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Farnesyltransferase (FTase) Inhibitors Increase Inhibition of KIT Mutants by Imatinib

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Abstract

Background: Mutations in the receptor tyrosine kinase KIT are the major cause of gastrointestinal stromal tumors. KIT-mediated activation of the RAS/RAF/MEK/ERK and PI3 kinase/AKT pathways plays an important role in KIT mutant-mediated cell transformation.

Methods: The frequently seen primary KIT mutations W557K558del and V560D, and the secondary KIT mutations V654A and N822K, in gastrointestinal stromal tumors were stably transfected into Ba/F3 cells. Cell proliferation was examined with a CCK kit, and cell survival and cell cycle were examined by flow cytometry. Cell signaling was examined by western blot.

Results: We found that farnesyltransferase inhibitors tipifarnib and lonafarnib, which inhibit RAS activity, inhibited ERK activation mediated by both wild-type and KIT mutants, which often occur in gastrointestinal stromal tumors. Correspondingly, both wild-type and KIT mutant-mediated cell survival and proliferation were inhibited by both inhibitors. Imatinib is used as the first-line targeted therapy for gastrointestinal stromal tumors in the clinic. In our study, both inhibitors increased imatinib-mediated inhibition of cell survival and proliferation induced by both wild-type and KIT mutants. Similar to the primary KIT mutations, secondary mutations of KIT-induced ERK activation and cell response were inhibited by both inhibitors.

Conclusions: Our results suggested the potential benefit of farnesyltransferase inhibitors either alone or combined with imatinib in the treatment of gastrointestinal stromal tumors carrying KIT mutations.

Keywords: Farnesyltransferase, Imatinib, KIT, RAS.

Introduction

KIT, a type III receptor tyrosine kinase, plays an important role in hematopoiesis, gametogenesis, and melanogenesis (1). Upon the binding with its ligand stem cell factor (SCF), KIT dimerizes and activates its kinase activity, which phosphorylates the tyrosines in the intracellular domain. Phosphorylated tyrosines further recruit and activate downstream molecules, which finally mediate cell survival, proliferation, and differentiation. Among the downstream signaling pathways, the RAS/RAF/MEK/ERK and PI3 kinase/AKT pathways play important roles in KIT-mediated cell response (2, 3).

Gain-of-function mutations of KIT are a group of substitution or deletion mutations of one or few amino acids in KIT. The mutations lead to ligand-independent KIT activation, and therefore induce cell transformation. KIT mutations occur in 70-80% of gastrointestinal stromal tumors (GISTs) and mastocytosis, and are also seen in acute myeloid leukemia, melanoma, and germ cell tumor (4-11). In primary GISTs, KIT mutations mainly map to exons 9 and 11, and to a lesser extent, exons 13

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and 14 (12). Imatinib inhibits the activation of most primary KIT mutations, is used as the first-line targeted therapy for GISTs, and has dramatically improved treatment outcomes (13). GISTs can develop resistance by gaining imatinib-resistant secondary mutations, while sunitinib, regorafenib, and ripretinib have been used as second-, third-, and fourth-line targeted GIST therapies, although they prolong patient survival by only a few months (14-16).

Due to the important role of the RAS/RAF/MEK/ERK pathway in KIT signaling (17-19), in order to know the possible use of inhibitors that can inhibit RAS activity in GISTs which is dominated by KIT mutants, in this study we tested FTIs that inhibit RAS activity. We found that both tested inhibitors inhibited ERK activation mediated by wild-type KIT, and primary and secondary KIT mutations, and inhibited cell survival and proliferation. These results provide a rationale to further test these inhibitors in malignancies carrying KIT mutants.

Materials and Methods

Reagents

Anti-phosphotyrosine antibody 4G10 was from Millipore (Billerica, MA), anti-KIT antibody was purified as previously described (20). Phospho-AKT (S473) antibody was from Cell Signaling Technology (Danvers, MA). AKT antibody, phospho-ERK (Thr202/Tyr204) antibody, ERK antibody, HRP-conjugated β-actin antibody, HRP-conjugated goat anti-rabbit antibody and HRP-conjugated goat anti-mouse IgG antibody were from Santa Cruz Biotechnology (Dallas, TX). Recombinant human stem cell factor (SCF) was from ORF genetics (Kópavogur, Iceland). Dynabeads™ Protein G was from Thermo Fisher Scientific (Waltham, MA). Tipifarnib and lonafarnib were from MedChemExpress (Monmouth Junction, NJ).

Cell culture

Ba/F3 cells were grown in RPMI 1640 medium plus 10% fetal bovine serum containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml IL-3. Ba/F3 cells expressing KIT were established as previously described (12). To examine the inhibition of FTase inhibitors on cell cycle, proliferation, and survival of Ba/F3 cells expressing KIT, or the signal transduction, cells were incubated with tipifarnib or lonafarnib at indicated concentration for 48 hours and proceeded for further detection.

Cell stimulation, immunoprecipitation, and western blotting

Ba/F3 cells were washed two times with PBS and starved in RPMI 1640 medium without serum and IL-3 for four hr before stimulation with SCF (100 ng/ml) for two min. After washing with cold PBS, cells were lysed on ice in a lysis buffer consisting of 1% Triton X-100, 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2 mg/ml Trasylol, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation for 15 min at 4 °C, cell lysates were proceeded for immunoprecipitation or western blot. For immunoprecipitation, cell lysates were incubated with indicated antibodies end-to-end for one hr at 4 °C, and then incubated with Dynabeads™ Protein G for 30 min at 4 °C. After washing two times with lysis buffer, immunoprecipitants were boiled in SDS-PAGE loading buffer for five min and electrophoresed for western blot. For western blot, proteins were separated by 8% SDS-PAGE followed by transfer to PVDF membranes. After blocking in PBS with 0.2% Tween-20 for one hr at room temperature, the membranes were incubated with indicated antibodies overnight at 4 °C and then incubated with HRP-conjugated secondary antibody for one hr at room temperature. After washing, the membranes were developed by ECL reagent.

Cell proliferation, cell survival, and cell cycle assay

Ba/F3 cells were washed two times with PBS and seeded in 6- or 96-well plates in Ba/F3 cell growth medium with 100 ng/ml SCF. After incubation for 48 hr, cells were proceeded as follows: for the cell proliferation assay, 10 μl CCK-8 reagent (Abmole Bioscience) were loaded into each well of96-well plates and absorbance was measured at 450 nm. For the cell apoptosis assay, cells were labeled with an Annexin V-PE apoptosis kit (BD Bioscience) according to the manufacturer's instructions

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followed by flow cytometry. For the cell cycle assay, cells were fixed in 70% ethanol overnight at 4 °C, washed two times with PBS, and stained with propidium iodide /RNase A mixture for 30 min in the dark followed by flow cytometry.

Statistical analysis

Data were presented as means ±standard deviations of three independent experiments. The differences between values were analyzed by Student's t-test. p values less than 0.05 were considered statistically significant.

Results

FTIs inhibited ERK activation mediated by KIT mutants

RAS/ RAF/ MEK/ ERK pathway plays an important role in KIT signaling. To determine a potential role for RAS inhibition in KIT mutation-related malignancies, we tested the RAS farnesyltransferase (FTase) inhibitors (FTIs) tipifarnib and lonafarnib-. The frequently seen GIST KIT mutations W557K558del and V560Ds were used as study models. Both inhibitors inhibited ERK activation mediated by either KIT/W557K558del or KIT/V560D without altering KIT activation (lanes 6, 8, 10, 12), while wild-type KIT mediated ERK activation was not significantly changed by either inhibitor (lanes 2 and 4) (Fig. 1). In addition, KIT activation and AKT activation were not changed by both inhibitors. These results suggested that FTIs inhibited RAS/RAF/MEK/ERK pathway activation mediated by KIT mutants.

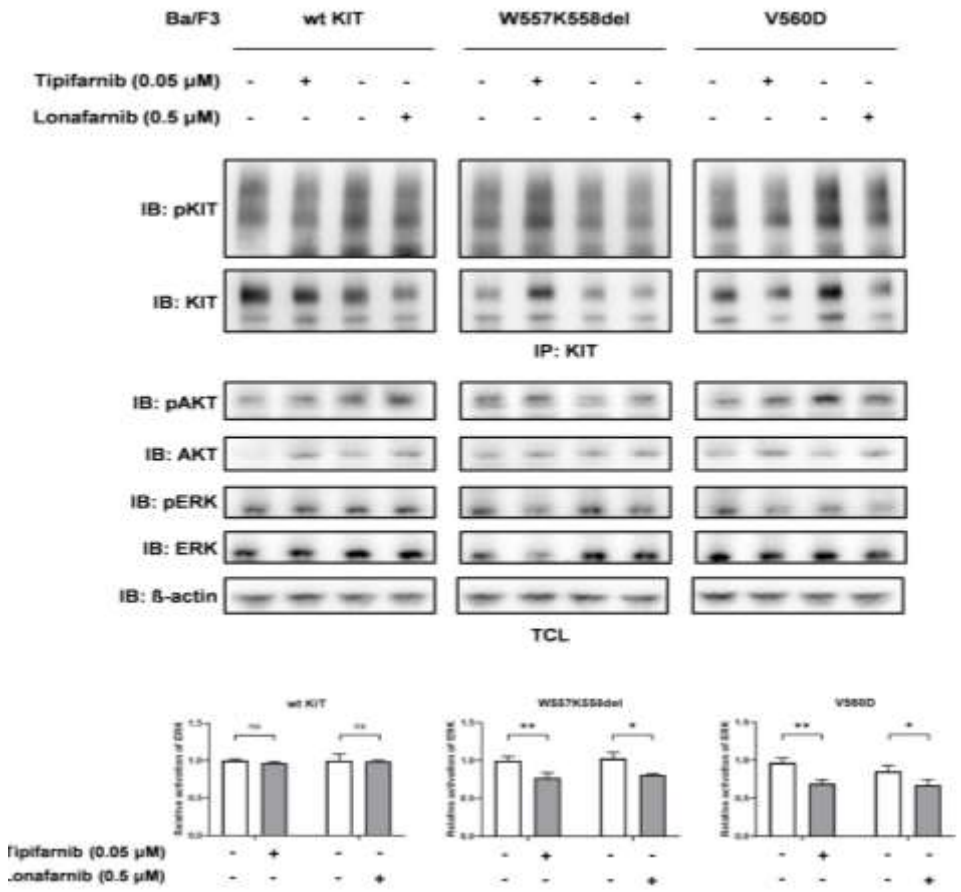

Fig. 1. Ba/F3 cells expressing wild-type KIT, KIT/W557K558del, or KIT/V560D were incubated with or without tipifarnib or lonafarnib for 48 hr. After washing with PBS, cells were resuspended in RPMI 1640 medium with or without tipifarnib or lonafarnib, and incubated with or without tipifarnib or lonafarnib, and incubated for four hr. Cells were stimulated with SCF for two min, lysed, and KIT was immunoprecipitated using KIT antibody to examine KIT activation by probing with 4G10 and KIT antibodies respectively. Total cell lysates (TCL) were probed with antibodies against pAKT, AKT, pERK, ERK and β-actin respectively. ERK activation was quantified and normalized by β-actin. pKIT, pAKT, and pERK = phosphorylated forms. (* = P< 0.05, ** = P< 0.01, *** = P< 0.001, **** = P< 0.0001

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FTIs inhibited cell survival and proliferation mediated by KIT mutants

Due to the key role of the RAS/RAF/MEK/ERK signaling pathway in the KIT-mediated cell response, we further tested the effects of tipifarnib and lonafarnib on KIT-mediated cell survival and proliferation. Both inhibitors inhibited cell proliferation, survival, and cycle progression in wild-type and the KIT/W557K558del and KIT/V560D mutants (Figs. 2A, 3B, and 3C, respectively), and as inhibitor concentration increased, proliferation decreased (Fig. 2A). The differences between the untreated and inhibitor-treated cells for all three cell lines was significant for both cell survival and cell cycle progression (Figs. 3B and 3C).

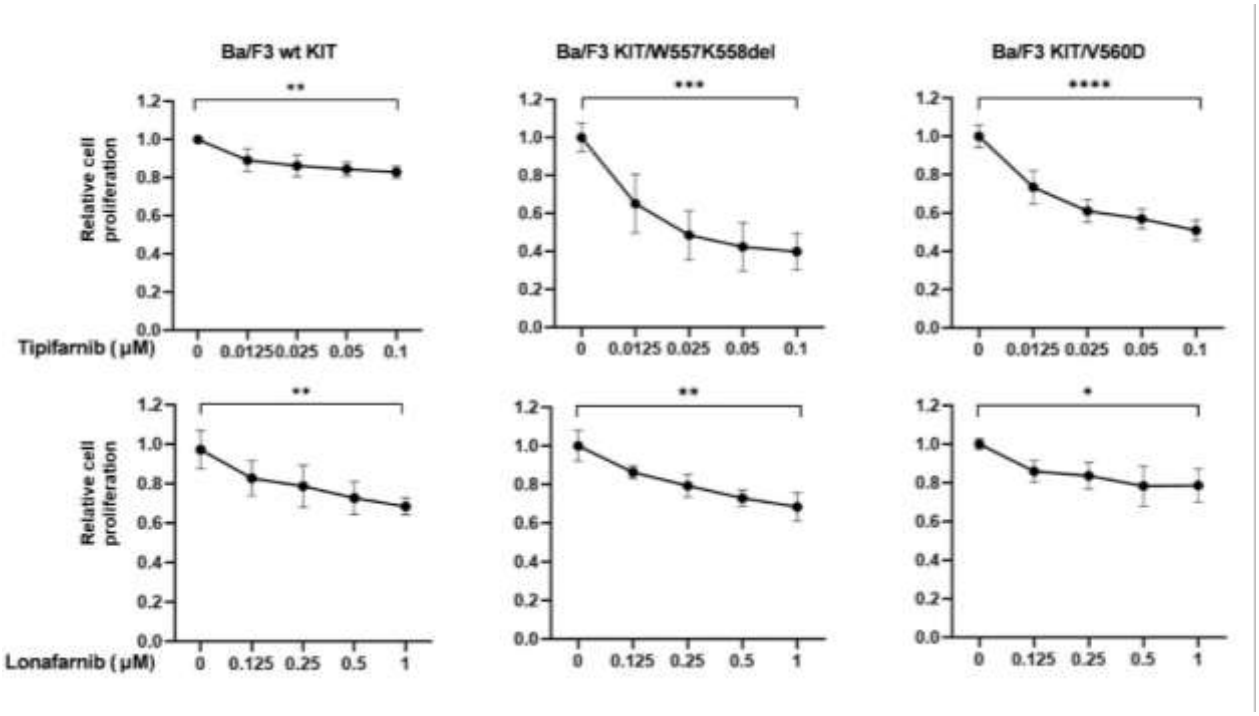

Fig. 2. Ba/F3 cells expressing wild-type KIT, KIT/W557K558del, or KIT/V560D were washed two times with PBS, resuspended in Ba/F3 cell growth medium containing 100 ng/ml SCF with or without tipifarnib or lonafarnib. After incubation for 48 hr, cell proliferation was examined using a CCK 8 kit. The proliferation of non-inhibitor treated cells was normalized as 1. (* = P< 0.05, ** = P< 0.01, *** = P< 0.001, **** = P< 0.0001).

FTIs increased imatinib-mediated inhibition of cell survival and proliferation mediated by both wild-type and mutant KIT

KIT mutations account for 70-80% of GISTs, and the KIT inhibitor imatinib is used as the first-line targeted therapy for GISTs. In this study, we tested imatinib and FTIs separately and together. We found that both FTIs significantly increased imatinib-mediated inhibition of cell proliferation in wide-type Ba/F3 KIT cells and the KIT/W557K558del and KIT/V560D mutants (Fig 3A), and flow cytometry showed similar results for cell survival and cell cycle progression (Figs. 3B, and 3C, respectively), suggesting a possible benefit of using FTIs combined with imatinib to treat GIST patients.

FTIs inhibited secondary KIT mutants

Gaining imatinib-resistant secondary KIT mutations is the major cause of relapse after initial response in GIST-targeted therapy(21). To test whether FTIs inhibited secondary KIT mutant-mediated activation of the RAS/RAF/MEK/ERK pathway and the cell response, we treated Ba/F3 cells expressing the frequently seen secondary KIT mutants W557K558del/V654A and W557K558del/N822K with tipifarnib or lonafarnib. We found that both inhibitors

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inhibited ERK activation mediated by either secondary KIT mutant (Fig. A: lane 2, 4, 6, 8). When used together with Imatinib, both inhibitors increased the inhibition of cell proliferation (Figs. 4B, 4C), cell survival (D), and cell cycle progression (4E) of both secondary KIT mutants expressing cells by Imatinib, indicating that the combination use of FTIs and Imatinib is beneficial to inhibit KIT mediated cellular responses.

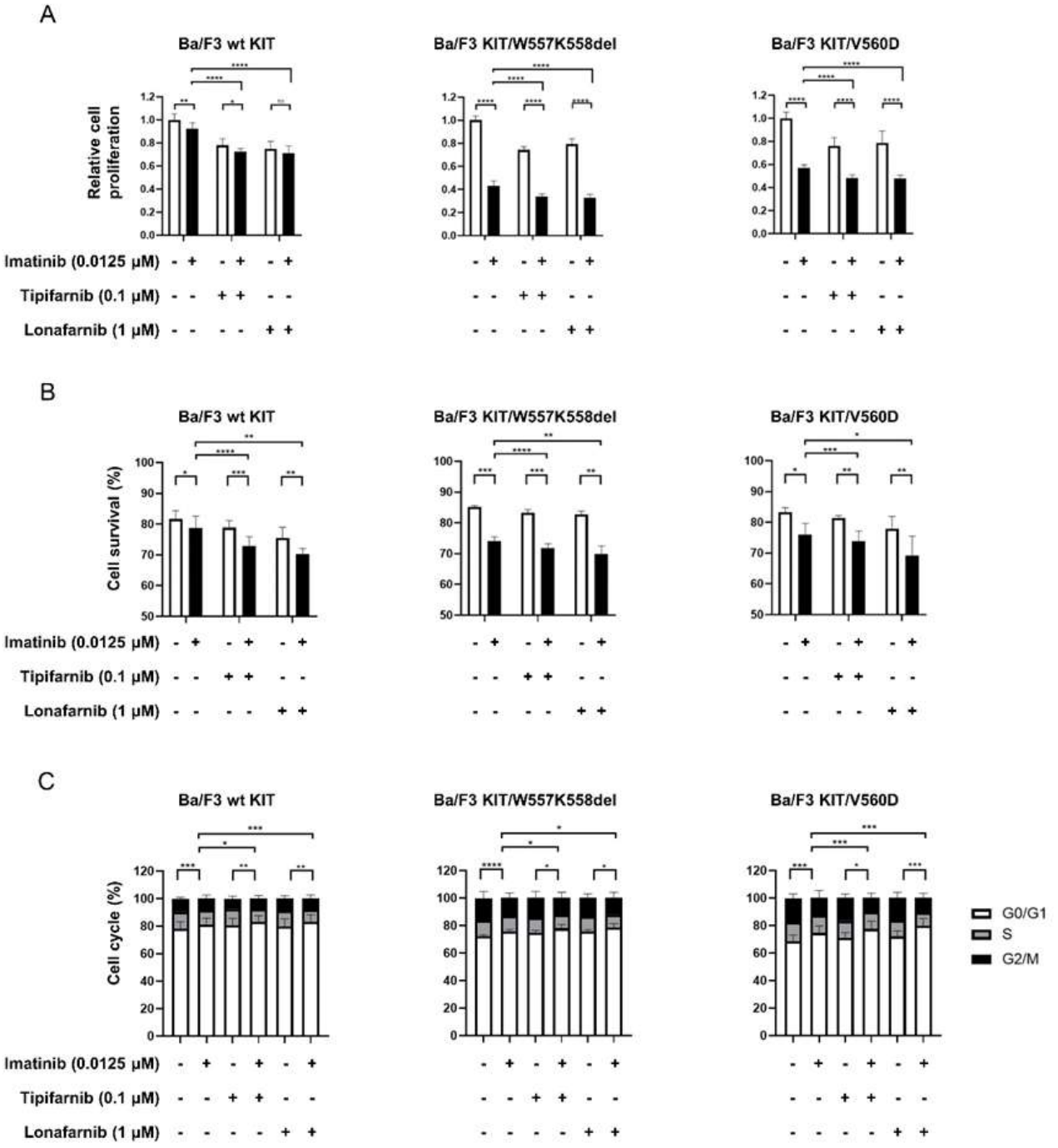

Fig. 3. Ba/F3 cells expressing wild-type KIT, KIT/W557K558del, or KIT/V560D were washed two times with PBS, resuspended in Ba/F3 cell growth medium with imatinib and/or tipifarnib or lonafarnib, and 100 ng/ml SCF. After incubation for 48 hr, cell proliferation (A) was examined using CCK 8 kit. The proliferation of non-inhibitor treated cells was normalized as 1. Cell apoptosis (B) was examined using an Annexin V-PE apoptosis kit and the cell cycle (C) was analyzed after labeling with propidium iodide (* = P< 0.05, ** = P< 0.01, *** = P< 0.001, **** = P< 0.0001).

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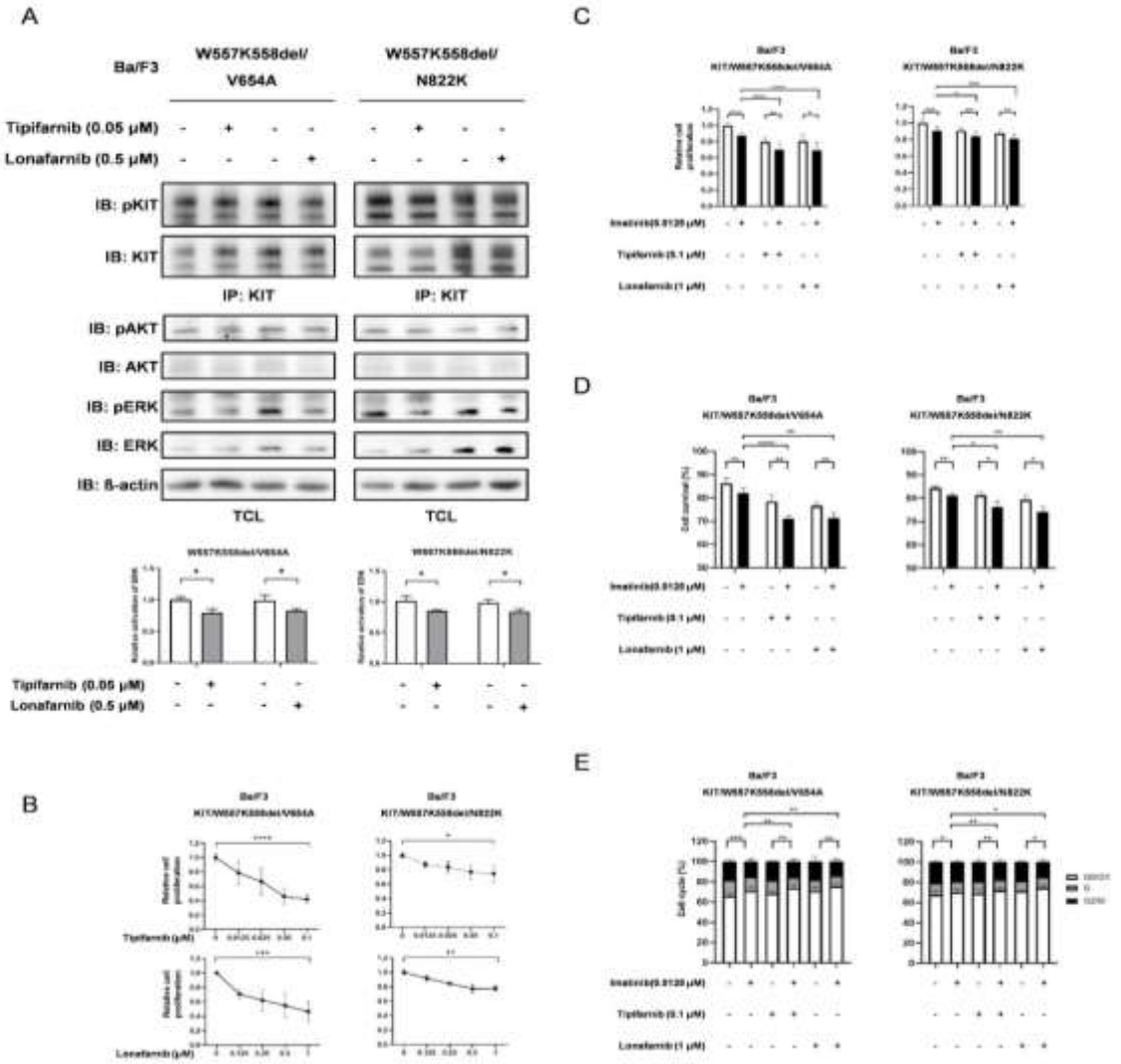

Fig. 4. (A) Ba/F3 cells expressing KIT/W557K558del/V654A or KIT/W557K558del/N822K were washed with PBS, resuspended in Ba/F3 cell growth medium with or without tipifarnib or lonafarnib, and incubated for 48 hr. After washing with PBS, cells were resuspended in RPMI 1640 medium with tipifarnib or lonafarnib and incubated for four hr. Cells were stimulated with SCF for two min, lysed, and KIT was immunoprecipitated using KIT antibody to examine KIT activation by probing with 4G10 and KIT antibody respectively. Total cell lysates were probed with antibodies against pAKT, AKT, pERK, ERK and β-actin respectively. ERK activation was quantified and normalized by β-actin. Cell proliferation (B, C) , cell apoptosis (D) and the cell cycle (E) of Ba/F3 cells expressing KIT/W557K558del/V654A, KIT/W557K558del/N822K were examined as described in previous figure (* = P< 0.05, ** = P< 0.01, *** = P< 0.001, **** = P< 0.0001).

Discussion

KIT mutations are the main cause of GISTs (4-7). To date, our KIT inhibitors have been approved for GIST targeted therapy, and these have dramatically improved outcomes (13-16). However, GISTs can develop drug resistance by gaining drug-resistant secondary KIT mutations (21) or activating alternative signaling pathways (22-24) to circumvent the KIT inhibition, leading to relapse and treatment failure. The RAS/RAF/MEK/ERK and PI3 kinase/AKT pathways are the two major signaling pathways that mediate KIT-induced cell survival and proliferation (12, 17-19, 25, 26). In this study, we tested two FTIs that target RAS in KIT signaling and KIT-mediated cell responses. Both inhibitors inhibited ERK activation mediated by wild-type KIT and

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primary KIT mutations. Correspondingly, the wild-type KIT and primary KIT mutations that mediated cell survival and proliferation were also inhibited by FTIs. Interestingly, tipifarnib showed stronger inhibition on the cell proliferation mediated by primary KIT mutants than wild-type KIT, suggesting possible specificity of tipifarnib against GISTs carrying KIT mutants but no other tissues carrying wild-type KIT. These results indicated the possible application of FTIs in the treatment of malignancies carrying KIT mutants. Furthermore, when used together with imatinib, which is used as the first-line targeted therapy for GISTs, the FTIs increased inhibition of proliferation, and cell survival, indicating a potential benefit from using FTIs and imatinib together.

A drug-resistant secondary KIT mutation is the major cause of treatment failure in imatinib-treated GIST patients (21). Although other KIT inhibitors can be used in the treatment of relapsed tumors, they generally only improve patient survival for few months (14-16), which is far from satisfaction. Our study found that FTIs inhibited secondary KIT mutant mediated ERK activation and cell survival and proliferation, suggesting that targeting the downstream KIT signaling pathway could be an alternative treatment when the treatment outcome of KIT inhibitor is limited. In addition, our results also suggested the possible beneficial effect of combining FTIs and KIT inhibitors. Mastocytosis is another malignancy dominated by KIT mutations. The D816V mutation in KIT exon 17 is the major mutation carried in mastocytosis (27, 28). In contrast, KIT mutations in GISTs mainly occur in exons 9 and 11. To date, no KIT inhibitor has been approved in the treatment of mastocytosis, with mastocytosis patients receiving only palliative treatment. Our results provided a rationale to further test drugs that can target downstream KIT signaling pathways in mastocytosis.

Small-molecule kinase inhibitors have been widely studied and approved to treat cancers and other illnesses. Unlike other kinases whose activity can be inhibited by small molecule inhibitors, RAS is difficult to target with these inhibitors due to the picomolar affinity of RAS proteins for GTP, the high concentration of GTP in the cells and the lack of a druggable pocket in the RAS proteins (29, 30). In this study, we tested FTIs that can inhibit RAS activity due to the fact that farnesyltransferase is necessary for the post-translation modification of RAS (31, 32). ERK activation, which occurs downstream of RAS, was inhibited by both tested inhibitors; however, we still see residential ERK activation, and cell proliferation and cell survival were only partially inhibited, meaning that there is still room for improvement in RAS targeting. We also tested FTIs in KIT signaling. Both inhibitors inhibited ERK activation mediated by wild-type KIT and primary and even secondary KIT mutants, and they can further increase imatinib-mediated inhibition of cell proliferation and survival, providing a rationale to further test these inhibitors in KIT-related malignancies.

The FTIs tipifarnib and lonafarnib inhibited both primary and secondary KIT mutant-mediated cell survival and proliferation. The inhibitors increased the inhibition of KIT mutants by imatinib, which is used as the first-line targeted therapy of GISTs. These findings suggest the potential use of FTIs alone or combined with imatinib in the treatment of GISTs carrying KIT mutations.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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