. Gelderblom, H. Maacke
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. L'Italien, O. Orozco, J. Desai, S. Schleyer, D. Skegro, S.T. Lee-Hoeflich
Writing, review, and/or revision of the manuscript: L. L'Italien, O. Orozco, A. Connor, J. Desai, H. Ebersbach, H. Gelderblom, K. Hoffmaster, E. Lees, H. Maacke, S. Schleyer, D. Skegro, S.T. Lee-Hoeflich
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. L'Italien, L. Cantagallo, H. Ebersbach
Study supervision: J. Desai, E. Lees, H. Maacke, S.T. Lee-Hoeflich

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