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Repurposing cabozantinib to GISTs: Overcoming multiple imatinib-resistant cKIT mutations including gatekeeper and activation loop mutants in GISTs preclinical models

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ABSTRACT

Despite of the great success of imatinib as the first-line treatment for GISTs, the majority of patients will develop drug-acquired resistance due to secondary mutations in the cKIT kinase. Sunitinib and regorafenib have been approved as the second and third line therapies to overcome some of these drug-resistance mutations; however, their limited clinical response, toxicity and resistance of the activation loop mutants still makes new therapies bearing different cKIT mutants activity spectrum profile highly demanded. Through a drug repositioning approach, we found that cabozantinib exhibited higher potency than imatinib against primary gain-of-function mutations of cKIT. Moreover, cabozantinib was able to overcome cKIT gatekeeper T670I mutation and the activation loop mutations that are resistant to imatinib or sunitinib. Cabozantinib demonstrated good efficacy in vitro and in vivo in the cKIT mutant-driven preclinical models of GISTs while displaying a long-lasting effect after treatment withdrawal. Furthermore, it also exhibited dose-dependent anti-proliferative efficacy in the GIST patient derived primary cells. Considering clinical safety and PK profile of cabozantinib, this report provides the basis for the future clinical applications of cabozantinib as an alternative anti-GISTs therapy in precision medicine.

1. Introduction

Gastrointestinal stromal tumors (GISTs) originate from the interstitial cells of Cajal (ICC) and are the most common mesenchymal tumors of the gastrointestinal tract [1,2]. cKIT kinase is a type III receptor tyrosine kinase that regulates cell proliferation, survival, apoptosis and differentiation through downstream signaling pathways including Ras/RAF/MEK/ERK, PI3K/AKT and STAT5. Approximately 75% GIST patients harbor activating mutations in the cKIT receptor tyrosine kinase. The mutations (such as V559A/D/G and L576P) are located in exon 11 of the cKIT gene, which encodes the juxtamembrane domain involved in activating dimerization, and accounts for the majority of the primary gain-of-function mutations [3–5]. Secondary drug resistant mutations usually occur in the kinase domain either in the ATP-binding pocket (such as T670I and V654A) due to steric hindrance which prevents full access of the drug to the ATP binding pocket [6–8] or in the activation loops (such as N822K, D816E/H/V, D820 E/G/Y and A829P) after drug treatment [9,10] which usually confer drug resistance by an unpredictable conformational change of the kinase [11].

Imatinib [12] is the first line clinical therapy for GISTs; however,

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drug resistance usually develops after continuous treatment due to heterogeneous secondary mutations including the gatekeeper and activation loop mutants [13–15]. Although latterly approved secondary and third line multi-target kinase inhibitors sunitinib [16,17] and regorafenib [18] could overcome some of the primary and secondary mutations, the poor overall drug response rate (7% ORR for sunitinib and 4.5% ORR for regorafenib) of them and the toxicity have greatly limited their clinical benefits [19]. Ponatinib is a multitarget kinase inhibitor that has been used in the clinic for treatment of the imatinib-resistant ABL-T315I mutant in CML; ponatinib was reported to be effective against a variety of the secondary imatinib-resistant cKIT mutants [19]. But the severe side effects also greatly affected its wide clinical application. Therefore, there is a great clinical demand for effective and safe drugs that can overcome the resistance due to the secondary mutants of the cKIT kinase that mediate imatinib-resistant GIST.

"Drug repurposing" may be the most efficient approach in the search for new available therapies for the unmet clinical demand. Using a functional isogenic BaF3 cell library and high-throughput screening facility, we found that cabozantinib [20–22], a multi-target kinase inhibitor approved for the treatment of advanced renal cell carcinoma (RCC) and progressive metastatic medullary thyroid cancer (MTC), displayed very potent inhibitory activity against cKIT wt. In addition, cabozantinib was able to overcome multiple imatinib resistant cKIT primary and secondary mutations including the gatekeeper mutant T670I as well as activation loop resistant mutants including N822K, D816E, D820 E/G/Y and A829P.

2. Materials and methods

2.1. Inhibitors

Cabozantinib, imatinib and sunitinib were purchased from a commercial chemical vendor (Haoyuan Chemexpress Inc.). Stock solutions (10 mmol/L) of all compounds were prepared in 100% dimethyl sulfoxide (DMSO).

2.2. cKIT protein purification

The sequences encoding cKIT wt and cKIT T670I residues 544–935 with a His tag were cloned into the baculovirus expression vector pFastHTa. The proteins were expressed by infecting SF9 cells with high titer viral stocks for 48 h. Cells were harvested and lysed in 25 mM Tris pH 7.4, 250 mM NaCl, and 1 mM PMSF. The supernatant was loaded onto a Ni-NTA column (Qiagen, Hilden, Germany). The proteins were eluted with the same buffer containing 250 mM imidazole. The eluted proteins were loaded on a Superdex-200 column equilibrated in 25 mM Tris (pH 7.4), 250 mM NaCl, 1 mM DTT, and 1 mM EDTA. Peak fractions were concentrated to 2 mg/ml, flash-frozen and stored at −80 °C.

2.3. Biochemical assay of kinase activity

To determine the inhibition of cKIT wt and relevant mutants by cabozantinib, imatinib and sunitinib, cKIT wt (12.5 ng/μL) or cKIT T670I (20 ng/μL) was incubated for 1 h at room temperature with the substrates poly (4:1 Glu, Tyr) peptide (0.4 μg/μL) (Promega, Madison, WI) and 100 μM ATP (Promega, Madison, WI) in 20 μL reaction buffer containing the compounds or DMSO. The reaction was stopped by the addition of the ADP-Glo reagent and samples were incubated for 40 min at room temperature. Then, the kinase detection reagent was added, and the samples were incubated for 30 min to produce a luminescence signal. The detection was performed with an automated plate reader (Envision, PE, USA). Data were analyzed and plotted using GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, CA).

2.4. Cell lines and cell culture

The human GIST-T1 cell line was purchased from Cosmo Bio Co. Ltd., Tokyo, Japan. GIST-882 and GIST-48B cell lines were obtained from Prof. Jonathan A Fletcher's group (Brigham and Women's Hospital, Boston, USA). GIST-5R cell line was provided by the group of Prof. Brian Rubin (Lerner Research Institute, Cleveland, USA). Isogenic BaF3 cell lines were maintained in RPMI 1640 medium (Corning, USA) supplemented with 2% L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. GIST-T1 and GIST-5R cells were cultured in DMEM (Corning, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. GIST-882 and GIST-48B cells were grown in IMDM (Gibco, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were cultured in a humidified incubator (Thermo, USA) at 37 °C under 5% CO_2. Adherent cells were grown in tissue culture dishes to 85–95% confluence before use. Suspension cells were collected by centrifugation at 700 rpm for 4 min prior to use.

2.5. BaF3 isogenic cell line generation

Functional BaF3 cell lines were generated as described previously [23]. Briefly, the cKIT wt and mutant genes were cloned into the pMSCVpuro retroviral vector for virus production in HEK-293T cells in combination with two helper plasmids. Virus-containing supernatant was used to infect BaF3 cells followed by puromycin selection and IL-3 withdrawal to obtain the stable cKIT-overexpressing BaF3 cells that are independent of IL-3 for cell survival and proliferation.

2.6. Cell proliferation assay

For suspension cells, 1–3 × 10^4 cells/mL were mixed with various concentrations of the compounds and transferred to a microplate at 100 μL per well. Adherent cells were grown in 96-well culture plates (2500–5000 cells/well) for 12 h, and various concentrations of the compounds were added. Cell proliferation was determined after the treatment with the compounds for 72 h. Proliferation of the primary cells was determined after the treatment with the compounds for 6 days. Cell viability was determined using the CellTiter-Glo (Promega, USA) and CCK-8 (MCE, USA) assays. Luminescence was determined using a multimode EnVision plate reader (PerkinElmer, USA) in the CellTiter-Glo assay. Absorbance was measured at 450 and 655 nm using a microplate reader (iMARK, Bio-Rad, USA) for the CCK-8 assay. Data were normalized to the control groups (DMSO) and are presented as the mean of three independent measurements with standard error < 10%. The GI_{50} values were calculated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

2.7. Molecular modeling

The crystal structure of the cKIT wt/imatinib complex (PDB ID: 1T46) and cKIT wt/sunitinib (PDB ID: 3G0E) were used for analysis and docking study. The protein was prepared by the Protein Preparation Wizard (Schrodinger Suites 2017). The structure of the cKIT T670I mutant was generated by site mutation with Maestro. The binding mode of the cKITwt/cabozantinib complex, cKIT T670I with imatinib, sunitinib and cabozantinib was obtained by docking with Glide and refined with Prime.

2.8. Signaling pathway studies

GIST-T1, GIST-882, GIST-5R and GIST-48B cells were treated with DMSO, serial dilutions of cabozantinib (0.03–10 μM), 1 μM imatinib and 1 μM sunitinib for 2 h. Cells were washed with cold 1 × PBS and lysed in a RIPA buffer (Beyotime, China) at 4 °C for 30 min. Western blotting was performed according to the routine protocols. The following antibodies were used at antibody concentrations recommended

by the corresponding manufacturers to probe for specific proteins: antibodies against phosphorylated and total cKIT (phospho-KIT Y703/Y719), ERK, AKT, STAT3, S6K and S6 were from Cell Signaling Technology; phospho-KIT Y823 antibody was from Invitrogen; GAPDH antibody was purchased from Transgen Biotechnology.

2.9. Apoptosis detection

GIST-T1, GIST-882, GIST-5R and GIST-48B cells were treated with DMSO, serial dilutions of cabozantinib (0.03–3 μM), 1 μM imatinib or 1 μM sunitinib for 24 h, 72 h (48 h), 24 h, and 48 h. Cells were collected and analyzed by Western blotting using PARP antibody was from Cell Signaling Technology. α-Tubulin antibody was from Santa Cruz Biotechnology. Cell apoptosis was also detected by Annexin V-FITC apoptosis detection Kit I (BD Pharmingen, SanDiego, CA, USA).

2.10. Cell cycle analysis

GIST-T1, GIST-882, GIST-5R and GIST-48B cells were treated with DMSO, serial dilutions of cabozantinib (0.03–1 μM), 1 μM imatinib or 1 μM sunitinib for 24 h, 48 h, 24 h, and 48 h. The cells were fixed in 70% cold ethanol, incubated at −20 °C overnight, and stained with a PI/RNase staining buffer (BD Pharmingen, SanDiego, CA, USA). Flow cytometry was performed using a FACS Calibur flow cytometer (BD, USA) and the results were analyzed by ModFit software.

2.11. GIST-T1 and GIST-5R xenograft and BaF3 cKIT-T670I and BaF3 cKIT-N822K allograft tumor models

All animal experiments were conducted according to the animal care regulations of Hefei Institutes of Physical Science, Chinese Academy of Sciences (Hefei, China). Experiments were performed in six-week-old female BALB/C-nu mice purchased from Nanjing Biomedical Research Institute, Nanjing University (Nanjing, China). Five million cells (GIST-T1 or GIST-5R) or one million cells (BaF3-tel-cKIT-T670I or BaF3-tel-cKIT-N822K) were suspended at a 1:1 ratio with Matrigel (BD Biosciences, USA) in PBS and injected subcutaneously on the right side of the mice. When tumors reached a size of 200–300 mm^3, animals were randomized into the treatment groups for efficacy studies. Cabozantinib was delivered daily in an HKI solution (0.5% methocellulose and 0.4% Tween 80 in ddH_2O) by oral gavage. Various doses of cabozantinib or the vehicle were administered as indicated in the figure legends. Body weight and tumor growth were measured daily after cabozantinib treatment. Tumor volumes were calculated as described previously [23].

2.12. GIST tumor sample processing

Human GIST samples were obtained with informed consent from the patients of the People's Liberation Army Joint Service Support Force No. 901 Hospital. All procedures were performed according to the hospital guidelines of the Research Ethics Committees and the World Medical Association Declaration of Helsinki. Fresh tumor tissue fragments were dissociated using collagenase/hyaluronidase and dispase (StemCell Technologies, Canada) at 37 °C for 2 h with shaking. Primary cells were placed in flasks coated with collagen I (Corning, USA) in the culture medium. The culture medium included DMEM/F12 medium freshly supplemented with 5% fetal bovine serum (Gibco, USA), Glutamax-I (Gibco, USA), primocin (Invivogen, USA), 25 μg/ml hydrocortisone (Sigma, USA), 5 μg/ml insulin (Gibco, USA), 125 ng/ml EGF (Sigma, USA), and 10 μM Rho kinase inhibitor Y27632 (Haoyuan Chem express Inc.). Cells were grown in humidified 37 °C incubators in the atmosphere of 5% CO_2 and 2% O_2. Medium was replaced every 3–4 days and the primary cells were cultured for a period of 3–4 weeks.

2.13. Immunofluorescence

Primary GIST cells were grown on coverslips and treated with various concentrations of the compounds for indicated time. Cells were washed once with PBS and fixed with 4% formaldehyde at room temperature for 20 min. The cells were blocked with AbDil-Tx (TBS-Tx supplemented with 2% BSA and 0.05% sodium azide) at room temperature for 1 h followed by overnight incubation with a cKIT antibody (Cell Signaling Technology, USA) at 4 °C. Secondary antibody was conjugated to Alexa Fluor 488 (Life Technologies, USA); then, samples were mounted using anti-fade ProLong Gold with DAPI (Life Technologies, USA). Fluorescence was imaged by a Leica DMI4000B fluorescence microscope. Images are representative of at least three independent experiments.

2.14. Immunohistochemistry staining

Tumor tissue samples were fixed in 10% neutral-buffered formalin and were processed for paraffin embedding. Six-micrometer thick tissue sections were prepared, deparaffinized, dehydrated, and stained with hematoxylin and eosin (H&E) using routine methods. After incubation in the casein block, anti-Ki-67 (ZSGB-BIO, China) was applied to the sections for Ki-67 staining. TUNEL staining was performed using a POD in situ cell death detection kit (Roche, USA). Standard procedures were performed as described previously [24].

3. Results

3.1. Inhibitory activity of cabozantinib against cKIT kinase mutations

We first examined the anti-proliferative effects of cabozantinib in comparison to imatinib and sunitinib against a panel of cKIT kinase transformed isogenic BaF3 cells whose proliferation depends on the cKIT wt or mutants (Fig. 1A, Supplementary Table 1). The results showed that cabozantinib was significantly more potent than imatinib and sunitinib in the cKIT wt cells (GI_{50}: 0.02 μM versus 0.18 μM and 0.12 μM, respectively). In the case of the cKIT primary gain-of-function mutations in the juxtamembrane domain, including V559A/D/G and L576P, sensitivity to cabozantinib in all mutants was higher than the sensitivity to imatinib and lower than sensitivity to sunitinib. In the case of the imatinib-resistant secondary mutants in the ATP binding pocket, including V654A and T670I as well as the mixed mutants V654A/V559D and T670I/V559D, cabozantinib and sunitinib were highly potent. Interestingly, the activation loop mutant D816E was sensitive to all three inhibitors, while D816H and D816V were resistant to all inhibitors. Cabozantinib was significantly more potent than imatinib and sunitinib against the D820 E/G/Y mutants. Moreover, cabozantinib was effective against the N822K and A829P activation mutants that were resistant to imatinib and sunitinib. Overall, these results demonstrated that cabozantinib can overcome most of the imatinib-resistant secondary mutants in the ATP binding pocket and activation loop except D816 H/V, which was also resistant to sunitinib.

Given that cabozantinib is a multiple-target kinase inhibitor, we examined its inhibitory effects on the autophosphorylation of cKIT wt/mutants to further confirm the on-target efficacy in the transfected BaF3 cells (Fig. 1B, Supplementary Table 2). The results demonstrated that cabozantinib could potently inhibit the autophosphorylation of cKIT Y823 site in the cKIT wt, V559A/D/G, T670I, V654A, D816E, D820 E/G/Y, N882K and A829P mutants, but much less potently in D816H mutant and nearly had no effect on the D816V mutant. The inhibition of autophosphorylation of Y703 and Y719 sites exhibited similar trends. The inhibition of autophosphorylation of Y823 (EC_{50}s) by cabozantinib were in accordance with the growth inhibition (GI_{50}s) observed from the transfected BaF3 cells, which confirmed that the anti-proliferative efficacy was from the on-target inhibition of the drug (Fig. 1C).

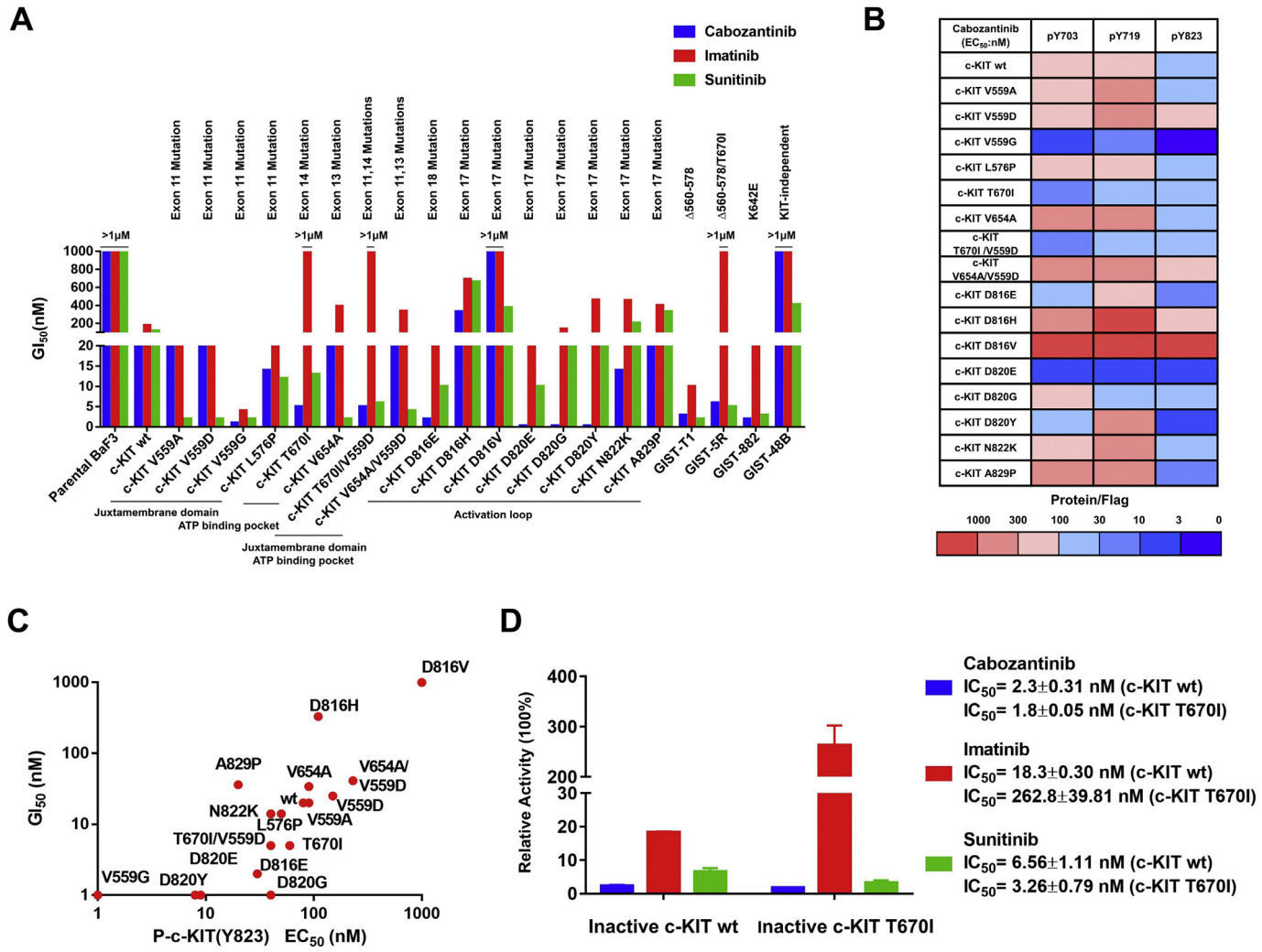

Fig. 1. Cabozantinib inhibits a panel of cKIT mutants in vitro.
A. GI_{50} values (nM) of cabozantinib, imatinib and sunitinib in four human GIST cell lines and BaF3 isogenic cell lines harboring mutants in the juxtamembrane domain, ATP-binding pocket and activation loop of cKIT kinase. B. Heatmap showing EC_{50} values calculated by quantification of the protein levels of various phosphorylation sites of cKIT relative to Flag in the BaF3 isogenic cell lines after cabozantinib treatment. Red color corresponds to high EC_{50} values, and blue color corresponds to low EC_{50} values. C. Correlation between GI_{50}s and EC_{50}s against various cKIT mutants in a panel of BaF3 isogenic cell lines. D. ADP-Glo biochemical assay of cabozantinib inhibition of cKIT wt and cKIT-T670I mutant proteins.

Next, we examined the inhibitory activity of cabozantinib against the purified cKIT wt/mutant proteins in the biochemical enzymatic assay (Fig. 1D). The results showed that cabozantinib was potent against cKIT wt (IC_{50}: 2.3 nM) and cKIT T670I mutant (IC_{50}: 1.8 nM). Comparably, sunitinib displayed similar potency to cabozantinib against these two mutants while imatinib was much less active. These data further confirmed the on-target effects of cabozantinib's anti-proliferative activity in the transfected BaF3 cells.

3.2. Structural basis of sensitivity of the cKIT-T670I mutants to cabozantinib

To understand the mechanism of cabozantinib to overcome the cKIT gatekeeper T670I mutant, we docked cabozantinib to the cKIT wt and T670I mutant structures (PDB ID: 1T46; the T670I mutant was obtained by substituting Ile for Thr670). In the crystal structure of the cKIT-imatinib complex (Fig. 2A), Thr670 provided a hydrogen bond donor towards imatinib. The mutation of Thr670 to the bulky Ile670 resulted in a loss of the key hydrogen bond and added a steric obstacle toward the aromatic ring of imatinib (Fig. 2B). As has been reported, sunitinib also preferred the DFG-out conformation of the cKIT kinase like imatinib did [10]. Since the indolinone moiety of sunitinib was away from Thr670 or Ile670 residue, sunitinib could retain the activity when Thr670 mutated to Ile670 (Fig. 2C–D). Cabozantinib has an O-linked hydrophobic phenyl ring in the gatekeeper area and does not form a hydrogen bond with Thr670 (Fig. 2E). Moreover, the O-link moiety of cabozantinib provided additional space that can accommodate bulky hydrophobic Ile670 without loss of activity (Fig. 2F).

3.3. Antiproliferative activity of cabozantinib in GIST cell lines harboring primary and secondary mutations

We examined the anti-proliferative effect of cabozantinib against a panel of established GIST cell lines (Figs. 1A and 3A and Supplementary Table 3). The results showed that cabozantinib and sunitinib displayed single digit nM GI_{50} values against GIST-T1 [25] cell line (primary Δ560–578 mutation in the JM domain) and GIST-882 [26] cell line (primary K642E mutation in the c-helix), which is relatively more potent than imatinib. Additionally, these two drugs were highly effective against the imatinib-resistant GIST-5R [27,28] cell line (GI_{50}: single digit nM), which was generated from GIST-T1 cell line and harbors Δ560–578 and gatekeeper T670I mutations. In the cKIT-independent

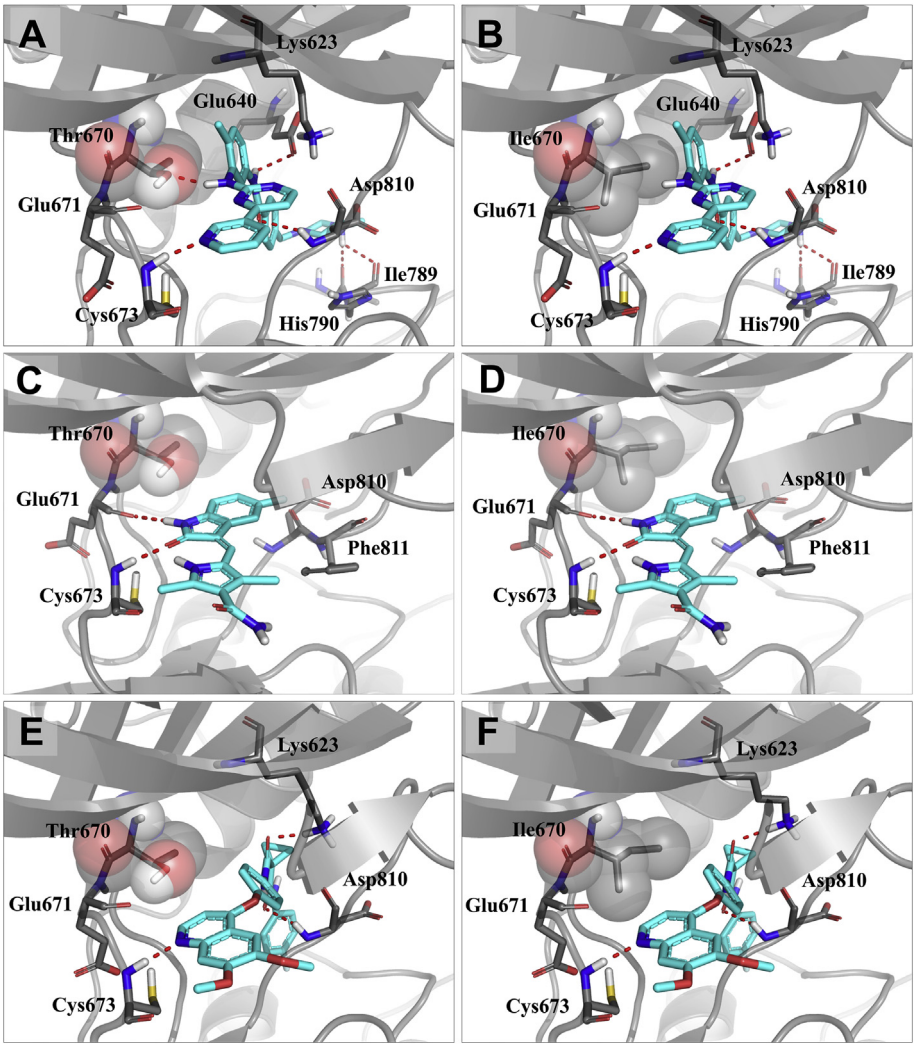

Fig. 2. Structure-based analysis of cabozantinib resistance towards the cKIT T670I mutant in comparison with imatinib and sunitinib.
A. Crystal structure of imatinib in complex with cKIT (PDB ID: 1T46). B. imatinib docked into cKIT T670I mutant (homology model built from PDB ID: 1T46). C. Crystal structure of sunitinib in complex with cKIT wt (PDB ID: 3G0E). D. Sunitinib docked into cKIT T670I mutant (homology model built from PDB ID: 3G0E). E. Cabozantinib docked into cKIT wt (PDB ID: 1T46). F. Cabozantinib docked into cKIT T670I mutant (homology model built from PDB ID: 1T46).

GIST cell line GIST-48B [29], imatinib completely lost its activity and cabozantinib showed weak potency (GI_{50}: 1.54 μM) while sunitinib was relatively more potent (GI_{50}: 0.41 μM), which reflected multiple target properties of cabozantinib and sunitinib. This result also indicated that cabozantinib may have a better therapeutic window than sunitinib, at least at the cellular level.

To confirm the on-target effect of cabozantinib in these cell lines, we determined the effects of cabozantinib on the cKIT-mediated signaling pathway (Fig. 3B). In the GIST-T1 and GIST-882 cells that harbor primary cKIT gain-of-function mutations, cabozantinib potently inhibited phospho-cKIT Y719, Y703, and Y823 and the downstream signaling mediators including phospho-AKT, S6K, S6 and ERK, similar to imatinib and sunitinib. In the imatinib-resistant cell line GIST-5R, cabozantinib strongly affected phospho-cKIT and downstream AKT, S6K, S6 and ERK. As expected, imatinib did not inhibit this signaling pathway. Interestingly, sunitinib was a potent inhibitor of all these signaling mediators. In the cKIT-independent cell line GIST-48B, however, the downstream mediators were not affected.

Next, we examined the effects of cabozantinib on cell cycle progression and apoptosis. As expected, cabozantinib blocked the cell cycle progression at the G0/G1 phase at 0.03 μM concentration in the drug-sensitive cell lines including GIST-T1 and GIST-882 and in imatinib-resistant GIST-5R, but not in the insensitive cell line GIST-48B (Fig. 3C, Supplementary Figs. 1–2). Cabozantinib also induced apoptosis in the cKIT mutant GIST cell lines in a dose-dependent manner, but it had no effect in GIST-48B (Fig. 3D, Supplementary Figs. 3–4). These results were consistent with the growth inhibition effects observed in various GIST cell lines.

3.4. Antitumor efficacy of cabozantinib in vivo against the cKIT kinase mutants

To further investigate potential clinical applications of cabozantinib, we examined in vivo efficacy of cabozantinib in several preclinical models. Oral administration of cabozantinib at various dosages (10, 20, 25, 40, 50 and 100 mg/kg/day) did not show any apparent toxicity and no weight loss was observed. In the GIST-T1 xenograft mouse model, cabozantinib suppressed tumor growth in a dose-dependent manner, and the TGI (tumor inhibition rate) was 94.6% (Fig. 4A) at 40 mg/kg/day dosage; cabozantinib displayed long-lasting response after the treatment withdrawal (Fig. 4A, Supplementary Fig. 5). In the GIST-5R xenograft mouse model, 20 mg/kg/day dosage of cabozantinib blocked the tumor progression and showed a TGI of 89.3% (Fig. 4B), while the same dosage of imatinib showed no apparent effect on tumor growth (Fig. 4B, Supplementary Fig. 6). Additionally, cabozantinib inhibited tumor growth in a dose-dependent manner, and even

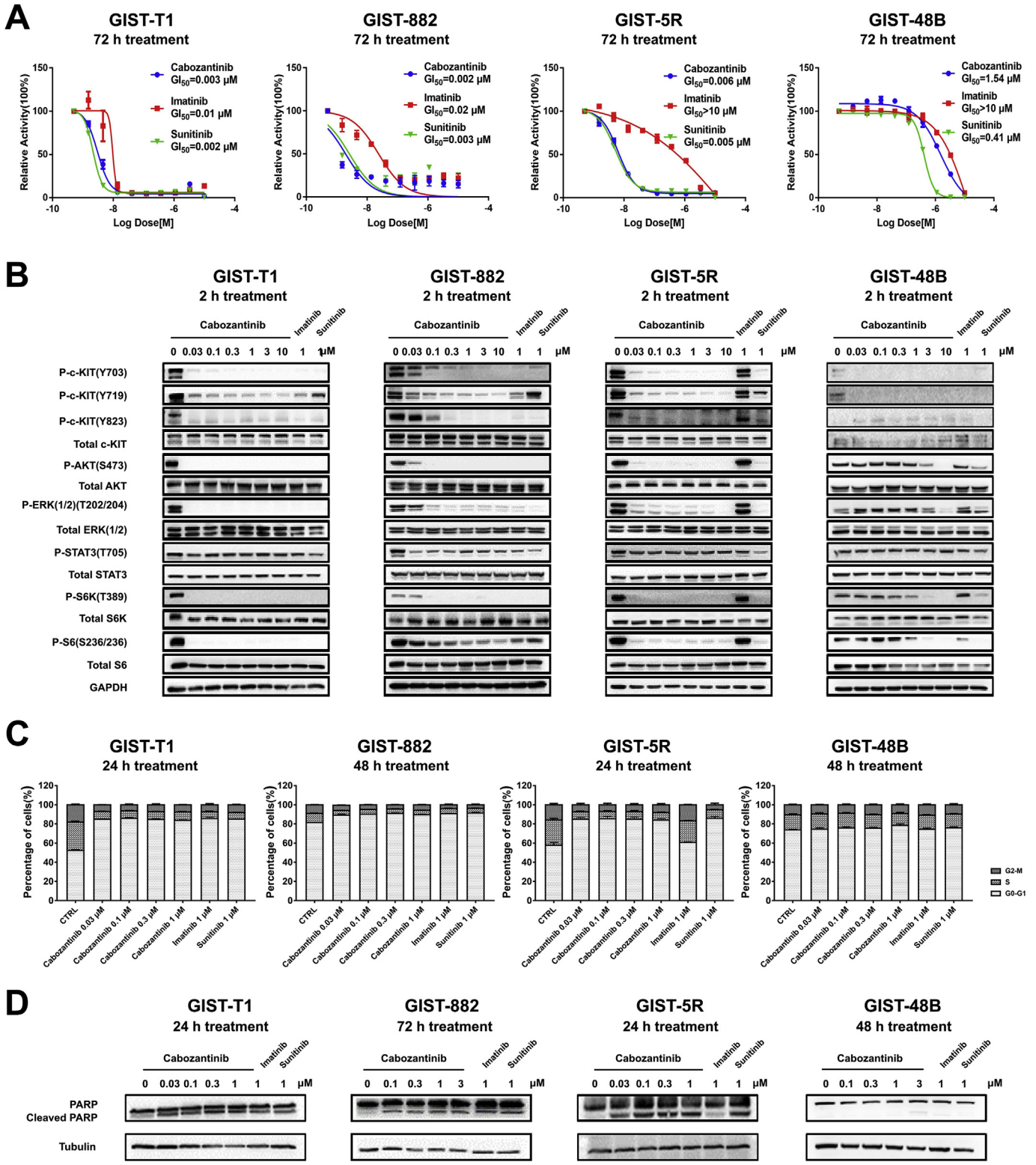

Fig. 3. Effects of cabozantinib on cell activity, cellular signaling, cell cycle progression and apoptosis in human GIST cancer cell lines. A. Effects of cabozantinib on proliferation of GIST-T1, GIST-882, GIST-5R and GIST-48B cell lines (CellTiter-Glo and CCK-8 assay). B. Effects of cabozantinib on the cKIT-mediated signaling pathways in GIST-T1, GIST-882, GIST-5R and GIST-48B cell lines (immunoblotting). C. Effects of cabozantinib on cell cycle progression in GIST-T1, GIST-882, GIST-5R and GIST-48B cell lines (flow cytometry). D. Effects of cabozantinib on apoptosis in GIST-T1, GIST-882, GIST-5R and GIST-48B cell lines (immunoblotting).

25 mg/kg/day dosage provided a TGI of 91.1% (Fig. 4C) in the cKIT-T670I mutant-transfected BaF3 cell allograft model, while imatinib did not inhibit tumor growth (Fig. 4C, Supplementary Fig. 7). Cabozantinib prevented the tumor progression in the cKIT-N822K mutant-transfected BaF3 cell allograft model, and even 25 mg/kg/day dosage achieved a TGI of 63.8% (Fig. 4D), while imatinib and sunitinib had no effect on tumor growth (Fig. 4D, Supplementary Fig. 8).

As expected, cabozantinib reduced phosphorylation of cKIT in

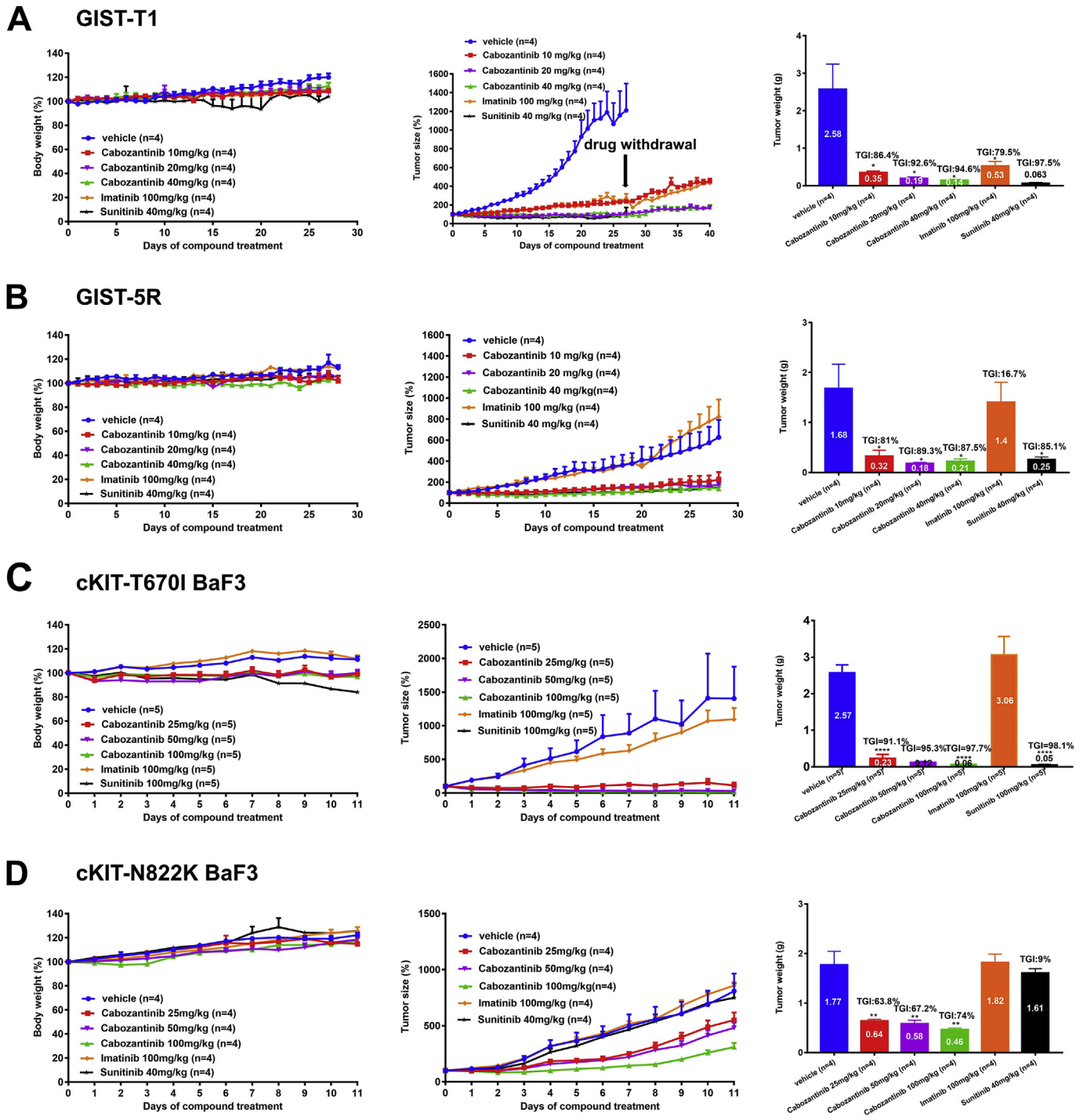

Fig. 4. Effect of cabozantinib in GIST-T1 and GIST-5R xenograft mouse models and in BaF3 cKIT-T670I and BaF3 cKIT-N822K allograft mouse models in vivo. Effect of cabozantinib on body weight (%), tumor size (%) and tumor weight (g) in various mouse models. A. GIST-T1 xenograft mouse model. B. GIST-5R xenograft mouse model. C. BaF3 cKIT-T670I allograft mouse model. D. BaF3 cKIT-N822K allograft mouse model. Data are shown for at least 4 mice in each group. Tumor-bearing animals were treated once daily by oral gavage with vehicle or the indicated dose of the drugs for the indicated dosing period. Mean tumor volume and SEM are plotted. Statistical significance was calculated using T-test to compare each treatment group to the corresponding vehicle control; significance is indicated by asterisks: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

tumors compared to that in the vehicle-treated controls. The results of immunohistochemical (IHC) staining showed that cabozantinib inhibited cell proliferation (Ki-67 staining) and induced apoptosis (TUNEL staining) in a dose-dependent manner. These results confirmed that the anti-tumor efficacy of cabozantinib was due to inhibition of the cKIT kinase activity (Supplementary Figs. 5–8).

3.5. Effects in human GIST patient-derived primary cells

We tested the effects of cabozantinib in a more pathologically-relevant setting of ex vivo culture of primary cells derived from 3 GIST patients (Supplementary Table 4) that expressed cKIT wt and the cKIT-K642E and cKIT-V559D mutations. Immunostaining confirmed that these cells express high levels of cKIT kinase after ex vivo culture

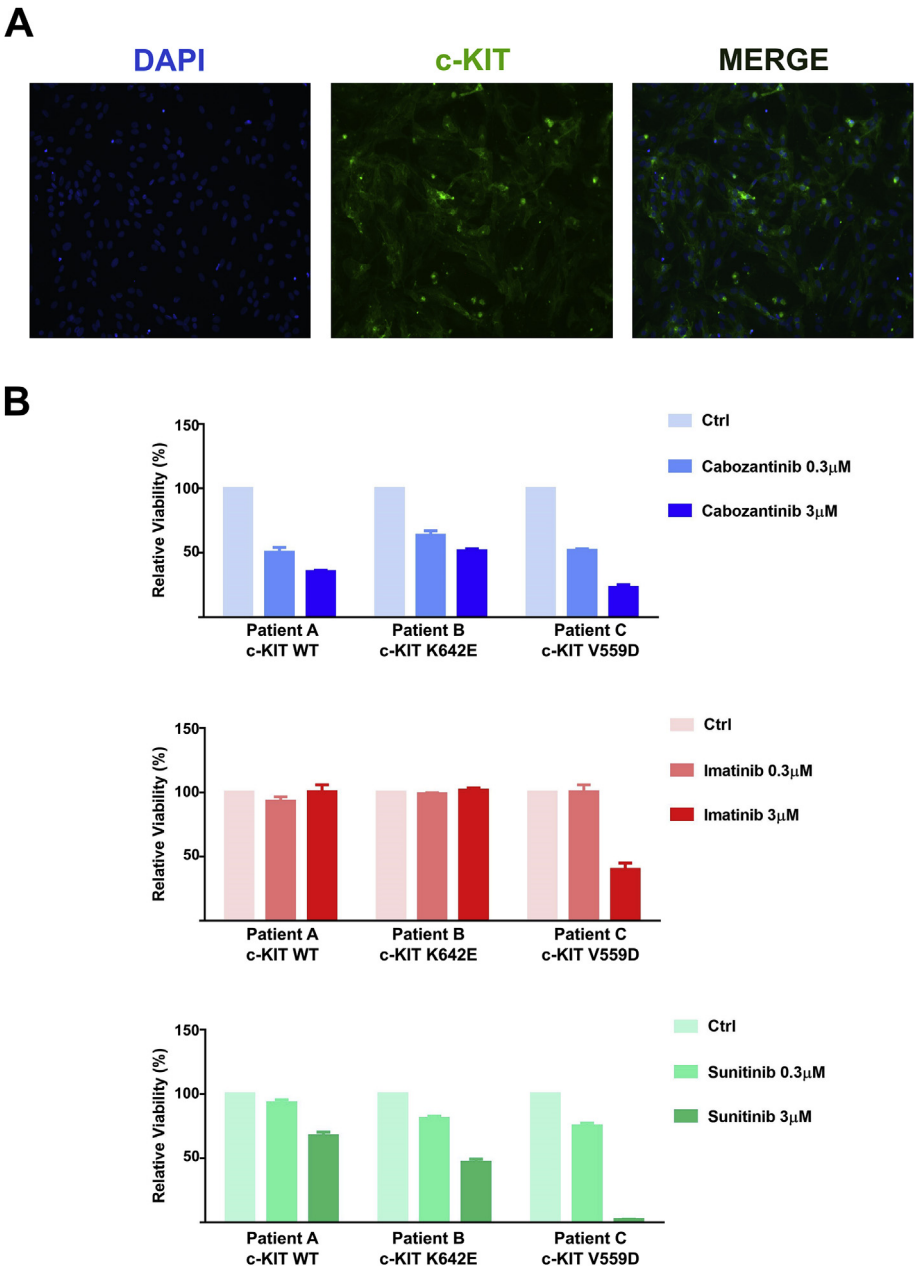

Fig. 5. Effect of cabozantinib, imatinib and sunitinib on GIST patient-derived primary cells.
A. Immunofluorescence staining for cKIT (scale bar: 50 μm) in GIST patient-derived primary cells. B. Cell activity of primary cells derived from 3 GIST patients relative to the DMSO-treated cells after 6 days of treatment with cabozantinib, imatinib and sunitinib at various concentrations determined using the Cell Titer-Glo assay. Data are shown as the mean ± SEM.

(Fig. 5A). The results of the proliferation assay demonstrated that cabozantinib dose-dependently inhibited the growth of all three patient samples at concentrations of 0.3 μM and higher (Fig. 5B). Interestingly, imatinib showed inhibitory activity only against the cKIT V559D sample but not the K642E mutant even at 3 μM; these data did not correlate with the results obtained in the established cell lines. Sunitinib exhibited a good dose-dependent inhibitory effect against all three patient samples.

4. Discussion

Targeted therapies against cancer have achieved great success in the past decade [30,31]. However, long-term clinical use and the progress in the next generation sequencing technology resulted in discovery of novel primary and secondary drug-resistant mutations [32,33]. Currently, it is unrealistic to develop a new drug for every new drug-resistant mutant considering the cost and time; however, in the precision medicine era, it is ideal to develop a specific drug for each specific gene mutation to enhance safety and efficacy. Drug repurposing strategy has been well recognized as a cost- and time-efficient approach for combating human diseases compared to the de novo development of new drugs [34,35]. Although three target therapies including imatinib, sunitinib and regorafenib have been approved clinically, all these drugs face primary and secondary drug resistances, toxicity and poor clinical responses [19]. Several new binding mechanism-based cKIT inhibitors such as avapritinib [36,37] and DCC-2618 [38] are under clinical investigation. More new pharmacophore-based cKIT inhibitors are still in preclinical development such as AZD3229 [39], CHMFL-KIT-110/8140 [24,40], etc. However, before completion of the full clinical evaluation, these new inhibitors cannot be used to fulfill the urgent clinical

demands. Cabozantinib, a multiple kinase inhibitor approved for treatment of RCC and MTC, showed different mutation sensitivity spectrum in comparison to the current therapies. More importantly, it not only exhibited better efficacy to the cKIT wt, but also showed great inhibitory effects to primary gain-of-function mutations, a variety of secondary drug resistance mutations in the ATP binding pocket (T670I and V654A imatinib resistant mutants) and the activation loops (imatinib resistant mutants including D820 E/G/Y, D816E, N822K, A829P, sunitinib resistant mutants N822K and A829P).

Interestingly, although cabozantinib, sunitinib and regorafenib are able to overcome the resistance of most activation loops mutants, none of these drugs were effective against the D816 H/V mutants. It has been reported that ponatinib was a potent inhibitor of the D816 mutant [19]. Since this mutation is outside of the ATP binding pocket and the activation loop is flexible, this effect is difficult to explain from the rational drug design point of view because imatinib, cabozantinib, regorafenib and ponatinib are all type II inhibitors that stabilize the inactive conformation of the cKIT kinase. Although sunitinib is a type I kinase inhibitor, but it has been reported to prefer the DFG-out conformation of the cKIT kinase like most of the type II kinase inhibitors [10]. Moreover, this also indicated that in comparison to other activation loop mutants like D820 and N822K, D816 residue mutant must bear different conformations which makes it disfavor most of the type II kinase inhibitors. However, determination of the detailed mechanism will require additional structural biology studies.

In the patient-derived primary cells, imatinib was not effective against the cKIT primary K642E mutant; this finding is not consistent with the results obtained in established cell lines. However, cabozantinib and sunitinib exhibited the expected responses. In addition, the patient's primary cells with cKIT V559D mutant did not respond to imatinib and cabozantinib. These may be due to heterogeneity of the genetic background of the primary cells. Some patients may harbor multiple mutants/signaling pathway dysregulation thus illustrate primary drug resistance [41]. Again, this further provides the basis for seeking new therapies bearing different mutants-sensitive profiles to provide additional clinical options.

Given the fact that cabozantinib has been clinically used and its safety profile and human PK profiles have been well documented, the findings in this report that it also displayed good inhibitory activity against the cKIT wt/mutants in the preclinical models and different sensitivity profile against the primary and secondary drug resistant mutants spectrum from the current clinically used therapies, it may have a great potential as a supplementary therapy in the anti-GIST battle.

Author contributions

Conception and design: Q. Liu, J. Liu; development of methodology: T. Lu, C. Chen, Z. Jiang, A. Wang, Z. Qi, Z. Hu, C. Hu; acquisition of data: T. Lu, C. Chen, Z. Jiang, A. Wang, Z. Qi, Z. Hu, C. Hu, F. Liu, W. Wang, H. Wu, B. Wang, L. Wang, S. Qi, J. Wu, W. Wang, J. Tang, H. Yan; writing, review, and/or revision of the manuscript: M. Bai, Q. Liu, J. Liu; administrative, technical, or material support: J. Tang, H. Yan, M. Bai; study supervision: Q. Liu, J. Liu.

Conflicts of interest

The authors declare no competing financial interest.

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Abbreviations

GIST	gastrointestinal stromal tumors
cKIT	stem cell factor receptor
PI3K	phosphatidylinositol 3-kinase
STAT	signal transducer and activator of transcription
RCC	renal cell carcinoma
MTC	medullary thyroid cancer
Wt	wild-type
DMSO	dimethyl sulfoxide
Tris	tris (hydroxymethyl) aminomethane
PMSF	phenylmethanesulfonyl fluoride
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
PI	propidium iodide
DAPI	4′,6-diamidino-2-phenylindole

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.024.

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