Increased KIT Inhibition Enhances Therapeutic Efficacy in Gastrointestinal Stromal Tumor

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Abstract

**Purpose:** Gastrointestinal stromal tumor (GIST) is the most common human sarcoma and a model of targeted molecular therapy. GIST depends on oncogenic KIT signaling and responds to the tyrosine kinase inhibitor imatinib. However, imatinib is rarely curative. We hypothesized that PLX3397, which inhibits KIT and colony-stimulating-factor-1 receptor (CSF1R), would be more efficacious than imatinib in GIST by also depleting tumor-associated macrophages, which are generally thought to support tumor growth.

**Experimental Design:** We treated KitV558del/+ mice that develop GIST or mice with subcutaneous human GIST xenografts with imatinib or PLX3397 and analyzed tumor weight, cellular composition, histology, molecular signaling, and fibrosis. In vitro assays on human GIST cell lines were also performed.

**Results:** PLX3397 was more effective than imatinib in reducing tumor weight and cellularity in both KitV558del/+ murine GIST and human GIST xenografts. The superiority of PLX3397 did not depend on depletion of tumor-associated macrophages, because adding CSF1R inhibition did not improve the effects of imatinib. Instead, PLX3397 was a more potent KIT inhibitor than imatinib in vitro. PLX3397 therapy also induced substantial intratumoral fibrosis, which impaired the subsequent delivery of small molecules.

**Conclusions:** PLX3397 therapy has greater efficacy than imatinib in preclinical GIST models and warrants study in patients with GIST. The resultant intratumoral fibrosis may represent one of the barriers to achieving complete tumor eradication. *Clin Cancer Res;* 20(9); 2350–62. ©2014 AACR.

Introduction

Gastrointestinal stromal tumor (GIST) is the most common subtype of human sarcoma (1) and is a model of targeted molecular therapy in solid tumors. GIST is driven by an activating mutation in the KIT oncogene encoding the KIT receptor tyrosine kinase (2) or, less frequently, the PDGFRα oncogene encoding the related platelet-derived growth factor receptor α (PDGFRα; ref. 3). Localized, primary GIST is treated with surgical resection, but historically up to 50% of patients developed tumor recurrence, after which median survival was less than 2 years (4). Molecular therapy has vastly improved the treatment of advanced GIST. Imatinib mesylate (Gleevec; Novartis) is a tyrosine kinase inhibitor that specifically targets oncogenic KIT and PDGFRα (5, 6) and benefits 80% of patients with advanced GIST (7). However, imatinib is rarely curative. Initially sensitive tumors acquire resistance at a median of only 18 months (8, 9), most often via a secondary KIT mutation (10). Sunitinib (Sutent; Pfizer; ref. 11) and regorafenib (Stivarga; Bayer; ref. 12) are two additional multikinase inhibitors that are approved by the U.S. Food and Drug Administration for patients who do not tolerate imatinib or whose tumors are imatinib-resistant. These drugs provide temporary benefit to some patients, but the overall median time to tumor progression is only 5 to 6 months (11, 12).

The limitations of available therapies underscore the need for more effective treatments in GIST. Approaches toward improving molecular therapy for cancers such as GIST include combination with other targeted inhibitors or conventional cytotoxic chemotherapy, alteration of the tumor microenvironment, and development of more potent molecular inhibitors. In a transgenic mouse model of GIST, Rossi and colleagues observed upregulation of Src family kinase signaling in response to imatinib and found modest histologic benefit from combining imatinib with dasatinib, a multikinase inhibitor that targets both KIT and Src (13). Focusing on the tumor microenvironment, we showed that GIST tumor cells suppressed antitumor CD8+ T-cell immunity via expression of indoleamine 2,3-dioxygenase (IDO) and that the T-cell activator anti–CTLA-4 was synergistic with imatinib (14).
Targeted molecular therapies have revolutionized the treatment of patients with oncogene-addicted tumors such as gastrointestinal stromal tumor (GIST), but patients almost always develop drug resistance and disease progression. We demonstrated that PLX3397, a KIT and colony-stimulating-factor-1 receptor (CSF1R) inhibitor, achieved substantially more tumor regression than imatinib in both a transgenic mouse model of GIST and human GIST xenografts. PLX3397 therapy was also associated with greater systemic KIT inhibition, including bone marrow suppression, and induced severe intratumoral fibrosis, which seemed to impair further drug delivery. Our findings warrant the investigation of more potent KIT inhibitors in human GIST and identify the stromal response as a potential barrier to maximal drug delivery.

PLX3397 (Plexxikon) is a novel, dual-specificity tyrosine kinase inhibitor that targets KIT and the related receptor tyrosine kinase CSF1R (c-fms) with nanomolar potency (15, 16). CSF1R signaling is critical for macrophage survival and function, and tumor-associated macrophages (TAM) are the most prevalent type of immune cell in human GIST (17). TAMs are thought to promote tumor growth through immunosuppressive, angiogenic, and pro-metastatic effects (18). Recent animal studies have reported that PLX3397-mediated tumor macrophage depletion caused direct tumor regression (19) or improved response to chemotherapy (15, 16) or radiation therapy (20). Therefore, we sought to evaluate combined KIT and CSF1R inhibition in GIST. In a transgenic mouse model of GIST and in human GIST xenografts, we demonstrate superior antitumor efficacy of PLX3397 compared with imatinib. However, tumor response was associated with substantial intratumoral fibrosis that seemed to limit further drug delivery.

Materials and Methods

**Mice and treatments**

Six- to 15-week-old KitV558del/+ mice (21) and C57BL/6j (B6) and NOD.Cg-Pkdcre-am/Il2rgtm1Jsw/J (NSG) mice (The Jackson Laboratory) were used. Mice were matched by age and sex. For xenograft experiments, 10<sup>6</sup> GIST-T1 cells in PBS were mixed 1:1 with growth factor–reduced Matrigel (BD Biosciences) and injected subcutaneously into the right flank of NSG mice. Treatment was initiated at a mean tumor volume of 100 mm<sup>3</sup> as measured by the ellipsoid formula (1/2 length × width × height). Imatinib (LC Laboratories) was administered in the drinking water at 600 mg/L. PLX3397 (15, 16) is a tyrosine kinase inhibitor that selectively inhibits KIT and CSF1R with biochemical half maximal inhibitory concentrations (IC<sub>50</sub>) of 10 and 20 nmol/L, respectively. PLX5622 (22) is a related compound with comparable potency against CSF1R (IC<sub>50</sub> < 10 nmol/L), but 60-fold less potency against KIT. Both inhibitors were provided by Plexxikon and administered as formulated diet, 290 mg/kg chow (PLX3397), 1,200 mg/kg chow (PLX5622), or control chow AIN-76A. Animals were maintained at Sloan-Kettering Institute (New York, NY), and procedures were approved by the Institutional Animal Care and Use Committee.

**Flow cytometry**

For tumor KIT<sup>+</sup> cell and macrophage analysis, tumors were minced, incubated in 12 mg/mL type 2 collagenase (Worthington Biochemical) plus 0.5 mg/mL DNAse I (Roche Diagnostics) for 30 minutes at 37°C, then washed through 100- and 40-μm nylon cell strainers (Falcon; BD Biosciences) with 1% fetal calf serum (FCS). For T-cell analysis, tumors and draining lymph nodes (DLN) were mechanically dissociated as described (14). Spleens were mashed through a 70-μm strainer, incubated in ammonium chloride lysing buffer (eBioscience), quenched in 1% FCS, then washed through a 40-μm strainer. Bone marrow was flushed from one tibia per mouse, pooled by treatment group, homogenized by repeated aspiration through an 18-gauge needle, and then washed in 1% FCS. All cells were analyzed on a FACSort (BD Biosciences) as described (14). Mouse-specific antibodies included CD45 (clone 30-F11), Kit (CD117; 2B8), CD11b (M1/70), CD3 (145-2C11), CD4 (GK1.5), Ly6C (M1/70), and Ly6G (1A8) from BD Biosciences; F4/80 (BM8) from Invitrogen; CD45 (30-F11), Kit (A2B5), CD8 (53-6.7), and FoxP3 (FJK-16s) from eBioscience. Intracellular staining for FoxP3 was performed using the FoxP3 Staining Buffer Set (eBioscience). Human-specific KIT antibody (YB5.B8) was purchased from BD Biosciences.

**Histology**

Mouse tumors were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Formalin-fixed, paraffin-embedded sections of surgical specimens were obtained from 17 patients with GIST who consented to tissue analysis under an Institutional Review Board (IRB) protocol. Hematoxylin and eosin (H&E) and Masson’s trichrome staining were performed using standard methods. Apoptosis was detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) using the ApopTag Red In Situ Apoptosis Kit (Millipore) as directed. Slides were digitized using Mirax Scan (Zeiss). TUNEL<sup>+</sup> cells were counted as a percentage of total 4’,6-diamidino-2-phenylindole positive cells per high power field in 4 to 5 representative fields per tumor (Photoshop; Adobe). Staining for mouse type I collagen (Calbiochem rabbit polyclonal; 1:60; overnight at room temperature) and mouse/human Ki67 (citrate antigen retrieval; DAKO rabbit polyclonal; 1:60; overnight at room temperature) was performed as before (23). Slides were analyzed on an Axioplan2 wide-field microscope (Zeiss).

**Western blot**

Protein from frozen GIST tissue or GIST-T1 cells was analyzed as before (23). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and total and...
phosphorylated KIT (Tyr719), AKT (Ser473), S6 (Ser235/236), STAT3 (Ser727), and ERK1/2 (Tyr202/Tyr204) were obtained from Cell Signaling Technology.

**Peripheral blood analysis**

Automated assessment of whole blood in EDTA was performed on an Idexx Procyte Dx Hematology Analyzer (Idexx Laboratories). Manual white blood cell differential was performed on 100 cells on a blood smear stained with Accustain Wright-Giemsa stain (Sigma-Aldrich).

**In vitro assays**

GIST-T1 cells have been described (24). HG129 cells were established under an IRB-approved protocol from an untreated primary gastric GIST harboring a KIT exon 11 mutation (45-bp insertion between phenylalanine 591 and glycine 592). GIST-T1 and HG129 cells were maintained in complete medium (RPMI with 10% FCS). NIH3T3 cells were provided by Dr. Jackie Bromberg (Memorial Sloan-Kettering Cancer Center, New York, NY) and maintained in Dulbecco’s Modified Eagle Medium with 10% FCS. To measure viability, GIST-T1 or HG129 cells were plated at $10^4$ cells per well in a 96-well flat-bottomed plate (Falcon) and cultured for 72 hours at 37°C in complete medium with imatinib, PLX3397, or H2O- or DMSO-only solvent control. Viability was then measured using the Cell Counting Kit-8 (Dojindo) as directed and normalized to control. IC$_{50}$ values were calculated using nonlinear regression in Prism 5.0 (GraphPad Software). For protein analysis, GIST-T1 cells were plated at $2 \times 10^6$ cells per well in a 24-well flat-bottomed plate (Falcon) and cultured for 72 hours at 37°C in complete medium with imatinib, PLX3397, or H$_2$O- or DMSO-only solvent control. Culture medium was then analyzed using the soluble collagen assay (Zyme BioSciences). Collagen quantification was measured in serum-free medium overnight, then changed to serum-free medium containing either 10 or 40 nmol/L of complete medium per well in a 6-well plate (BD Biosciences) in serum-free medium overnight, then changed to serum-free medium containing either 10 or 40 nmol/L of control or PLX3397, or DMSO-only solvent control.

**Imaging and pharmacodelivery assays**

MRI was performed and analyzed as described (14). For tumor uptake and biodistribution studies, 100 μCi of $^{99m}$Tc-Sestamibi (Nuclear Diagnostic Products) in 100 μL was administered via tail vein injection to Kit$^{V558del}$ mice that had been treated with control, imatinib, or PLX3397 for 2.5 weeks, then taken off treatment for 3 days. Radioactivity in the syringe before and after administration was measured in an energy-calibrated dose calibrator (CRC-15R; Capintec), and exact quantity received by each animal was determined. Mice were then sacrificed at 2 hours by CO$_2$ asphyxiation. Tissue was collected in preweighed tubes, weighed, and counted for radioactivity using a gamma counter (PerkinElmer 1480 Wizard 3 Auto Gamma counter) with an autodynamic energy window near peak position 140 keV, which covered 90% of counts. The counts were then converted to activity (mCi) using a calibration factor, and percentage of injected dose per gram (%ID/g) was calculated by dividing by decay-corrected injected activity and organ weight. For fluorescent tracer studies, Kit$^{V558del}$+ mice were treated with control, imatinib, or PLX3397 for 10 weeks, then injected with a single intravenous dose of 15 mg/kg Hoechst 33342 dye (Molecular Probes; Invitrogen) or PBS control 50 minutes before sacrifice, followed by a single intravenous dose of 2 mg/kg fluorescein-conjugated Locop snelation scutelatum lectin (Vector Laboratories) or saline control 5 minutes before sacrifice. Tumors and spleens were embedded in Tissue-Tek O.C.T. Compound (Sakura) and cut into 5-μm thick cryosections. Slides were digitized as above, and 3 representative 0.6 mm$^2$ fields per tumor were analyzed in MetaMorph software (Molecular Devices). Average cellular Hoechst intensity was calculated with threshold area measurements and normalized to control-treated mice. Total lectin-staining vessel area per field was similarly calculated and normalized. Baseline autofluorescence was subtracted from each field analyzed.


**Statistical analysis**

Data were analyzed using Prism 5.0. An unpaired two-tailed Student's *t* test or one-way ANOVA with Bonferroni post-test comparison was performed as applicable. When comparing tumor KIT⁺ cell number between groups, cell counts were first log-transformed, and then analyzed by one-way ANOVA. A *P* value of <0.05 was considered significant.

**Results**

**PLX3397 has greater antitumor effects than imatinib in Kit\(^{V558del}\) mice**

To investigate the efficacy of PLX3397 in GIST, we used transgenic Kit\(^{V558del/+}\) mice, which spontaneously develop cecal GIST that is responsive to imatinib (14, 21, 25). Four weeks of PLX3397 reduced tumor weight and size to an even greater extent than imatinib (Fig. 1A and B). In addition, PLX3397 therapy resulted in 90% fewer Kit⁺ tumor cells than imatinib (Fig. 1C) and a further decrease in tumor cell size and granularity, suggestive of apoptosis and necrosis (Fig. 1D). Histology confirmed the dramatic effects of PLX3397, as hypocellularity, necrosis, and fibrosis were more pronounced after PLX3397 therapy compared with imatinib (Fig. 1E). Consistent with our findings, PLX3397 also resulted in greater tumor apoptosis than imatinib (Fig. 1F). Overall, PLX3397 was more effective than imatinib in Kit\(^{V558del/+}\) mice.

**CSF1R inhibition does not account for the efficacy of PLX3397**

PLX3397 and imatinib both inhibit Kit with nanomolar potency (15, 26). However, PLX3397 inhibits CSF1R more potently than imatinib (15, 26, 27), and CSF1R signaling contributes significantly to the maintenance and function of many types of macrophages, including TAM (18). In Kit\(^{V558del/+}\) mice, we found that PLX3397 depleted tumor macrophages to a much greater degree than imatinib (Fig. 2A), while less substantially affecting the frequency of other immune cells in the tumor, DNL, and spleen (Supplementary Fig. S1A and S1B). We therefore hypothesized that increased CSF1R inhibition and greater tumor macrophage depletion accounted for the superior efficacy of PLX3397 in GIST. To test this, we combined imatinib with PLX5622 (22), a CSF1R-specific inhibitor of equal potency to PLX3397 that does not appreciably inhibit Kit. Despite comparable levels of tumor macrophage depletion as PLX3397 therapy (Fig. 2B), treatment with PLX5622 did not enhance the effect of imatinib on tumor weight, cell number, or histology, and PLX3397 remained the best therapy (Fig. 2C–E). These results suggested that tumor macrophage depletion was not responsible for the efficacy of PLX3397 in Kit\(^{V558del/+}\) mice.

**PLX3397 achieves greater KIT inhibition than imatinib in preclinical models of GIST**

Aside from CSF1R, Kit is the only other kinase for which PLX3397 has a biochemical IC\(_{50}\) less than 100 nmol/L (15). Given the noncontributory effect of CSF1R inhibition on tumor size in *vivo*, we hypothesized that stronger Kit inhibition could explain the greater efficacy of PLX3397 compared with imatinib. In tumors from Kit\(^{V558del/+}\) mice treated for 1 or 4 weeks, there was no significant difference in phospho-KIT to total KIT expression in PLX3397-treated tumors, at least in the residual tumor cells (Fig. 3A). However, PLX3397 reduced total KIT expression on a per cell basis more effectively than imatinib (Fig. 3B). In addition, PLX3397 decreased tumor levels of phospho-Akt and the phospho-STAT3β isofrm, both of which are downstream mediators of Kit signaling (Fig. 3A). PLX3397 did not reduce expression of the downstream mediator phospho-ERK1/2 (Fig. 3A), suggesting possible activation of compensatory signaling pathways, as has been demonstrated in other tumor models (28). Additional systemic manifestations of PLX3397-mediated Kit inhibition included hair depigmentation (Fig. 3C) and decreased erythropoiesis (Fig. 3D), two Kit-dependent processes that were not affected by a similar duration of treatment with imatinib. PLX3397 also decreased other hematopoietic lineages in the bone marrow to a greater extent than imatinib (Supplementary Fig. S1C), but was similar to imatinib in the degree of peripheral blood neutrophil and monocyte reduction (Fig. 3E and F). Collectively, these data suggested stronger inhibition of oncogenic as well as wild-type Kit during PLX3397 versus imatinib treatment in *vivo*.

To exclude potential pharmacokinetic differences between PLX3397 and imatinib, we compared the drugs in *vivo* against 2 human GIST cell lines that harbor an imatinib-sensitive, activating Kit exon 11 mutation similar to our Kit\(^{V558del/+}\) mouse model. Indeed, PLX3397 decreased viability in both cell lines with 2-fold greater potency than imatinib, with an IC\(_{50}\) of 8 to 18 nmol/L versus 42 nmol/L (Fig. 4A. *P* < 0.05). At concentrations similar to the IC\(_{50}\) of each drug, i.e., 10 and 40 nmol/L, PLX3397 also decreased phospho-KIT relative to total KIT more effectively than imatinib in *vivo* (Fig. 4B). Thus, PLX3397 was a stronger Kit inhibitor than imatinib in *vivo*. To translate these findings to an *in vivo* model of human GIST, we administered PLX3397 or imatinib to mice with established subcutaneous GIST-T1 xenografts. Analogous to our *in vitro* findings, PLX3397 reduced tumor size and Kit⁺ tumor cells more dramatically than imatinib (Fig. 4C–F). Although GIST-T1 cells contain a Kit exon 11 mutation and were sensitive to imatinib in *vivo* (Fig. 4A), imatinib was only cytostatic in *vivo* (Fig. 4C), as has been seen previously (29). In contrast, PLX3397 therapy caused substantial regression of GIST-T1 xenografts. Altogether, PLX3397 demonstrated greater antitumor efficacy than imatinib in murine and human GIST.

**KIT-targeted therapies may be limited by stromal accumulation in GIST**

Although PLX3397 was clearly more effective than imatinib, our results raised the question of why PLX3397 could not achieve tumor eradication in our transgenic Kit\(^{V558del/+}\) mouse model, in which presumably there is relative tumor...
homogeneity. One possibility was that the stromal reaction we observed by H&E staining (Fig. 1E) was limiting further drug delivery to remaining tumor cells. To further analyze the stromal response to PLX3397 therapy, we examined tumors by Masson’s trichrome staining, which stains collagen blue (Fig. 5A). By 1 week, PLX3397 substantially increased tumor collagen, and by 17 weeks, collagen accumulation was dramatic. Imatinib-treated tumors, on the other hand, accumulated collagen more slowly and to a lesser extent, and untreated tumors maintained only a constant, low level of collagen. Interestingly, human GISTs that were responsive to long-term imatinib therapy (e.g., 6 months) demonstrated comparable collagen staining with PLX3397-treated mouse tumors, whereas untreated human tumors or those that had acquired resistance to imatinib did not (Fig. 5B). Therefore, the stromal reaction may not be specific to PLX3397 therapy.

Immunohistochemistry demonstrated the presence of fibrillar type I collagen within the stroma of PLX3397-treated tumors (Fig. 5C). Surprisingly, tumors were minimally infiltrated with fibroblast activation protein alpha (FAP)–expressing cells (not shown), which have been reported in other tumor models to represent activated cancer-associated fibroblasts, the major cellular source of collagen in epithelial-derived carcinomas (30). Instead, type I collagen transcripts were more highly expressed in Kit⁺ tumor cells than in other intratumoral cells after PLX3397 treatment (Fig. 5D). Furthermore, GIST cell lines produced soluble collagen in vitro, with a trend toward increased collagen in cells treated with imatinib or PLX3397 (Fig. 5E). Taken together, these findings suggested a final common pathway of tumor cell–mediated collagen deposition and tissue remodeling caused by KIT inhibition in GIST.

Next, we assessed the viability of the residual tumor cells and adequacy of drug delivery. In KitV558del/+ mice treated with imatinib or PLX3397 for as long as 17 weeks, residual tumor cells maintained intact nuclei and Kit expression...
Fig. 6A), similar to imatinib-responsive human GISTs (7, 31), suggesting viability despite chronic drug exposure. We proved in our KitV558del/þ mouse model that when PLX3397 was discontinued after 4 weeks of therapy, tumors regrew over the next 11 weeks (Fig. 6B). To model drug delivery, we injected treated mice intravenously with the radioactive small-molecule tracer 99mTc-sestamibi or the fluorescent DNA dye Hoechst 33342, both of which have similar molecular weight (approximately 780 and 620) to imatinib and PLX3397 (approximately 490 and 400). Indeed, there was markedly less tumor uptake of 99mTc-sestamibi in PLX3397-treated mice compared with control- or imatinib-treated mice (Fig. 6C, Supplementary Fig. S2). However, 99mTc-sestamibi results only reflected average tracer uptake per gram of tumor, and not the relative amounts of intracellular versus extracellular matrix-bound tracer. To better assess drug delivery per tumor cell, we used Hoechst 33342. Consistent with our 99mTc-sestamibi findings, PLX3397 therapy markedly decreased tumor cell uptake of Hoechst dye compared with control or imatinib treatment (Fig. 6D). On the other hand, imatinib, and, to a lesser extent, PLX3397, increased tumor perfusion as estimated by lectin staining, compared with control. Overall, KIT inhibition induced tumor fibrosis in both murine and human GIST to a degree that was proportional to the potency or duration of KIT inhibition, and the resultant stromal accumulation seemed to impede small-molecule delivery to residual tumor cells.

Discussion

That molecular therapy rarely cures cancer is a major clinical problem and a driving force behind current oncologic research. Here, we demonstrate two unexpectedly related explanations for why KIT inhibition cannot completely eradicate GIST, despite the tumor’s exquisite sensitivity to KIT inactivation, namely, suboptimal potency of currently available inhibitors and stromal impedance of drug delivery. Weinstein first introduced the theory of oncogene addiction to explain how the inactivation of a single driving oncogene can halt tumorigenesis despite the presence of numerous other genetic alterations (32).

Figure 3. PLX3397 achieves stronger Kit inhibition than imatinib in vivo. A, Western blot of tumors from KitV558del/þ mice treated as indicated. B, Kit expression, shown as mean fluorescence intensity (MFI), was measured by flow cytometry of CD45/Kitþ cells from tumors of KitV558del/þ mice treated for 4 weeks. C, representative photograph of KitV558del/þ mice treated with either imatinib or PLX3397 for 17 weeks. Peripheral red blood cells (RBC; D), neutrophils (E), and monocytes (F) were quantified by automated complete blood count with manual white blood cell differential in KitV558del/þ mice treated for 4 weeks (*x* denotes single B6 control mouse). Data, 3 to 6 mice per group and are shown as mean ± SEM. *, P < 0.05.
Studies in conditional transgenic mouse models have indeed demonstrated that transcriptional or translational silencing of a single oncogene can cause complete regression of tumors as diverse as lymphoma (33), osteogenic sarcoma (34), and melanoma (35), through mechanisms including cell cycle arrest, apoptosis, differentiation, and senescence. Unfortunately, in GIST (9), as in other solid human tumors presumed to be oncogene-addicted, such as BRAF-mutated melanoma and epidermal growth factor receptor–mutated non–small cell lung cancer, pharmacologic inhibition has not been able to recapitulate the same degree of tumor regression. One of the simplest explanations for this discrepancy may be insufficient oncoprotein inhibition by currently available drugs. In the present study, we show that increased driver inhibition can cause even greater tumor regression than the first-line agent, imatinib, in both a transgenic mouse model of GIST and human GIST xenografts. The enhanced tumor cell eradication by PLX3397 depended on more potent KIT inhibition and not coincidental CSF1R-mediated macrophage depletion. The rapid kinetics of drug-induced apoptosis remain to be further investigated. Overall, our results reiterate the importance of driver inhibition in GIST and lend rationale to developing and testing more potent inhibitors, not only in GIST, but in other solid tumors with identified driver oncoproteins.

Notably, compared with imatinib, PLX3397 not only exhibited better antitumor efficacy, but also appeared to achieve greater systemic inhibition of wild-type KIT in follicular melanocytes, which require KIT signaling for melanogenesis (36), and in a subset of hematopoietic stem cells, particularly erythroid precursors, which require KIT for normal red blood cell development (37). Depigmentation has been observed previously in mice (19) and humans (38) with PLX3397 but not imatinib. Such systemic manifestations reflect stronger KIT inhibition by PLX3397 but likely also signal the upper limit of the therapeutic index of KIT inhibition. Thus, although more potent KIT inhibitors could theoretically achieve even greater tumor responses than what we report here, such drugs would probably be limited by KIT-related toxicities such as bone marrow suppression. Notably, PLX3397 therapy did not predispose mice to increased infection. PLX3397 may therefore represent the most clinically attractive KIT inhibitor for GIST, achieving the highest level of KIT inactivation within an acceptable range of systemic toxicity.
From a broader perspective, the tumor microenvironment is gaining increasing recognition for its role in tumorigenesis and potential for therapeutic targeting. Relevant to the current study, resistance to molecular therapy can arise not only from mechanisms intrinsic to the tumor cell, but also from a variety of cell-extrinsic mechanisms. Of particular concern in solid tumors is impaired drug delivery, which can result from abnormal tumor perfusion, increased tumor interstitial hydrostatic pressure, and extracellular matrix remodeling, all of which contribute to poor drug penetration into the tumor, subtherapeutic drug levels within regions of the tumor, and ultimate treatment failure (39). Here, we demonstrate that potent KIT inhibition induces tumor fibrosis in a KIT-driven mouse model of GIST, with histologic changes reminiscent of the stromal accumulation observed in patients with GIST responding to imatinib (31, 40). Importantly, we show that this fibrotic response is not just a correlate of tumor cell death, but potentially an obstacle to further small-molecule delivery in treatment-responsive tumors. It was initially surprising that a KIT inhibitor as potent as PLX3397, for treatment durations as long as 1 month to greater than 1 year, could eradicate most but not all tumor cells in our genetically homogeneous mouse model. Instead, residual tumor cells persisted, maintained the ability to resume growth off treatment, and retained drug sensitivity after regrowth (not shown). Such observations implicate reversible mechanisms of drug resistance, which could include cell-intrinsic...
mechanisms, such as drug-induced quiescence, or cell-extrinsic mechanisms, such as subtherapeutic drug delivery. Using injected Hoechst dye to image tumor cell proximity to blood vessels in a subcutaneous lung carcinoma model, Chaplin and colleagues found that Hoechst-dim cells, which were multiple cell layers away from the nearest vessel, demonstrated less response than Hoechst-bright cells to a systemic injection of Adriamycin. These findings suggested that Hoechst fluorescence correlated with drug delivery and that delivery on a per cell basis affected drug efficacy (41). In the current study, we predicted that uniformly decreased Hoechst uptake in PLX3397-compared with control-or imatinib-treated tumors resulted from decreased tissue penetration due to extracellular matrix accumulation. The suspected impairment in drug delivery and tumor cell response is likely analogous to the results of Chaplin and colleagues. We acknowledge that our pharmacokinetic studies are limited by the use of small-molecule tracers that are similar in molecular weight to our drugs of interest but obviously different in water and lipid solubility, molecular structure, and charge, all of which influence molecular flux through cells and tissue (39). Restricted by the lack of labeled drug forms that can be imaged with high enough resolution, we cannot directly measure imatinib or PLX3397 delivery to tumors on a per cell basis. However, we demonstrated the consistent finding of decreased small-molecule uptake in PLX3397-treated tumors, regardless of chemical properties unique to 99mTc-sestamibi or Hoechst dye. Extrapolating our results to the agents under investigation, we propose that impaired drug penetration of
treatment-responsive tumors may contribute to tyrosine kinase inhibitor resistance in GIST. Additional optimization of drug delivery will likely be necessary to maximize drug efficacy, and the rapid kinetics of PLX3397-mediated tumor fibrosis may serve as a useful model for future studies.

Elucidating the specific mechanisms of the fibrotic response to KIT inhibition may reveal new therapeutic targets in GIST. However, unlike most epithelial carcinomas, in which extracellular matrix derives from infiltrating fibroblasts (30), we have found that FAP+ fibroblasts comprise only 1% of intratumoral cells in KitV558del+ tumors (not shown). Functional studies of collagen production are ongoing in KitV558del+ mice crossed to FAP-DTR mice, which express the diphtheria toxin receptor under the FAP promoter (42). However, we report here that GIST tumor cells themselves seem to be the most abundant source of collagen in the setting of Kit-targeted therapy. Thus, the very agents that ablate the majority of GIST tumor cells paradoxically induce a stromal response that may prevent complete tumor cell elimination. With no additional cell type to target, it may prove beneficial to modulate the stroma by either increasing degradation or inhibiting production of pathologic collagen. In a conditional transgenic mouse model of pancreatic adenocarcinoma, Provenzano and colleagues (43) and Jacobetz and colleagues (44) demonstrated that desmoplastic tumor stroma was rich in hyaluronic acid, a proteoglycan that in excess led to exceedingly high interstitial fluid pressures within the tumor, and that degradation of hyaluronic acid with systemically administered PEGylated hyaluronidase not only reduced tumor stroma but also improved chemotherapeutic delivery and response. Intratumoral collagenase injection has similarly been reported to improve therapeutic delivery in various solid tumors (45), but it is impractical for the treatment of intraabdominal tumors in the clinical setting, and a systemically administrable, nontoxic form of collagenase is not currently available. A trial of alternative anti-fibrotic agents, e.g., collagen synthesis inhibitors, warrants consideration in combination with KIT inhibition in GIST.

Although we hypothesized that collagen accumulation in PLX3397-treated tumors was in part due to the loss of macrophages, combined treatment with imatinib and macrophage depletion only resulted in mild tumor fibrosis. Therefore, despite the major physiologic role that macrophages play in homeostatic extracellular matrix degradation and remodeling, their loss in the setting of PLX3397 therapy did not seem to affect stromal accumulation. Such lack of effect suggests that the net state of extracellular matrix turnover in GIST depends more on the activity of the tumor cells, and less on traditionally implicated stromal cells such as macrophages and fibroblasts. That macrophage depletion did not affect overall tumor cell survival was also surprising and may highlight a limitation of our model, i.e., that KitV558del+ murine GISTs do not metastasize or display tumor outgrowth in the setting of chronic therapy, two clinical problems in which depletion of tumor-promoting macrophages may actually improve outcome. Alternatively, sufficient oncprotein inactivation may halt tumorigenesis to an extent that renders tumor immune responses less biologically relevant. A more aggressive GIST model is needed to investigate these questions.

We herein demonstrate that increased KIT inhibition translates into enhanced antitumor effects in clinically relevant mouse and xenograft models of GIST, but that such therapies may ultimately be limited by KIT-related systemic toxicities and, importantly, tumor cell-mediated fibrosis and probable impairment of drug delivery. Complete tumor eradication of nonoperable GIST may require a combination of potent KIT inhibition, stromal modulators, and, perhaps in the setting of reduced fibrosis, immune activators. A potent KIT inhibitor such as PLX3397 may be most effective as first-line therapy in treatment-naïve tumors, which remain the most sensitive to oncogene inactivation and have likely acquired fewer genetic and nongenetic mechanisms of drug resistance. Sufficient tumor cell eradication upstream may minimize the risk of residual cell persistence and later disease progression. Our results therefore have immediate applicability to the management of patients with GIST and similarly oncogene-addicted cancers.

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

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The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCI or NIH.

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