Ripretinib and MEK Inhibitors Synergize to Induce Apoptosis in Preclinical Models of GIST and Systemic Mastocytosis

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ABSTRACT

The majority of gastrointestinal stromal tumors (GIST) harbor constitutively activating mutations in KIT tyrosine kinase. Imatinib, sunitinib, and regorafenib are available as first-, second-, and third-line targeted therapies, respectively, for metastatic or unresectable KIT-driven GIST. Treatment of patients with GIST with KIT kinase inhibitors generally leads to a partial response or stable disease but most patients eventually progress by developing secondary resistance mutations in KIT. Tumor heterogeneity for secondary resistant KIT mutations within the same patient adds further complexity to GIST treatment. Several other mechanisms converge and reactivate the MAPK pathway upon KIT/PDGFRA–targeted inhibition, generating treatment adaptation and impairing cytotoxicity. To address the multiple potential pathways of drug resistance in GIST, the KIT/PDGFRA inhibitor ripretinib was combined with MEK inhibitors in cell lines and mouse models. Ripretinib potently inhibits a broad spectrum of primary and drug-resistant KIT/PDGFRα mutants and is approved by the FDA for the treatment of adult patients with advanced GIST who have received previous treatment with 3 or more kinase inhibitors, including imatinib. Here we show that ripretinib treatment in combination with MEK inhibitors is effective at inducing and enhancing the apoptotic response and preventing growth of resistant colonies in both imatinib-sensitive and -resistant GIST cell lines, even after long-term removal of drugs. The effect was also observed in systemic mastocytosis (SM) cells, wherein the primary drug–resistant KIT D816V is the driver mutation. Our results show that the combination of KIT and MEK inhibition has the potential to induce cytotoxic responses in GIST and SM cells.

Introduction

Gastrointestinal stromal tumors (GIST) originate from the interstitial cells of Cajal and are the most common malignant mesenchymal tumors of the gastrointestinal tract (1). GIST is driven by mutually exclusive mutations in two receptor tyrosine kinases (KIT and PDGFRα), which result in their constitutive activation. Eighty percent to 85% of GIST have mutations in KIT and an additional 5%–10% have mutations in PDGFRα (2). Imatinib mesylate, a small-molecule tyrosine kinase inhibitor (TKI) effectively inhibits KIT activity, and is used as a first line of therapy for treatment of metastatic GIST (3).

First-line imatinib has proven to be effective in stabilizing disease progression in most GIST patients, but only 2% of patients experience complete regression (4). While median progression-free survival (mPFS) in the first-line setting is 18.9 months, most patients with imatinib-treated GIST experience progressive disease and no longer respond to imatinib treatment (5–8). Imatinib treatment does not induce tumor eradication and long-term treatment with imatinib results in a cellular quiescent state and tumor cells start to grow upon imatinib withdrawal (9, 10). GIST most often becomes imatinib-resistant after acquiring secondary mutations in the KIT kinase domain that directly abrogate the binding of imatinib to KIT or that lead to conformational escape to type I conformations to which imatinib does not bind (11). Many secondary mutations are found in the ATP-binding pocket or the activation loop. Sunitinib and regorafenib, approved as second- and third-line therapy for GIST, respectively, target only a subset of secondary resistant mutations (12–15). Different resistant mutations can arise in metastatic tumor sites, and individual patients often acquire multiple secondary mutations in KIT during the course of therapy (16). Although the majority of resistance mutations occur within the KIT gene, recent genomic data has shown that mutations arise in other genes as well, such as SDH, Kras, Braf, Nf1, P13Kca, and Pten (17–19). Imatinib treatment can activate the MAPK pathway through receptor tyrosine kinase switch (20) or reactivate MAPK signaling through a positive feedback circuit wherein MAPK activation downstream of KIT stabilizes the transcription factor Etv1, leading to a positive feedback loop upregulating KIT expression (21). Combined targeting of Etv1 stability by imatinib and the MEK inhibitor binimetinib resulted in increased growth suppression in imatinib-sensitive disease (21). Hence, novel therapeutic approaches are needed to treat patients with KIT mutational heterogeneity and KIT independent pathway mutations that result in reactivation of the MAPK pathway. Given the convergence of these mechanisms feeding into the RAS/MEK pathway, a potential treatment strategy would be to combine a broad-spectrum KIT inhibitor with an inhibitor of the MAPK pathway. In addition, combination therapy provides the potential to increase cytotoxic activity compared with cytostatic responses to KIT monotherapy.

Ripretinib was designed to target a broad spectrum of primary and secondary drug-resistant mutations in KIT and PDGFRα and is currently in clinical trials for advanced GIST (22, 23). Ripretinib is...
a “switch-control” kinase inhibitor designed to force even aggressively activated mutants into an inactive conformation. It inhibits KIT activity of highly resistant KIT mutations including D816V (22). In the randomized, double-blind, placebo-controlled INVICTUS phase III clinical study of ripretinib in patients with advanced GIST previously treated with ≥3 prior TKIs, ripretinib demonstrated a significant improvement in mPFS compared to placebo [6.3 vs. 1 months, respectively; HR, 0.15 (95% confidence interval (CI), 0.09–0.25); P < 0.0001] and had a favorable overall safety profile; the ORR was 9.4% vs. 0.0% (P = 0.0504) for ripretinib and placebo, respectively (NCT0335373; ref. 23). In May 2020, the FDA approved ripretinib for the treatment of adult patients with advanced GIST who have received prior treatment with 3 or more kinase inhibitors, including imatinib (24).

Herein, we studied whether ripretinib, like other KIT-targeted therapies, has cytostatic activity and if it can be turned into cytotoxic response by combination therapy. We used ripretinib in combination with three FDA approved MEK inhibitors (MEKi) in cellular assays of growth and apoptosis in both imatinib-sensitive and -resistant GIST cell lines. GIST cell lines have been shown to be excellent models of GIST tumor growth and response to drug treatment in vivo (14, 15, 25).

We also investigated the prevention of drug resistance in a cellular model and in vivo efficacy of the ripretinib and MEKi combination. Imatinib was used as a comparator in these studies. We also evaluated this combination in the systemic mastocytosis (SM) cell line HMC1.2. SM is driven by a KIT D816V mutation in 90% of patients and is resistant to most KIT inhibitors in clinical practice (26, 27). The ripretinib/MEKi combination was also evaluated in HMC1.2 cells stably transfected with mutant NRASG12D; an acquired mutation found in advanced SM (28, 29).

**Materials and Methods**

**Cell lines**

GIST-T1 (RRID:CVCL_4976) and its derivative cell lines GIST-T1/816 (with D816E mutation; RRID:CVCL_A9N0) and GIST-T1/670 (with T670I mutation; RRID:CVCL_A9M9) were obtained and cultured as described previously (22). HMC1.2 (KIT-V560G/D816V) cell line was obtained from Millipore-Sigma (SCC062; RRID:CVCL_H205). The BaD-KIT-V560D cell line was described previously (22). Cells were not further authenticated upon receipt. Mycoplasma tests were performed using the MycoAlert Mycoplasma Detection Kit (Lonza, LT07–318). Cells were expanded and frozen at low passage number and were passedaged <40 times before thawing a fresh vial. Cells were grown in Iscove’s Modified Dulbecco’s Medium (IMDM; Life Technologies, 12240–046) with 10% FBS (Life Technologies, 16000–046) and penicillin–streptomycin–L-Glutamine (Life Technologies, 10378–016). NRASG12D was cloned in PLVX-IRES Clontech Laboratories, 632183) and virus was packaged in HEK293T cells with pVSVG and pCMVΔR8.2 packaging plasmids. GIST-T1 and HMC1.2 cells were infected with lentivirus containing either empty vector (EV) or NRASG12D and selected in media containing 1 µg/mL puromycin (Sigma, P9620).

**Chemicals**

Imatinib mesylate, ripretinib, and regorafenib (synthesized internally at Deciphera Pharmaceuticals according to published protocols), sunitinib (LC Laboratories, S-8803), trametinib (LC Laboratories, T-8123), cobimetinib (Selleck Biochemicals, S8041), and binimetinib (Selleck Biochemicals, S7007) were prepared as 10 mmol/L stocks in DMSO.

**Western blot assays**

Cells were treated with drugs for 48 hours and cell lysates were prepared as described previously (22). Electrophoresis was carried out in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Bands were detected by incubating with Immobilon Forte Western HRP substrate (Millipore-MERCK KGaA) and captured by chemiluminescence with Amersham Image 600 (GE Healthcare Life Science). Primary antibodies to phospho-KIT Y703 (3073; RRID:AB_1147635), Bim (2819; RRID:AB_10692515), phospho-Histone H2AX Ser139 (9718; RRID:AB_2118809), phospho-ERK Thr202/Tyr204 (9101; RRID:AB_331646), total ERK (9102; RRID:AB_330744) were purchased from Cell Signaling Technology. Total KIT antibody (A4502; RRID:AB_2335702) was from DAKO and actin (A700; RRID:AB_476730) was from Sigma-Aldrich-MERCK (KGaA).

**Annexin V staining**

Cells were treated with various concentrations of drugs as shown in figures for 24, 48, or 72 hours. A total of 1 × 10⁶ cells were resuspended in Hanks balanced salt solution (HBSS, Thermo Fisher Scientific, 14025–092) and incubated with annexin V-FITC antibody (BD Biosciences, 550475; RRID:AB_2868885) for 15 minutes in the dark at room temperature. Cells were incubated with propidium iodide at a final concentration of 10 µg/mL (Sigma-Aldrich, P4170) for 5 minutes at room temperature and immediately analyzed by flow cytometry with BD FACSCalibur from BD Biosciences. 10,000 cells were recorded and analyzed in all conditions.

**Caspase assays**

GIST cells at 10,000 cells/well were plated in 96-well white flat-bottom plates and drugs were added 24 hours later. Apoptosis was evaluated by Caspase Glo-3/7 assay system (Promega, G8092) after 24 hours of treatment with inhibitors as per manufacturer’s instructions. A matrix of various concentrations of drugs was used to measure caspase activity (10) and the Bliss synergy score was calculated with Combenefit software using 1/(fold caspase induction) with 100% being no induction and 0% being maximum induction of caspase activity.

**Clonogenic assays**

GIST-T1 and GIST-T1/D816E cells at 100 cells/well and GIST-T1/T670I cells at 500 cells/well were seeded in 6-well plates and treated with various concentrations of drugs alone or in combination for 2 weeks. The drugs were washed off with PBS twice and fresh complete media without drug was added to the wells and incubated for 10 days to check for any outgrowth of colonies. If there were no visible colonies, plates were incubated for additional 10 days with change of fresh media.

**Soft-agar colony assays**

Soft-agar colony formation assay was set up in 6-well plates with 2 mL base layer of 1% Difco noble agar (BD Biosciences, 214220) and 2 mL top layer of 0.36% agar with 10,000 cells/well in IMDM media. Agar was allowed to solidify for 2 hours at RT and 2 mL of IMDM complete media with or without drug was added to each well. The media was carefully removed after 10 days of incubation. Wells were washed twice with PBS and fresh media without drug was added and incubated for 5 days. If no colonies were observed under the microscope, cells were incubated for additional 8 days with fresh change of media. Cells were stained with crystal violet (0.01% w/v in methanol) and colonies were photographed and counted.
Saturation mutagenesis

Ba/F3 cells stably transfected with V560D KIT kinase were treated with the mutagen ethylnitrosourea (ENU) for 18 hours (15). Cells were washed several times, then 10,000 cells/well were plated in 96-well plates containing various concentrations of ripretinib or imatinib either alone or with 10 nmol/L of trametinib for 28 days. Wells showing cell growth were sequenced for KIT mutations as described previously (22).

Results

Combination of ripretinib and MEKi induces apoptosis in GIST cell lines

To investigate whether blockade of the MAPK and KIT signaling pathways induces apoptosis, cells were treated with the FDA-approved MEKi trametinib, cobimetinib and binimetinib as single agents and in combination with ripretinib. Imatinib was used as a comparator. A multi-dose combination matrix was used for each drug combination (as shown in Fig. 1; Supplementary S1) and caspase activity was measured after 24 hours of treatment. Ripretinib or trametinib as a single agent induced a dose-dependent increase (up to ~1.2–1.8-fold) in apoptosis in GIST-T1/D816E and 2–4 fold increase in apoptosis in GIST-T1/T670I cells (Fig. 1B and C). The combination of ripretinib and trametinib was synergistic in inducing apoptosis in both cell lines while combination of imatinib and trametinib did not show synergy (Fig. 1D).

Similarly, combinations of ripretinib with MEKi cobimetinib or binimetinib exhibited significant synergy in GIST-T1/D816E and GIST-T1/T670I cells, whereas combinations with imatinib did not exhibit synergy (GIST-T1), modest synergy (GIST-T1/T670I), or led to antagonism (GIST-T1/D816E; Supplementary Fig. S1).

Apoptosis was also evaluated by Annexin V/PI staining in GIST-T1 and GIST-T1/T670I cell lines. GIST-T1 showed an increase (18% → 40%) in apoptotic cells with imatinib or ripretinib alone after 72 hours of treatment compared with DMSO control, and apoptosis increased to approximately 50% in combination with 25 nmol/L of trametinib with both drugs (Fig. 1E).

Imatinib or ripretinib alone did not induce apoptosis in the imatinib-resistant GIST-T1/T670I cell line, and the imatinib/trametinib combination failed to induce apoptosis as well, whereas the ripretinib/trametinib combination significantly increased apoptosis to 56% compared with 18% in DMSO control (Fig. 1F).

MAPK activation is a major component of the KIT signaling pathway and is important for GIST cell survival. To corroborate the enhanced apoptotic effect of the combination of ripretinib and trametinib, we evaluated the phosphorylation of ERK1/2 and levels of prosapoptic proteins BimEL and p-H2Ax. Phosphorylated H2Ax has previously (22) been shown to be upregulated after imatinib-induced apoptosis in GIST cells (30). Ripretinib treatment resulted in greater inhibition of Kit and ERK1/2 phosphorylation (pERK) in both imatinib-resistant cell lines GIST-T1/D816E and GIST-T1/T670I compared with imatinib (Fig. 1G). Likewise, ripretinib as single agent induced higher apoptosis induction, as assessed by the expression of BimEL and p-H2AX in imatinib-resistant cell lines. The addition of trametinib, in contrast to the imatinib and trametinib combination, increased levels of both BimEL and p-H2AX apoptosis markers in the imatinib-resistant cell lines.
Ripretinib and MEKi Synergize in Preclinical Models

Figure 1.
Ripretinib and trametinib combinations induce apoptosis in GIST cell lines. Heatmap of caspase activity in GIST-T1 (A), GIST-T1/D816E (B), GIST-T1/T670I (C) cell lines with ripretinib and trametinib combination after 24 hours of treatment. Values represent average of three replicates. P values for drug combination versus each drug alone were <0.001. BLISS scoring combination synergy graphs (D) of ripretinib (top) or imatinib (bottom) in combination with trametinib in GIST-T1, GIST-T1/D816E and GIST-T1/T670I cell lines. Apoptosis induction in GIST-T1 (E) and GIST-T1/T670I (F) cells as measured by AnnexinV/PI staining. Cells were treated with compounds at indicated concentrations for 24, 48, and 72 hours and stained with Annexin V and PI. G, Immunoblot of pKIT, pERK, BimEL, and pH2AX levels in imatinib-sensitive (GIST-T1) and imatinib-resistant (GIST-T1/D816E and GIST-T1/T670I) cells treated with imatinib (IM, 100 nmol/L) or ripretinib (Ripr, 100 nmol/L) in the absence or presence of trametinib (Tram, 10 nmol/L) for 48 hours. BL, baseline sample.
Combination of ripretinib with trametinib synergize to inhibit colony outgrowth of GIST cells. One-hundred cells for GIST-T1 and GIST-T1/D816E and 500 cells for GIST-T1/T670I were plated in 6-well plates and treated with compounds for 2 weeks. Drugs were washed off and cells were grown in complete media for an additional 10 days. If no colonies were visible, cells were revived for an extra 10 days. Colonies were stained with crystal violet and counted. A, Colony outgrowth in GIST-T1 cell line treated with ripretinib or imatinib in combination with trametinib. B, Average colony counts from three wells. Arrows indicate no visually determined colony outgrowth. C, Colony outgrowth in GIST-T1/D816E cell line treated with ripretinib or imatinib in combination with trametinib. D, Average colony counts from three wells. Arrows indicate no visually determined colony outgrowth. E, Colony outgrowth in GIST-T1/T670I cell line treated with ripretinib or imatinib in combination with trametinib. F, Average colony counts from three wells. Arrows indicate no visually determined colony outgrowth. G, Average colony counts for GIST-T1 cell line stably transfected with empty vector or NRASG12D. Colony outgrowth studies were done in presence of 100 nmol/L of imatinib or 50 nmol/L of ripretinib in combination with 50 or 100 nmol/L of trametinib. The values represent average from three wells. Arrows indicate no visually determined colony outgrowth.
Both imatinib (250 nmol/L) and ripretinib (100 nmol/L) as single agents demonstrated a lack of cytotoxic activity in GIST T1/D816E leading to approximately 61%–72% colony growth compared with vehicle control (Fig. 2C and D). A combination of ripretinib (100 nmol/L) and trametinib (100 nmol/L) completely inhibited the outgrowth of colonies and only 2 colonies outgrew with extra revival of 10 days (a total of 20 days), whereas the imatinib/trametinib combination showed a modest decrease in outgrowth of colonies by approximately 40%–60% (Fig. 2C and D).

In GIST-T1/T670I, neither imatinib (250 nmol/L) nor ripretinib (100 nmol/L) as single agents led to a significant reduction in colony outgrowth after removal of drug. Treatment with 100 nmol/L ripretinib in combination with 100 nmol/L trametinib eradicated colony outgrowth, with no detectable colonies even after 20 days of revival (Fig. 2E and F, arrow). Imatinib (250 nmol/L) in combination with trametinib (100 nmol/L) resulted in a significant reduction of colony outgrowth, but did not lead to complete inhibition and approximately 8%–10% colonies still outgrew after a total of 20 days of revival compared with vehicle control. Collectively, these results demonstrated that the combination of ripretinib is more effective than a combination with imatinib in eradicating tumor cell outgrowth in all GIST cell lines tested.

To mimic potential resistance due to MAPK reactivation, colony outgrowth experiments were also performed in GIST-T1 stably transfected with NRASG12D. In these cells, combined treatment with 50 nmol/L of ripretinib and 50 nmol/L or 100 nmol/L trametinib led to complete or near complete inhibition of colony outgrowth after 10 days after drug removal (Fig. 2G, right panel arrows). Although colonies formed were bigger in size in NRASG12D-transfected cells, no significant difference in colony count was seen when compared with EV-transfected cells.

Other MEKi inhibitors, cobimetinib and binimetinib, were also used in combination with imatinib and ripretinib. Ripretinib exhibited greater activity than imatinib in combination with cobimetinib and binimetinib (Supplementary Fig. 2SA–2SL). Of the MEKi inhibitors tested, trametinib showed greater efficacy than binimetinib, followed by cobimetinib.

**Combination of ripretinib and MEKi synergistically increases apoptosis in the HMC1.2 mast cell line**

Systemic mastocytosis is a rare neoplasm wherein KIT D816V is found in 90% of patients, and associated with an aggressive clinical manifestation (14). The HMC1.2 cell line has a primary mutation in KIT D816V and NRASG12D, which is found in 90% of patients, and associated with an aggressive clinical manifestation (14). The HMC1.2 cell line has a primary mutation in KIT D816V and NRASG12D, which is found in 90% of patients, and associated with an aggressive clinical manifestation (14).

Ripretinib (25 nmol/L and 50 nmol/L) and trametinib (1, 10, or 25 nmol/L) combination also led to decreased colony outgrowth of NRASG12D-transfected HMC1.2 cells, whereas the single agent treatments resulted in colony outgrowth at all concentrations (Fig. 4A). Combination of ripretinib (50 nmol/L) with trametinib (1 nmol/L) resulted in a significant reduction in colony outgrowth compared with single-agent treatment of either drug. Combination of ripretinib (50 nmol/L) with trametinib (10–25 nmol/L) dramatically led to eradication of outgrowth of viable HMC1.2-NRASG12D-transfected cells to the limit of detection as determined by visualization with 5X objective microscopy after 5 days of drug removal (Fig. 4B and C, arrows). Further extension of colony outgrowth for 8 additional days (total of 13 days after drug removal) still maintained the inhibitory effect. Similar results were obtained in EV-transfected cells except that combination of 50 nmol/L ripretinib and 10 nmol/L of trametinib showed approximately 20–25 colonies after extended period of revival. NRASG12D-transfected cells were also more sensitive to colony growth inhibition than EV-transfected cells. These results demonstrated that both KIT-dependent and -independent mechanisms can be effectively inhibited with combination treatment of ripretinib and trametinib.
Similar results were observed with ripretinib in combination with cobimetinib (Supplementary Fig. S4A–S4C).

Ripretinib/trametinib combination prevents emergence of drug-resistant colonies

Ba/F3 cells stably transfected with KIT-V560D were treated with the mutagen ethylnitrosourea (ENU) to induce random mutations. Ripretinib as a single agent showed outgrowth of a few colonies at 25 and 100 nmol/L (Fig. 5A, left), although no secondary KIT mutations were observed (Fig. 5A, right), indicating that the outgrowth of colonies with single-agent ripretinib treatment was due to another mechanism, likely involving signaling pathway-induced resistance. Ripretinib (25 nmol/L) in combination with trametinib (10 nmol/L) led to the outgrowth of only 2 colonies, whereas combination of trametinib (10 nmol/L) with ripretinib (100 or 250 nmol/L) eliminated outgrowth of any resistant colonies. In contrast, secondary resistant mutations in KIT were identified in cells treated with imatinib alone at all three concentrations tested (100, 250, and 500 nmol/L) as shown here and described previously (22). With imatinib/trametinib combination, approximately 25–30 colonies outgrew at all concentrations of imatinib tested, but no secondary resistance mutations in KIT were observed (Fig. 5B, right).

In vivo antitumor studies demonstrate in vivo efficacy of ripretinib + trametinib combination

GIST-T1 tumors were staged for 10 days and then mice were fed on formulated diet containing ripretinib or control diet until day 27 and then monitored for additional 40 days on control diet. In mice treated with single-agent trametinib (0.5 mg/kg orally, twice daily), no significant difference in tumor growth was observed when compared with control. Mice on single-agent ripretinib showed significant tumor regression at both doses (Fig. 6A). At the high dose of ripretinib, 6 of 10 mice had complete tumor regression, while the remaining 4 of 10 mice had partial tumor regression during the dosing period. Combination treatment with the higher dose of ripretinib and trametinib showed complete regression in 10 of 10 mice after 18 days of dosing; however, combination treatment at the high dose resulted in significant body weight loss that was recovered quickly after the end of treatment. Combination treatment at the lower dose was well tolerated. Tumors exhibited slow regrowth after the end of the dosing period with ripretinib alone and slower regrowth with the combination of ripretinib and trametinib. Four mice had partial regression and one mouse showed complete regression after 40 days of revival period in the higher dose combination group, versus two

Figure 3.

Combination treatment induces apoptosis and inhibits colony outgrowth in HMC1.2 (V560G/D816V KIT) cells. A, Heatmap of caspase activity in HMC1.2 cells treated with ripretinib alone or in combination with trametinib at indicated concentrations for 24 hours. Values represent an average of three replicates each from two different experiments. B, BLISS synergy graph of ripretinib and trametinib combination at indicated concentrations. C, HMC1.2 cells were grown in soft agar for 10 days in the presence of ripretinib or trametinib alone and in combination. Drug treatments were washed off and cells were grown for 5 days in complete media. If no colonies were visible, cells were revived for extra 8 days. Colonies were stained and photographed. D, Average colony counts from three wells. Arrows show no colony outgrowth.
partial regressions in the ripretinib high dose group and no partial regressions in the trametinib group. Body weight changes for ripretinib treated group were similar to vehicle.

In two imatinib-resistant KIT-mutant mastocytosis models, (P815 mouse D814Y KIT and HMC1.2 human V560D/D816V KIT), the ripretinib combination with trametinib also showed greater tumor growth inhibition than either single agent (Fig. 6C and D), with the combination showing synergistic activity in the P815 allograft model. Treatments were tolerated though body weight loss was observed in the combination group in both models (Fig. 6E and F). Body weight changes for the ripretinib single-agent-treated groups were similar to vehicle.

Discussion

The development of secondary resistance mutations in KIT has been a major challenge for the treatment of patients with GIST. These
mutations often arise in exons 13, 14, 17, and 18 after treatment with imatinib, sunitinib, and/or regorafenib. A major focus of research in the GIST field has been to develop novel and more potent inhibitors that can target most, if not all, of the possible combination of primary and secondary mutations. Other mechanisms of resistance have also been reported, such as cells entering quiescent state indefinitely by undergoing autophagy (10) or existence of KITlow stem/progenitor tumor cells that fail to respond to KIT inhibitors (31). With the advent of next-generation sequencing of GIST tumors in the clinic and in clinical trials, a new landscape for GIST has emerged. It has become evident that in some cases, GISTs can also become dependent on other signaling pathways besides KIT/PDGFRA (18, 32). Activating mutations have been found in PI3K/AKT/mTOR and RAS/RAF/MAPK signaling pathways in KIT-mutated or in wild-type GIST and in patients on later lines of therapy (17–19, 32–35). Mutations in BRAF, KRAS, or PIK3CA, which encode downstream effectors of KIT/PDGFRA, are associated with poor prognosis and patients progress on imatinib (34–36). Cell lines with KRAS or BRAF mutations concomitant with KIT/PDGFRA mutations failed to respond to imatinib (35). Activation of the ERK signaling pathway through alternate receptor tyrosine kinases such as FGFR1 or FGFR2, AXL and c-MET can also induce adaptation to imatinib treatment (37, 38). These studies demonstrate that both KIT-dependent and -independent pathways are active in some cases of GIST and these patients may not respond to KIT inhibition alone. Recent studies have shown that transcription factor ETV1 activates a positive feedback mechanism for KIT transcription and its levels are maintained by MAPK signaling pathway and PDGFRA signaling in KIT-mutant GIST (21, 39, 40). Because most of the KIT-independent pathways appear to act via MAPK signaling, identifying new strategies that target the full range of KIT/PDGFRA mutations and MAPK signaling pathway are of paramount importance.

In this study, ripretinib or imatinib was used in combination with FDA-approved MEK inhibitors in imatinib-sensitive and -resistant GIST cell lines. The data showed that combining imatinib with MEKi has a distinct advantage over treatments with imatinib alone in killing the cancer cells. This is in accordance with recent reports where imatinib or pexidartinib (a multikinase inhibitor including KIT) used in combination with binimetinib has shown better efficacy in advanced GIST tumors (21, 41). Ripretinib showed superior efficacy to imatinib
in combination with MEKi in the imatinib-sensitive and particularly, in the imatinib-resistant cell lines tested. Ripretinib combined with MEKi completely abrogated the residual MAPK signaling that was not inhibited by KIT inhibitors alone and exhibited strong synergy in inducing apoptosis. The ripretinib-MEKi combination was also better than imatinib-MEKi combination at inhibiting colony outgrowth even after long-term removal of drug. Ripretinib/MEKi combinations were also tested in GIST-T1 stably transfected with NRASG12D to mimic the mutations found in some patients. Colony outgrowth assay showed NRASG12D-transfected cells were efficiently inhibited by combination treatment.

The combination of ripretinib with trametinib completely prevented outgrowth of resistant colonies in saturation mutagenesis studies, although there are limitations to the experiment. Ethylnitrosurea treatment leads to predominantly A to G transitions, and based on the number of cells mutagenized, not all secondary KIT mutations or mutations in other genes that would lead to resistance would be generated. However, resistant clones were isolated following imatinib treatment, suggesting that ripretinib/MEKi combination treatment warrants further study in clinic as a method for slowing disease progression due to drug resistance in patients. Combination of imatinib/trametinib resulted in outgrowth of several resistant colonies, possibly due of activation of other pathways (42) or overexpression of certain transcription or translation factors. On the basis of these data, imatinib could be a weaker inhibitor of KIT compared with ripretinib, and therefore might allow some colonies to go into quiescent or cytostatic phase, leading to later stage activation of different pathways.

Another disease which is dependent on KIT is SM, where 90% of the indolent and aggressive SM patients carry the KIT D816V mutation (27). These patients are at a greater risk for disease progression (29). So far, the only FDA approved D816V KIT inhibitor for SM is midostaurin (43). Advanced SM patients also can have NRAS

Figure 6. Ripretinib and trametinib combination treatment in KIT mutant models in mice. A, Mice bearing GIST T1 tumors were treated as indicated. Dosing started on day 10 and ended on day 27. Tumor growth was monitored for additional 40 days. B, Body weight change in mice on study. Data are represented as mean ± SEM. C, Mice bearing PB15 tumors were treated as indicated. Dosing started on day 5 and ended on day 28. D, Mice bearing HMC1.2 tumors were treated as indicated. Dosing started on day 10 and ended on day 28. E, Body weight change in mice on PB15 study. Data are represented as mean ± SEM. F, Body weight change in mice on HMC1.2 study. Data are represented as mean ± SEM.
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mutations (29, 44), which may indicate the need to target both driver mutations. We demonstrate here that ripretinib and MEKi combination synergistically induced apoptosis in HMC1.2 cells with complete inhibition of colony outgrowth observed at much lower concentrations of each drug in long term assays. The combination was more potent in HMC1.2 NRASG12D cells at lower concentrations, demonstrating that combination warrants further study in the clinic as an option for advanced SM patients.

Because MEKIs have shown some toxicity in the clinic (45, 46), lower doses needed for combination treatment might be better tolerated. The high-dose combination of ripretinib with trametinib led to body weight loss in mouse studies, but treatment cessation led to recovery in body weights within 2 days in the GIST T1 model. Alternatively, binimetinib could be a better combination partner with ripretinib because of its higher clearance compared to trametinib, and no toxicity has been reported in GIST T1 xenograft studies when used in combination with imatinib (41).

In summary, our data suggest that further clinical study of combination therapy is warranted for advanced GIST and SM patients. Ripretinib potently inhibits a broad spectrum of primary and drug-resistant KIT/PDGFR Alpha mutants and is approved by the FDA for the treatment of advanced GIST who have received previous treatment with 3 or more kinase inhibitors, including imatinib. Studies from several clinical trials have shown that some patients with KIT exon 11 mutations do not respond to imatinib treatment, and analysis of KRAS and BRAF mutations together with KIT mutations has been suggested for profiling patients (35). In that respect, dual inhibition of KIT and the MAPK pathway may be highly beneficial for both imatinib-naïve and heavily treated patients. This strategy has the potential to overcome the MAPK pathway reactivation compensatory resistance mechanisms in this continuously evolving disease. One major advantage of using ripretinib as a combination partner with MEKI over imatinib is that ripretinib treatment did not result in the generation of secondary KIT resistance mutations in preclinical studies. A combination therapy, if successful in clinical trials, could be used as a first- or second-line of therapy for patients with GIST.

Authors’ Disclosures

A. Gupta reports a patent for US 16/943,821 pending and a patent for US 16/943,871 pending. C. Serrano reports grants from Deciphera Pharmaceuticals, Pfizer, and Bayer; personal fees from Blueprint Medicines, Immunac AB, and personal fees from Deciphera Pharmaceuticals during the conduct of the study; non-financial support from Pharmamar, Novartis, Lilly, Bayer, and non-financial support from Pfizer outside the submitted work. D.L. Flynn reports other from Deciphera Pharmaceuticals outside the submitted work, in addition, D.L. Flynn has a patent for US 8,461,179 issued, and Deciphera Pharmaceuticals has a relationship with the following GIST support groups: (i) The Life Raft Group; (ii) Gist Support International. B.D. Smith reports other from Deciphera Pharmaceuticals outside the submitted work; in addition, B.D. Smith has a patent for US8461179 issued, a patent for US8188113 issued, a patent for US: 16/943821 pending, and a patent for US: 16/943871 pending. No disclosures were reported by the other authors.

Authors’ Contributions

A. Gupta: Conceptualization, data curation, investigation, methodology, writing-original draft, writing-review and editing. J. Singh: Data curation, investigation, methodology, writing-review and editing. A. Garcia-Valverde: Data curation, investigation, methodology. C. Serrano: Conceptualization, data curation, investigation, methodology, writing-review and editing. D.L. Flynn: Conceptualization, methodology, writing-original draft, writing-review and editing. B.D. Smith: Conceptualization, data curation, investigation, methodology, writing-original draft, writing-review and editing.

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