INTRODUCTION

Gastrointestinal stromal tumors (GIST) are the most common type of sarcoma found in the gastrointestinal tract and are typically characterized by the expression of KIT (1, 2). Hirota and colleagues (3) first described gain-of-function KIT mutations that result in constitutive activation of the kinase and downstream signaling in GISTs. Activating mutations in KIT or PDGFRA are present in approximately 90% of gastrointestinal stromal tumors (GIST). Although treatment with the KIT and PDGFR inhibitor imatinib can control advanced disease in about 80% of GIST patients, the beneficial effect is not durable. Here, we report that ligands from the FGF family reduced the effectiveness of imatinib in GIST cells, and FGF2 and FGFR1 are highly expressed in all primary GIST samples examined. The combination of KIT and FGFR inhibition showed increased growth inhibition in imatinib-sensitive GIST cell lines and improved efficacy in patient-derived GIST xenografts. In addition, inhibition of MAPK signaling by imatinib was not sustained in GIST cells. An ERK rebound occurred through activation of FGF signaling, and was repressed by FGFR1 inhibition. Downregulation of Sprouty proteins played a role in the imatinib-induced feedback activation of FGF signaling in GIST cells.

SIGNIFICANCE: We here show that FGFR-mediated reactivation of the MAPK pathway attenuates the antiproliferation effects of imatinib in GISTs. The imatinib-induced ERK rebound can be repressed by the FGFR inhibitor BGJ398, and combined KIT and FGFR inhibition leads to increased efficacy in vitro and in patient-derived xenografts. Cancer Discov; 5(4): 438–51. ©2015 AACR.
imatinib significantly increased overall survival and progression-free survival in patients with unresectable or metastatic GISTs (5–8).

Although imatinib has revolutionized the treatment of advanced GISTs, the beneficial effects are not durable, as the majority of patients develop resistance to imatinib within 2 to 3 years. Different molecular mechanisms are responsible for primary and secondary imatinib resistance in GISTs. Primary resistance is defined as evidence of progression of disease within 6 months of imatinib treatment and is strongly associated with mutations in $KIT$ exon 9 or $PDGFRA$ exon 18 and with GISTs that are wild-type for both genes (9–11). Secondary resistance is observed in patients who respond to imatinib treatment initially and experience progression after 6 months of therapy. Newly acquired mutations in $KIT$ or $PDGFRA$ are frequently found in GISTs with secondary resistance (12–15). The acquired mutations that confer resistance to imatinib are not randomly distributed and are always located in exons encoding the kinase domains (12, 14, 15). A recent study has also shown that signaling cross-talk between KIT and FGFR3 promoted resistance to imatinib (16), suggesting that resistance to imatinib evolves by mechanisms other than acquired secondary mutations in $KIT$ or $PDGFRA$. In addition, complete responses are rarely achieved (≤5%), even in patients responding favorably to imatinib (8). Importantly, viable tumor cells can be found in most patients who undergo tumor resections during imatinib therapy (17), suggesting that the residual GIST cells may exhibit adaptation to KIT or PDGFR inhibition through the activation of other pathways and the effect of imatinib on these cells is cytostatic rather than cytotoxic.

Previous studies have shown that kinases that mediate uncontrolled proliferation cause feedback inhibition of other mitogenic signaling pathways in tumor cells. Pharmacologic inhibition of these kinases relieves the feedback and induces the activation of multiple pathways (18–24). The adaptive changes in the signaling network following treatments with selective inhibitors attenuate the antitumor effects of the therapies. Here, we report that inhibition of KIT by imatinib caused a feedback activation of FGF signaling and resulted in a rebound in ERK phosphorylation in GIST cells. The FGFR1–3 inhibitor BGJ398 repressed the ERK rebound and enhanced the antitumor activity of imatinib in GISTs.

RESULTS
Secretome Screening Suggests That Ligands from the FGF Family Reduce the Antitumor Activity of Imatinib in GISTs

In addition to the mutationally activated kinases, cancer cells typically express multiple wild-type receptor tyrosine kinases (RTK) that mediate proliferation and survival signals through common downstream effectors, such as PI3K and MAPK. Ligand-dependent activation of RTKs may mitigate the effects of inhibitors targeting oncogenic kinases (25). To identify the growth factors that potentially attenuate the antiproliferative effects of imatinib in GISTs, 317 cDNA constructs encoding 220 unique secreted or single-pass transmembrane proteins were transfected into HEK293T cells in 384-well plates in which each well was expected to contain a defined secreted protein. Secreted proteins accumulated in the media of these cultures were added to two imatinib-sensitive GIST cell lines, GIST-T1 and GIST882, treated with the KIT inhibitor imatinib. GIST-T1 cells harbor a heterozygous deletion of 57 bases in exon 11 of $KIT$ (26), and GIST882 cells harbor a homozygous missense mutation in $KIT$ exon 13 (4), resulting in KIT-dependent growth. The effect of the secreted proteins on proliferation was assessed after incubation of GIST-T1 and
GIST882 cells with imatinib and conditioned media from the secretome library. The majority of the secreted proteins have shown a minimal effect on altering cell growth. However, a small subset of ligands partially rescued the effect of KIT inhibition and reduced the imatinib sensitivity, most notably the ligands of the FGF family (Fig. 1). A recent study has also shown that FGF2 rescued GIST cell lines from KIT inhibition (16).

**Figure 1.** FGF ligands reduce the effectiveness of the KIT inhibitor imatinib in GIST-T1 and GIST882 cells. Secreted ligands were collected from the conditioned medium of 293T cells transfected with cDNA constructs encoding 220 unique secreted proteins. Each dot represents the average of 3 replicate wells of GIST cells treated with imatinib (0.5 μmol/L) and the conditioned medium derived from a single cDNA construct. Gene symbols are in alphabetical order on the x-axis, and readouts of relative cell growth quantified using CellTiter-Glo on day 6 are on the y-axis. DMSO, dimethyl sulfoxide; mEGF, mouse epidermal growth factor; mEGF, mouse epidermal growth factor.
FGF2 and FGFR1 Are Highly Expressed in Primary GISTs

The data in Fig. 1 suggest that FGF signaling may decrease the effectiveness of KIT inhibition by imatinib in GISTs. To determine the expression levels of FGF ligands and receptors in GIST, we examined the transcriptional profiles of 30,527 tumor tissue samples of 42 histologic types and 36 primary sites (Supplementary Table S1). These publicly deposited datasets contain expression of 90 GIST samples. Examination of individual tumor samples revealed that FGF2 and FGFR1 are highly expressed in GISTs (Fig. 2A). We did not
find uniform and high expression of the genes encoding the other FGF ligands and receptors (Supplementary Fig. S1A) in GISTs. Gene microarray studies have identified a number of genes that demonstrate elevated mRNA expression in GISTs compared with other tumors (27–30). In agreement with previous observations, our data have shown high expression of genes known to be highly expressed in GISTs, such as KIT, ETv1, PRKCQ, and ANO1 (Fig. 2A and Supplementary Fig. S1A). Next, we compared mRNA levels of FGF2 and FGFR1 in four GIST cell lines with a large panel of human cancer cell lines characterized in the Cancer Cell Line Encyclopedia (CCLE) project (Supplementary Table S2; ref. 31).

The Sensitivity of GIST Cells to Imatinib Is Reduced by FGF2 and Can Be Restored by Imatinib and BGJ398 Combination

To test the ability of FGF2 to reduce sensitivity to imatinib, cell proliferation assays were performed in GIST-T1 and GIST882 cells with FGF2 plated at four different concentrations in the presence of imatinib. FGF2 at 20 ng/mL reduced the maximum percentage inhibition from 85% to 60% in GIST-T1 cells and from 65% to 25% in GIST882 cells without changing the imatinib IC_{50} values significantly (Fig. 3A). To examine whether the FGF2-mediated rescue was acting through its cognate receptors, we next used BGJ398, a selective and potent FGFR1/2/3 inhibitor that is currently in clinical development (32, 33), to attempt to restore the sensitivity of the two GIST cell lines to imatinib. As expected, the antiproliferative activity of imatinib in GIST cells was restored in the presence of added FGF2 by cotreatment with BGJ398 (Fig. 3B). In addition, FGF2 alone had little or no effect on the growth of the GIST cells, excluding the possibility of a primary growth effect exerted by FGF2 (Fig. 3A and 3B). To explore whether FGFR activation and the downstream signals commonly engaged by RTKs were associated with the observed rescue effects, we assessed the effects of imatinib, FGFR2, and BGJ398 treatments on the levels of protein phosphorylation by immunoblotting of the relevant protein extracts. In the absence of added FGF2, imatinib, but not BGJ398, strongly inhibited the phosphorylation of KIT and downstream singling outputs, including ERK and AKT (Fig. 3C). The addition of FGF2 activated FGFR signaling, as measured by the presence of Tyr-phosphorylated FGFR substrate 2 (FRS2) that is a downstream substrate of the FGFRs, and at the same time reactivated MAPK signaling with little effect on AKT phosphorylation in the presence of imatinib (Fig. 3C). BGJ398 profoundly down-regulated the phosphorylation of FRS2 and suppressed the reactivation of MAPK (Fig. 3C). MAPK reactivation was not due to reactivation of KIT, as KIT autophosphorylation remained suppressed by imatinib after FGF2 cotreatment (Fig. 3C).

BGJ398 Enhances the Inhibition of GIST Cell Growth by Imatinib in the Absence of Exogenous FGF Ligands

To further evaluate the enhanced antiproliferative effect of imatinib in combination with BGJ398 in the absence of added FGF ligands, we applied long-term viability assays using GIST-T1 and GIST882 cell lines. Cells were exposed to imatinib, BGJ398, or the combination for 6 days, and stained with crystal violet. As depicted in Fig. 4A, the imatinib and BGJ398 combination had a greater antiproliferative effect and was more efficacious in killing both GIST-T1 and GIST882 cells than imatinib alone. The combination effect was also confirmed with a real-time cell proliferation assay using the xCELLigence system. The growth of cells was monitored over the course of 6 days in the presence of imatinib, BGJ398, or imatinib plus BGJ398. In agreement with the colony formation assay, a greater antiproliferative effect was observed for the combination than for imatinib alone in the absence of exogenous FGF ligands (Fig. 4B). BGJ398 had no effect on cell growth as a single agent, suggesting that KIT-addicted GIST cells are not initially dependent on FGFRs for survival and proliferation (Fig. 4A and B).

Treatment of GIST Cells with Imatinib Is Associated with Activation of FGF Signaling and a Rebound in ERK Phosphorylation

To address the molecular mechanism underlying the enhanced inhibition of the growth of GIST cells by imatinib in combination with BGJ398, we assessed the activated states of components of the KIT and FGF signaling pathways of GIST-T1 and GIST882 cells in both short-term and long-term cultures. We observed that treatment of both cell lines with imatinib for 3 to 7 days led to a strong induction of FRS2 phosphorylation at tyrosine 436 (Fig. 5A), indicating activation of FGFR receptors. After initial inhibition of ERK phosphorylation in both cell lines, long-term treatments resulted in a profound rebound in ERK phosphorylation, accompanying the increased phosphorylation of FRS2 (Fig. 5A). The addition of BGJ398 for the last 4 hours of the long-term treatments suppressed the activation of FGFR and the rebound in ERK phosphorylation (Fig. 5A). Knockdown of FGFR1 in GIST-T1 and GIST882 cells markedly inhibited the induction of FRS2 phosphorylation and diminished phosphorylated ERK (pERK) rebound (Fig. 5B), consistent with the observation that FGFR1 is the primary FGF receptor expressed in GIST-T1 and GIST882 cells (Fig. 5C) and in GIST tissues (Fig. 2A and Supplementary Fig. S1A). These results suggest that a feedback activation of FGFR is elicited by KIT inhibition, and provide a rationale for the enhanced antiproliferation activity when imatinib and BGJ398 are administered in combination.

To begin to address how inhibition of KIT causes activation of FGF signaling, we sequenced poly(A)-selected RNA from mock-treated, imatinib-treated, or imatinib and BGJ398-treated GIST-T1 and GIST882 cells on an Illumina HiSeq 2500 System. Next, we applied the DESeq algorithm to identify genes that were differentially expressed in GIST-T1
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**Figure 3.** Exogenous FGF2 attenuates the antiproliferation activity of imatinib, and the reduced sensitivity can be restored by the FGFR inhibitor BGJ398. **A,** effects of added FGF2 at various concentrations on the cell viability of GIST cells treated with imatinib for 72 hours. **B,** reversal of rescue mediated by exogenous FGF2 with BGJ398. BGJ398 was tested at 0.5 μmol/L final concentration. **C,** Western blot analysis of KIT, FRS2, AKT, and ERK phosphorylation in extracts from GIST-T1 and GIST882 cells that were treated for 4 hours with the indicated compounds in the presence and absence of added FGF2.
and GIST882 cells treated with imatinib for 4 hours, as compared with mock-treated cells (34). When the threshold was set at a fold change greater than 4 or less than 0.25 and a false discovery rate (FDR) less than 0.01, this method identified 131 and 53 genes that comprise the KIT-dependent transcriptional output in GIST-T1 and GIST882 cells, respectively (Supplementary Table S3). The expression of 27 genes was changed significantly in both cell lines after imatinib treatment for 4 hours, with 21 genes downregulated and 6 genes upregulated (Table 1). Among these genes were previously described targets of ERK signaling, including multiple transcription factors (FOS, FOSL1, and EGR1) and genes involved in the feedback inhibition of MEK/ERK signaling (DUSP4, DUSP5, DUSP6, SPRY2, and SPRY4; refs. 35, 36). Imatinib treatment led to significant reduction of their expression at the 4-hour time point (Fig. 5D and Supplementary Table S4). However, a rebound of the expression of these ERK-dependent genes was observed 3 days after imatinib treatment, accompanying the reactivation of ERK (Fig. 5D and Supplementary Table S4). The addition of BGJ398 4 hours before the end of the 3-day imatinib treatment suppressed the restored expression of these genes, consistent with the re-suppression of ERK phosphorylation by BGJ398 (Figs. 5A and D and Supplementary Table S4). No significant alterations in the expression of FGF ligands and receptors were observed after short-term and long-term imatinib treatments (Supplementary Table S4).

The genes that were dramatically downregulated 4 hours after imatinib treatment encode multiple targets of ERK signaling, such as DUSP6 and Sprouty (SPRY) proteins, which are negative regulators of the FGF signal transduction pathway (37–40). SPRY proteins act as repressors of growth factor–induced ERK activation, in part by binding to RAF1 and to GRB2 and sequestering the GRB2–SOS complex (41, 42). SPRY4 inhibits FGF–induced but not EGF–induced ERK activation (40). The gene whose expression was most significantly altered by short-term imatinib treatment was SPRY4 (Table 1), and it is overexpressed in GIST cell lines and primary GIST tissues (Fig. 5E and F). These data suggest that low FGF signaling activity is due to high levels of ERK signaling in untreated GIST cells.

Figure 4. BGJ398 enhances the antiproliferation activity of imatinib in GIST cells in the absence of exogenous FGF ligands. A, crystal violet staining of GIST-T1 and GIST882 cells. Cells were treated with imatinib (1 μmol/L), BGJ398 (0.5 μmol/L), or the combination for 3 days. For each cell line, all dishes were fixed, stained, and photographed at the same time. B, the growth in real time of GIST-T1 and GIST882 cells treated with the indicated compounds. Electric impedance was monitored as a measure of cell proliferation. Imatinib and BGJ398 were used at fixed concentrations of 1 and 0.5 μmol/L, respectively. Treatment with BGJ398 alone has no effect on viability.
FGFR Feedback Attenuates the Effect of Imatinib in GIST

We hypothesized that SPRY proteins and other negative regulators mediate ERK-dependent feedback in GIST cells and cause them to be less responsive to FGF ligands. Inhibition of KIT by imatinib leads to inhibition of pERK and results in relief of ERK-dependent feedback inhibition of FGF signaling.

To test this hypothesis, we first assessed the ability of added FGF2 to activate FGF signaling in GIST cells treated with imatinib at various times. SPRY2 and SPRY4 levels were diminished and pKIT and pERK were quite low in GIST-T1 and GIST882 cells after 24 hours of exposure to imatinib in the absence of exogenous FGF2 (Fig. 5G). Next, FGF2 (4 ng/mL) was added at various times after imatinib treatment, and ERK activation was examined 15 minutes after FGF2 addition. Although pKIT and pERK were markedly inhibited by imatinib within 30 minutes, no significant induction of pERK by added FGF2 was observed after 1 hour of KIT inhibition (Fig. 5G). FGF2 stimulation of ERK phosphorylation rose gradually after 2 to 8 hours of KIT inhibition (Fig. 5G). After 24 hours of KIT inhibition, FGF2 significantly stimulated pERK (Fig. 5G). The ability of FGF2 to induce pERK was associated with a decrease in SPRY2 and SPRY4 levels (Fig. 5G). No exogenous FGF2 was required to observe the activation of FGF signaling and the rebound in ERK phosphorylation after 3 to 7 days of imatinib treatment (Fig. 5A). No correlation between induction of RAS-GTP and decreasing levels of SPRY2 and SPRY4 proteins was observed, suggesting that the relief of the feedback inhibition acting downstream of RAS was an early event (Fig. 5A). To test the effect of KIT inhibition on FGF receptor activation, GIST-T1 and GIST882 cells were treated with imatinib for 24 hours and then stimulated with FGF2 for 15 minutes. Induction of FRS2 phosphorylation was increased, as compared with mock-treated cells (Fig. 5H). These data show that the transduction of the signal from FGF receptors is markedly potentiated through enhanced activation of the receptors and enhanced signaling downstream of RAS.

SPRY proteins may play a role in blocking FGF-induced FGFR activation and signal transduction in GISTs. To test this hypothesis, we transfected GIST882 cells with siRNAs against SPRY2 and SPRY4 to knockdown their expression. Transfected cells were cultured for 96 hours and then treated

Figure 5. Inhibition of KIT induces activation of FGF signaling and ERK rebound through relief of ERK-dependent feedback in GIST cell lines. A, activation of FGF signaling and rebound in pERK in GIST-T1 and GIST882 cells after imatinib treatment. Cells were harvested after short-term or long-term imatinib treatment, and whole-cell lysates were analyzed by Western blotting. Long-term KIT inhibition resulted in strong upregulation of Tyr 436 FRS2 phosphorylation. Rebound in pERK was associated with induction of pFRS2. Addition of BGJ398 4 hours before the end of the long-term imatinib treatment resulted in complete inhibition of pFRS2 and pERK. B, abolished activation of FGF signaling and rebound in pERK in GIST-T1 and GIST882 cells by knocking down the expression of FGFR1 proteins. GIST-T1 and GIST882 cells were transfected with control and FGFR1 siRNAs. Transfected cells were cultured for 72 hours and harvested after imatinib treatment for 4 hours or 3 days. Whole-cell lysates were subjected to Western blotting to determine the induction of pFRS2 and rebound in pERK. C, basal transcription levels of FGFRs in GIST-T1 and GIST882 cells. Expression of FGFR1-4 was quantified by RNA-Seq FPKM, fragments per kilobase of transcript per million fragments mapped. D, modulation of SPRY4 gene transcription by imatinib treatment. GIST-T1 and GIST882 cells were treated with imatinib (1 µmol/L) for 4 or 72 hours and expression of SPRY4 was analyzed by RNA-Seq. (continued on next page)
Imatinib and BGJ398 Combination Leads to Improved Efficacy In Vivo

A GIST882 xenograft model was used to further evaluate the in vitro finding of FGF signaling activation via KIT inhibition by imatinib. GIST882 tumor-bearing mice were treated with imatinib for 2 hours. FGF2 (4 ng/mL) was added for 15 minutes before cell lysis. Whole-cell lysates were analyzed by Western blotting to assess the phosphorylation of KIT, FRS2, and ERK and the protein levels of SPRY2 and SPRY4.

Figure 5. (Continued) E and F, universally high expression of SPRY4 in GISTs. E, expression of SPRY4 in multiple primary tumor types. Normalized gene expression was calculated as the Z-score. Each dot represents an individual primary tumor sample. More than 30,000 primary tumor tissue samples were sorted by the expression level of the gene and plotted. Ninety primary GIST tumor tissue samples are indicated by blue arrows. F, expression of SPRY4 in cell lines of multiple lineages. Normalized gene expression was calculated as the Z-score. Each dot represents an individual cell line. More than 1,000 cell lines were sorted by the expression level of the gene and plotted. Four GIST cell lines are indicated by blue arrows.

with imatinib for 2 hours. FGF2 (4 ng/mL) was added for 15 minutes before cell lysis. Downregulation of either SPRY2 or SPRY4 induced pFRS2 and increased FGF2 induction of pERK (Fig. 5I). Knockdown of both SPRY2 and SPRY4 resulted in greater induction of pFRS2 and pERK than knockdown of SPRY2 or SPRY4 alone (Fig. 5I). These findings indicate that constitutive activation of KIT in GIST cells suppresses FGF signaling through ERK-dependent feedback inhibition partially mediated by SPRY proteins. Relief of this feedback by imatinib enhances FGFR activation and intracellular transduction of FGF-induced signal.
FGFR Feedback Attenuates the Effect of Imatinib in GIST

Table 1. Genes significantly changed in expression upon imatinib treatment for 4 hours

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DISCUSSION

Kinases constitutively activated by oncogenic mutations reprogram the signaling network and induce feedback inhibition of other pathways in cancer cells. Inhibition of these kinases by selective inhibitors may relieve the feedback and lead to the activation of alternative pathways. The adaptive responses of tumors to target therapies occur in a relatively short period of time and diminish their effectiveness. This process may also facilitate the selection of tumor clones that harbor acquired mutations that confer greater drug resistance. In this study, we have shown that long-term imatinib treatments induced FGFR-mediated reactivation of MAPK signaling in KIT-mutant GIST cells (Fig. 5A), which may result in reduced sensitivity. We also found that inhibition of FGFR by BGJ398, a selective and potent FGFR1/3 inhibitor, abrogated the rebound in ERK activity (Fig. 5A), and combined KIT and FGFR inhibition led to increased sensitivity in vitro and improved efficacy in vivo (Fig. 4 and 6B).

A number of recent studies have shown that BRAFV600E inhibition by vemurafenib caused feedback activation of RTKs in melanoma, colon cancers, and thyroid cancers, resulting in reactivation of ERK signaling (20–22, 24). However, the magnitude of ERK reactivation varies across different lineages. The degree of ERK rebound is greater in BRAFV600E colon and thyroid cancers than it is in melanoma, consistent with the findings that colon and thyroid cancer cells are less sensitive to vemurafenib than melanoma cells. In addition, the RTKs engaged after relief of the feedback by BRAFV600E inhibition are lineage specific. Treatment of BRAFV600E colon and thyroid cancer cell lines with vemurafenib causes a rapid rebound in ERK activity through increased signaling from HER kinase family members, specifically EGFR in colon and HER3 in thyroid cancer cells (20, 22, 24). In melanoma, it seems that multiple RTKs are activated when ERK-dependent feedback is relieved by RAF inhibition (21). These findings suggest that an understanding of lineage-specific determinants of adaptive response to selective target inhibition is critical to guide design of appropriate combination therapies.

One limitation of cotargeting the RTK induced by the drug treatment is that the adaptation process may depend on activation of multiple RTKs. However, it is possible that this process may be preferentially dependent on a specific receptor in a particular lineage. FGFR1 and FGFR2 are ubiquitously expressed at high basal levels in primary GIST tissues (Fig. 2A and Supplementary Fig. S1A), although it is likely that FGFR signaling is inactive before imatinib treatment. The HER kinase family receptors and MET were expressed at low levels in primary GIST tissues and cell lines (Supplementary Fig. S1A and S1B). Strong expression of the insulin-like growth factor 1 receptor (IGF1R) was detected only in GISTs lacking
activating mutations in KIT and PDGFRA (46). In GIST cell lines, a subset of the ligands of the FGF family rescued the effect of KIT inhibition and reduced the sensitivity of imatinib (Fig. 1 and 3). In the absence of added FGF ligands, prolonged exposure of KIT-mutant GIST cells to imatinib was associated with ERK signaling reactivation that was accompanied by FGFR activation and the ERK rebound was repressed by the FGFR inhibitor BGJ398 (Fig. 5A). These findings suggest that the majority of, if not all, KIT-mutant GISTs will exhibit adaptation to KIT inhibition through the activation of FGF signaling. Although reactivation of ERK signaling in response to imatinib was mediated by FGFR in GIST cell lines, we did not observe induction of FGF ligand and receptor expression after imatinib treatment (Supplementary Table S4). Instead, we have shown that negative feedback suppressed FGF signaling in GISTs, and imatinib relieved this feedback inhibition. ERK outputs, such as SPRY proteins, may act as the mediators that suppress ligand-induced FGF signaling and block signaling downstream of the activated receptors in the feedback loop. KIT inhibition results in the inactivation of ERK signaling and relieves the negative feedback effects on FGF signaling, leading to the diminished dependence on KIT. The induced FGF signaling reactivates ERK, resulting in the formation of a new steady state in the presence of the drug. Consistent with this model, we observed that SPRY2 and SPRY4 levels decreased after treatment with imatinib, and FGF2 can potently activate its receptor and the downstream ERK signaling only after 24 hours of KIT inhibition when SPRY2 and SPRY4 were depleted (Fig. 5G). In addition, knockdown of SPRY in GIST cells increased FGR activation and downstream signaling (Fig. 5I). We also observed that knockdown of SPRY4 led to the downregulation of KIT (Fig. 5I). One possible explanation for this observation may involve the ubiquitin ligase CBL that binds to the phosphorylated tyrosine on the activated RTK.

Figure 6. Combination of imatinib and BGJ398 results in improved efficacy and increased apoptosis in patient-derived GIST xenografts. A, activation of FGF signaling and rebound in pERK in GIST882 xenograft model after imatinib treatment. Tumor tissues treated with vehicle or imatinib at 250 mg/kg twice daily for 1 day or 4 days were recovered 4 and 8 hours after the last dose treatment and analyzed by Western blot analysis. B, the improved in vivo efficacy when imatinib was combined with BGJ398. KIT-mutant GIST xenografts derived from 2 patients were treated with vehicle, imatinib (60 mg/kg), BGJ398 (20 mg/kg), or both inhibitors in combination for 21 days. Treatments were withdrawn on day 22, and tumors were allowed to regrow until the tumors in the imatinib single-agent treatment group reached the initial tumor volume in the GC-GIST09-0210 xenografts. Average change in tumor volume relative to initial tumor volume and SEM are shown as a function of time. C, the increased apoptosis in tumors treated with combination of imatinib and BGJ398. Tumor tissue from GIST xenografts treated for 3 days as indicated above was evaluated by IHC for p-histone H2A.X (Ser139). Tumors were harvested 2 hours after dosing on day 3.
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and mediates its degradation through the preosomal and lysosomal pathways. SPRY proteins sequester CBL, thus inhibiting receptor ubiquitination and degradation (47). SPRY4 may bind to CBL and inhibit the degradation of activated KIT in GISTs. Recent studies have also demonstrated the role of SPRY proteins in ERK-dependent feedback inhibition of various RTKs in BRAF-mutant melanoma and colon cancers (20, 21). However, it is unlikely that loss of SPRY alone can account for the full effect of ERK-dependent feedback. Further studies are required to elucidate the molecular mechanism responsible for the adaptive responses induced by target therapies.

An understanding of how tumors adapt to target inhibition can guide selection of appropriate combination therapies. The model outlined above suggests that an FGFR inhibitor, when combined with imatinib, can abrogate the adaptive response that occurs after KIT inhibition in imatinib-sensitive GISTs. This combination is unlikely to be effective after the emergence of imatinib-resistant clones that contain acquired mutations in KIT kinase domains. The findings described here support clinical trials to determine whether combining imatinib and FGFR inhibitors will delay or prevent the emergence of imatinib-resistant clones in patients with KIT-mutant GISTs.

METHODS

Cell Lines and Culture

The GIST882 cell line was obtained from Brigham and Women’s Hospital (Boston, MA) in 2010. It was established from an untreated human GIST with a homozygous missense mutation in KIT exon 13, encoding a K642E mutant KIT protein (4). The GIST-T1 cell line was obtained from Kochi Medical School (Kochi, Japan) in 2010. It was established from a metastatic human GIST with a heterozygous deletion of 57 bases in exon 11 of KIT (26). The GIST882 cell cultures were cultured in RPMI-1640 (ATCC) supplemented with 15% FBS and 1% L-glutamine, and GIST-T1 cells in DMEM (ATCC) supplemented with 10% FBS. The identity of the cell lines was confirmed periodically throughout the study using SNP fingerprinting, and the KIT mutations were confirmed with RNA sequencing.

Reagents

Imatinib and BGJ398 were synthesized in the Global Discovery Chemistry Department at NIBR (Novartis). For both compounds, 10 mmol/L stock solutions were prepared in 100% dimethyl sulfoxide (DMSO). Antibodies against phosphorylated and total KIT, ERK, AKT, and FGFR1 and phospho-FRS2 and phospho-histone H2A.X (Ser139) were obtained from Cell Signaling Technology; FGF2 and AKT, and FGFR1 and phospho-FRS2 and phospho-histone H2A.X (Ser139) were obtained from Cell Signaling Technology; FGF2 and total FRS2 from Santa Cruz Biotechnology; SPRY2 from Abcam; (Ser139) were obtained from Cell Signaling Technology; FGF2 and SPRY4 from R&D Systems.

Cell Viability and Proliferation Assays

To determine the dose–response relationship between the dose of imatinib and the magnitude of its effect on cell proliferation, cells were plated in 96-well plates at a density of 10,000 cells per well in a volume of 100 μL, and grown for 24 hours before treatment. Cells were then treated with DMSO or imatinib at concentrations ranging from 1.5 mmol/L to 10 μmol/L (3-fold dilutions) in the presence and absence of FGF2. After 3 days, cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and Victor4 plate reader (PerkinElmer). Percentage inhibition was calculated relative to median DMSO signal. For colony formation assays, cells were plated into 10-cm tissue culture dishes (5–10 × 10^4 cells/dish) in a total volume of 10 mL. After 24 hours, DMSO, imatinib, BGJ398, or both compounds were added to the dishes at final concentrations of 1 μmol/L for imatinib and 0.5 μmol/L for BGJ398. At the endpoints of the assays, cells were fixed, stained with crystal violet, and photographed.

A real-time cell proliferation assay was also conducted to continuously monitor growth of GIST cells using the xCELLigence system (Roche). This system uses measurement of impedance to quantify cell proliferation (48). Cells were seeded at a density of 10,000 cells per well. After 24 hours, DMSO, imatinib, BGJ398, or both compounds were added to the wells at final concentrations of 1 μmol/L for imatinib and 0.5 μmol/L for BGJ398. On the basis of the measured impedance, a unit-less parameter called cell index was determined and normalized to the baseline reading at time point 0 to represent the cell number. Impedance was measured every 2 hours for approximately 6 days.

SECRETOME SCREEN

Secretome screening was performed as described previously (21, 49). In brief, 317 cDNA constructs that represent 220 unique secreted proteins were reverse transfected into HEK293T cells individually and incubated for 4 days to allow accumulation of secreted proteins in the supernatant. The supernatant was transferred to the assay cells, followed by addition of imatinib to a final concentration of 0.5 μmol/L. Cell proliferation was measured using CellTiter-Glo after 96 hours. Cells treated with DMSO in the absence of conditioned media and with imatinib alone were used as controls.

RNA Interference

siRNA pools against SPRY2, SPRY4, FGFR1, and negative control were purchased from Dharmacon. Cells were plated into 6-well plates at 50% of confluence and transfected with Lipofectamine RNAiMAX (Invitrogen) as indicated in the manufacturer’s instructions. Transfected cells were cultured for 96 hours and then treated with imatinib (1 μmol/L) and/or FGFR2 (4 ng/mL) for 15 minutes.

Microarray Expression Data Mining

Gene expression data of primary tumor tissues were extracted from OncExpress. OncExpress is a Novartis internal database and application for management and analysis of expression data characterized by Affymetrix GeneChip Human Genome U133A 2.0 Array and Affymetrix GeneChip Human Genome U153 Plus 2.0 Array. The database contains 30,527 publicly deposited datasets available from the Gene Expression Omnibus (GEO), representing baseline expression profiles of primary tumor tissue samples. The microarray data accession numbers of the primary tumor samples in GEO are listed in Supplementary Table S1. Each sample is annotated with the COSMIC classification system (50) as well as other clinical variables. The microarray data accession numbers of CCLE and GIST cell lines in GEO are GSE36139 and GSE64762, respectively. Probe sets from the Affymetrix gene expression datasets were normalized using MAS5 with a trimmed-mean target of 150 and log-transformed.

RNA Sequencing, Data Processing, and Differentially Expressed Gene Detection

Total RNA was isolated from cells using the Qiagen RNeasy Kit. RNA integrity and purity were assessed with the Agilent RNA 6000 Nano kit on an Agilent 2100 BioAnalyzer. One microgram of high-quality total RNA with an RNA integrity number (RIN) score >7 was processed using the Illumina TruSeq RNA Sample Prep Kit according to the manufacturer’s instructions. The libraries were sequenced on an Illumina HiSeq 2500 using a 100 nucleotide paired-end indexed run and the standard Illumina primers. Transcriptomic alignments and gene expression quantification were performed using Array Studio 6.0 (OmicSoft). Transcript counts were estimated using HTSeq-Published OnlineFirst February 11, 2015; DOI: 10.1158/2159-8290.CD-14-0763
The tumors were harvested 2 hours after the last dose for imatinib, BGJ398, or the combination as described above for 3 days. Experiments in GIST882 xenograft model were performed in outbred athymic (nu/nu) female mice (Harlan Laboratories). Animals at 7 to 8 weeks of age were injected subcutaneously in the right axillary region with 1 × 10^7 GIST882 cells suspended in a 1:1 mixture of cold PBS and Matrigel in a total volume of 150 μL. When tumors reached 400 to 500 mm³, animals were treated with vehicle or imatinib at 250 mg/kg twice daily for 1 day or 4 days (6 animals per group). Plasma and tumors were collected at 4 and 8 hours after the last dose for Western blot analysis.

Patient-derived GIST xenograft models were established as described previously (43). Prior written informed consents were obtained from patients. The study also received ethics board approval from the National Cancer Centre of Singapore as well as the Singapore General Hospital. Treatment groups were assigned by stratification of measured tumor volume before treatment commencement. To investigate the antitumor effects of imatinib, BGJ398, and the combination on the growth of GIST xenografts, mice bearing GC-GIST09-0210 and GIST26-0208A tumors (8 per group) were orally administered daily either 200 μm of imatinib, 20 mg/kg BGJ398, or 60 mg/kg imatinib plus 20 mg/kg BGJ398 for 21 days. Treatment started when the tumors reached the size of approximately 1,200 mm³ (curative). Treatments were withdrawn on day 22 and tumors were allowed to regrow until they reached the initial tumor volume. Bidimensional measurements were performed twice a week and tumor volumes were calculated on the basis of the following formula: tumor volume = (length × width^2) / 2. The data were plotted as means and standard errors for each treatment group versus time.

To investigate the effects of imatinib, BGJ398, and the combination on apoptosis of GC-GIST09-0210 and GIST26-0208A xenografts, mice bearing tumors (3 per group) were treated with imatinib, BGJ398, or the combination as described above for 3 days. The tumors were harvested 2 hours after the last dose for immunohistochemistry.

**Immunohistochemistry**

Tumor tissue samples were processed for paraffin embedding and 5-μm sections were prepared. The sections were stained with the anti-phospho-histone H2A.X (Ser139) antibody to assess apoptosis, as described previously (52).

**Disclosure of Potential Conflicts of Interest**

R. Schlegel has ownership interest (including patents) in Novartis. J.E. Monahan is an employee of Novartis Institutes for BioMedical Research. A. Huang is an employee of Novartis. No potential conflicts of interest were disclosed by the other authors.

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FGFR Feedback Attenuates the Effect of Imatinib in GIST


FGFR-Mediated Reactivation of MAPK Signaling Attenuates Antitumor Effects of Imatinib in Gastrointestinal Stromal Tumors

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