KRAS Secondary Mutations That Confer Acquired Resistance to KRAS G12C Inhibitors, Sotorasib and Adagrasib, and Overcoming Strategies: Insights From In Vitro Experiments

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ABSTRACT

Introduction: KRAS mutations have been recognized as undruggable for many years. Recently, novel KRAS G12C inhibitors, such as sotorasib and adagrasib, are being developed in clinical trials and have revealed promising results in metastatic NSCLC. Nevertheless, it is strongly anticipated that acquired resistance will limit their clinical use. In this study, we developed in vitro models of the KRAS G12C cancer, derived from resistant clones against sotorasib and adagrasib, and searched for secondary KRAS mutations as on-target resistance mechanisms to develop possible strategies to overcome such resistance.

Methods: We chronically exposed Ba/F3 cells transduced with KRASG12C to sotorasib or adagrasib in the presence of N-ethyl-N-nitrosourea and searched for secondary KRAS mutations. Strategies to overcome resistance were also investigated.

Results: We generated 142 Ba/F3 clones resistant to either sotorasib or adagrasib, of which 124 (87%) harbored secondary KRAS mutations. There were 12 different secondary KRAS mutations. Y96D and Y96S were resistant to both inhibitors. A combination of novel SOS1 inhibitor, BI-3406, and trametinib had potent activity against this resistance. Although G13D, R68M, A59S and A59T, which were highly resistant to sotorasib, remained sensitive to adagrasib, Q99L was resistant to adagrasib but sensitive to sotorasib.

Conclusions: We identified many secondary KRAS mutations causing resistance to sotorasib, adagrasib, or both, in vitro. The differential activities of these two inhibitors depending on the secondary mutations suggest sequential use in some cases. In addition, switching to BI-3406 plus trametinib might be a useful strategy to overcome acquired resistance owing to the secondary Y96D and Y96S mutations.

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Introduction

Patients with metastatic NSCLC harboring driver gene mutations and fusions occurring either in the EGFR, ALK, ROS1, BRAF, NTRK, RET, or MET gene receive respective kinase inhibitors as a standard of care for the first-line treatment, most of which are already approved in the United States, Japan, and many other countries. Furthermore, targeted therapies against HER2, NRG1, and EGFR exon 20 insertion are actively being developed.

Among driver oncogenes in NSCLC, the KRAS gene mutation is the oldest one that was identified, in 1982. The KRAS protein acts as a molecular switch in the growth factor signaling pathway. KRAS toggles between its guanosine diphosphate (GDP)-bound inactive conformation and guanosine triphosphate (GTP)-bound active one. When KRAS receives an upstream signal from receptor tyrosine kinases by means of guanine nucleotide-exchange factors, such as SOS1, GDP of inactive KRAS is exchanged with GTP, resulting in an active form of GTP-bound KRAS that activates downstream pathways. GTP-bound KRAS returns to GDP-bound inactive KRAS through its intrinsic guanosine triphosphatase (GTPase) activity. GTPase activating proteins (such as RAS-GAPs and NF1) enhance the GTPase activity by several orders of magnitude.

Mutations occurring at G12, G13, or Q61 impair GTPase activity and affect the guanine exchange rate of KRAS in some cases, resulting in accumulation of GTP-bound KRAS and persistent activation of downstream signals. KRAS mutations are present in approximately 15-30% of patients with NSCLC, respectively. According to the cBioPortal database (searched on March 15, 2020), most of the KRAS mutations in NSCLC occur at codon 12 and approximately half of them are glycine-to-cysteine (G12C) substitution (Fig. 1A). In contrast, G12C is relatively rare in colon or pancreatic cancer.

Despite its high frequency, the development of targeted therapy against KRAS-mutated cancer has long been unsatisfactory. Recently, however, several KRASG12C inhibitors have been developed and early clinical trial results are promising. KRASG12C inhibitors are designed to bind covalently to cysteine at codon 12 to occupy the cryptic pocket near the switch II region of GDP-bound inactive form of KRAS to lock KRAS in its GDP-bound inactive form. Among several KRASG12C inhibitors, sotorasib and adagrasib have already revealed promising results in early phase clinical trials for patients with NSCLC. In a phase 1/2 study of previously treated patients with locally advanced or metastatic NSCLC carrying the KRAS G12C mutation (CodeBreak-100), sotorasib achieved an objective response rate (ORR) of 32.2% (19 of 59) and median progression-free survival of 6.3 months in a phase 1 cohort and an ORR of 37.1% (46 of 124) and median progression-free survival of 6.8 months in a phase 2 cohort, respectively. Adagrasib also revealed an ORR of 45% (23 of 51) and a disease control rate of 96% (49 of 51) in an NSCLC cohort in a phase 1/2 clinical trial for patients with solid tumors harboring KRAS G12C mutation.

Acquired resistance is inevitable in targeted therapies for NSCLC, despite an initial dramatic response. Acquisition of secondary mutations of the target gene (off-target mechanisms), activation of bypass and accessory pathway (off-targeted mechanisms), and histologic transformation are known as the three major molecular mechanisms. For KRASG12C inhibitors, in preclinical studies with NSCLC cell lines, intrinsic and acquired resistance was reported owing to resynthesis of KRAS that immediately associates with GTP and an epithelial-to-mesenchymal transition.

We hypothesized that secondary mutations in the KRAS gene would confer acquired resistance to KRASG12C inhibitors as in the case of many other targeted therapies for NSCLC. Therefore, we created an in vitro model using a Ba/F3 cells transduced with mutated KRAS, derived resistant cell lines to sotorasib or adagrasib, and searched for secondary KRAS mutations. We also aimed to find possible strategies to overcome these types of resistances.

Material and Methods

Cell Lines and Reagents

NSCLC cell lines expressing KRAS G12 mutations (National Cancer Institute [NCI]-H358 [G12C], NCI-H23 [G12C], NCI-H2122 [G12C], NCI-H2009 [G12A], and NCI-H441 [G12V] cells) were kindly provided by the late Dr. Adi F. Gazdar. A549 (G12S) and SK-LU1 (G12D) cells were kind gifts by the late Dr. Hikotaka Osada. The murine pro-B cell line Ba/F3 was obtained from the RIKEN Bio Resource Center (Tsukuba, Japan).

Cells except for SK-LU1 cells were cultured in Roswell Park Memorial Institute 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 1% penicillin and streptomycin (PS, Wako) at 37°C with 5% CO2. SK-LU1 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) with 10% FBS and 1% PS. We maintained parental Ba/F3 cells in the presence of murine interleukin-3 (IL-3) under sterile conditions. Mycoplasma contamination was checked using the
TaKaRa polymerase chain reaction mycoplasma detection set (Takara, Kusatsu, Japan).

The KRAS<sup>G12C</sup> inhibitors, sotorasib and adagrasib, were purchased from MedChemExpress (Monmouth Junction, NJ). BI-3406, a SOS1 inhibitor, was provided by Boehringer Ingelheim (Ingelheim, Germany). TNO155 was purchased from Selleck Chemicals (Houston, TX). The drugs were dissolved in DMSO (Sigma-Aldrich) at 10 mM and stored at −80°C.

**Introduction of KRAS Mutations Into Ba/F3 and H358 Cells**

Introduction of G12C, G12D, and G12V mutations into Ba/F3 or H358 cells was conducted using a retrovirus system as previously reported. These three mutations account for approximately 80% of KRAS mutations in NSCLC (Fig. 1A). Briefly, we introduced each KRAS mutation using a Prime STAR mutagenesis basal kit (Takara) with designed primers into the pBABE-puro-
KRas construct (Addgene, Cambridge, MA; Supplementary Table 1). Retroviral particles were generated by co-transfection of each pBabe-puro-KRAS construct and pSV-S-G vector (Clontech, Fremont, CA) into gp-IRESC293 cells with FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Viral particles were concentrated using a retrovirus concentration kit (Clontech). Ba/F3 (3 x 10^5) or H358 cells (1 x 10^5) were transfected with each retrovirus and cultured at 37°C for a few days. The transfected Ba/F3 or H358 cells were selected with 0.8 to 1.0 μg/mL or 2.0 μg/mL of puromycin, respectively. After puromycin selection, the presence of each KRAS mutation was confirmed as follows: total RNA was extracted from cells using a mirVana microRNA isolation kit (Qiagen, Hilden, Germany), and cDNA was generated by reverse transcription using ReverTra Ace (TOYOBO, Osaka, Japan). The KRAS coding sequence was amplified by polymerase chain reaction with the designed primer sets (Supplementary Table 2). The KRAS nucleotide sequence was checked by Sanger sequencing using the 3130 or 3500XL Genetic Analyzer (Applied Biosystems, Waltham, MA).

**Cell Growth Assay and Growth Inhibition Assay**

Ba/F3 cells (3 x 10^4) expressing one of the KRAS mutations were plated in six-well plates and cultured in the absence of IL-3. Nontransfected Ba/F3 cells were cultured with or without IL-3 as a control. The number of cells was counted in triplicate every 24 hours until 96 hours using a OneCell Counter (Biomedical Medical Science, Tokyo, Japan).

In the two-dimensional (2D) growth inhibition assay, 5 x 10^3 cells were cultured in 96-well plates for 24 hours and treated with reagents at 10 different concentrations for 72 hours. Cell viability assays were performed using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). We measured the amount of formazan dye, which reflects cell viability, by reading the absorbance at 450 nm using a multiplate reader (Tecan, Mannedorf, Switzerland). In the three-dimensional (3D) growth inhibition assay, 5 x 10^3 cells were embedded in growth factor-reduced Matrigel (Corning, NY) and cultured in Roswell Park Memorial Institute 1640 medium with 10% FBS and PS in 96-well plates. After 72 hours of incubation, cells were treated with the indicated concentrations for 72 hours, and a cell viability assay was performed as described previously. Half maximal (50%) inhibitory concentration (IC_{50}) values were determined by a nonlinear regression curve fit using a variable slope model with normalized response in GraphPad Prism version 8 (GraphPad Software, San Diego, CA).

**Establishment of Clones Resistant to KRAS G12C Inhibitors**

We performed N-ethyl-N-nitrosourea (ENU, Sigma-Aldrich) mutagenesis to generate clones resistant to sotorasib and adagrasib, as previously reported.28,29 A total of 1 x 10^6 Ba/F3 cells harboring KRAS G12C were exposed to 100 μg/mL ENU for 24 hours. After washing with PBS, cells were cultured for 48 hours and plated in 96-well plates with the indicated concentrations of KRAS_{G12C} inhibitors. We treated the cells for 14 to 21 days, and medium and drug were changed every third to fifth day. Clones resistant to KRAS_{G12C} inhibitors were generated in the course of the treatment.

In addition, we exposed the H358 cells to increasing concentrations of sotorasib (up to 10 μM) or adagrasib (up to 1 μM) to obtain resistant clones. After the establishment of resistant clones, secondary KRAS mutations were sought in a similar fashion.

**Immunoblotting**

Preparation of the cell lysates and immunoblotting were conducted in a standard manner. After treatment with the indicated concentrations of KRAS_{G12C} inhibitors or DMSO, cell pellets were dissolved in lysis buffer. We applied a total of 20 to 30 μg protein to each well of the 5% to 20% acrylamide gel and electrophoresed. Separated proteins were transferred to membranes using a Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA). After blocking with Blocking Buffer (TaKaRa), the membranes were probed with antibodies against the following proteins overnight at 4°C: phospho (p)-p44/42 MAPK (Thr202/Tyr204, #9101s, Cell Signaling Technologies [CST], Danvers, MA), p44/42 MAPK (#9128s, CST), pMEK1 (S298, #9146s, CST), MEK1 (#9102s, CST), p44/42 MAPK (Thr202/Tyr204, #9101s, Cell Signaling Technologies [CST], Danvers, MA), p44/42 MAPK (#9128s, CST), pMEK1 (S298, #9146s, CST), MEK1 (#9101s, Cell Signaling Technologies [CST], Danvers, MA), p44/42 MAPK (#9101s, CST), pMEK1 (S298, #9146s, CST), MEK1 (#9102s, CST), p56 (S235/236, #4858s, CST), S6 (#2217, CST), KRAS (Y53270s, CST), and β-actin (#4970s, CST). As a secondary antibody, horseradish peroxidase-conjugated antirabbit immunoglobulin G (Y7040, CST) was incubated with the target protein and primary antibody complex for two hours. For chemiluminescence assays, ECL solution (GE Healthcare, Chicago, IL) was added to the membrane and scanned in an Amersham Imager 680 (GE Healthcare) to detect the expression of the target protein.

**Results**

**Growth-Inhibitory Effects of Sotorasib and Adagrasib in Cells With KRAS Mutations**

First, we evaluated the growth-inhibitory activities of sotorasib and adagrasib, in seven NSCLC cell lines carrying KRAS G12 mutations (three G12C and four non-G12C) (Fig. 1B). All non-G12C cell lines were resistant as expected. In KRAS G12 C cells, although H358 and
H2122 were sensitive, H23 cells were resistant to sotorasib. This discrepancy was probably owing to the difference of KRAS dependence of these cell lines, that is, H358 and H2122 are classified as KRAS dependent with an epithelial phenotype, whereas H23 is classified as KRAS independent with a mesenchymal phenotype.30,31 The sensitivity of H23 cells to adagrasib may be attributable to the off-target effect of adagrasib.

In Ba/F3 cell lines driven by specific KRAS G12 mutant variants, the experimental results were clearer. Although Ba/F3 cells expressing either KRAS G12C, G12V, or G12D lost their IL-3-dependence for their growth, indicating the oncogenic activities of all these three KRAS mutations (Fig. 1C), only Ba/F3 cells expressing KRAS G12C were sensitive to sotorasib and adagrasib with IC50 values of 12.4 nM and 1.3 nM, respectively (Fig. 1D and Supplementary Table 3). Downstream signaling as analyzed by ERK1/2 and S6 phosphorylation was reduced in Ba/F3 cells with KRAS G12C but not those with G12D on treatment with either 100 nM sotorasib or adagrasib (Fig. 1E).

**Exploration for Secondary KRAS Mutations That Caused Resistance to Sotorasib or Adagrasib**

To identify secondary mutations that would confer acquired resistance to sotorasib or adagrasib, we conducted ENU mutagenesis screening (Fig. 2A). The minimum concentrations of sotorasib (100 nM) and adagrasib (20 nM) were determined as the lowest concentrations that suppressed the growth of parental G12C Ba/F3 cells in long-term (>2 wk) exposure. The maximum concentrations of sotorasib (2000 nM) and adagrasib (1000 nM) were determined to exceed greater than 100 times of IC50 of each drug.

We generated a total of 142 resistant clones. Among these clones, secondary KRAS mutations were identified in 124 of 142 (87.3%) (Fig. 2B, Supplementary Fig. 1). In the clones treated with sotorasib, 52 of 68 resistant clones harbored secondary KRAS mutations. A59T (n = 6), R68M (n = 3), and Y96D (n = 1) mutations were common after treatment with high concentrations (>1000 nM), whereas G13D (n = 12), A59S (n = 11), R68M (n = 8), and Q61L (n = 5) mutations were common after treatment with lower concentrations (Fig. 2B). After adagrasib treatment, 72 of 74 resistant clones harbored secondary KRAS mutations. Y96D mutation was the sole secondary mutation in cells established with high concentrations of adagrasib (>200 nM; n = 11), whereas Q99L (n = 38), R68S (n = 10), V8E (n = 5), M72I (n = 5), and A59S (n = 3) were detected in cells treated with lower concentrations. A59S and Y96D were the only mutations that were shared between cells

![Figure 2](image_url)
treated with sotorasib and adagrasib, whereas others were unique to each drug (Fig. 2B). R68 was the other shared site of the secondary mutation; however, R was substituted for M and S on sotorasib and adagrasib treatment, respectively.

We also established two sotorasib-resistant H358 clones and one adagrasib-resistant H358 clone (H358-SR1, -SR2, and -AR) by treatment with increasing concentrations of the drug without ENU (Supplementary Fig. 2). Nevertheless, no secondary mutation was identified in exons 2 to 3 of the KRAS gene in these cell lines.

**Cross Resistance of Secondary Mutations Developed Against Sotorasib or Adagrasib**

To evaluate cross resistance, we conducted growth inhibition assays with sotorasib (Fig. 3A) and adagrasib (Fig. 3B) for resistant clones generated by the ENU mutagenesis. Because the IC50 values of sotorasib and adagrasib for KRAS G12C parental Ba/F3 cells were different (12.4 nM and 1.3 nM, respectively), we used the resistance index (RI) to clearly compare the degree of resistance of each secondary mutation. RI was defined as a ratio of the IC50 of each drug for each resistant clone (Supplementary Table 4) to the respective IC50 of each drug for the parental Ba/F3 cells with KRAS G12C.

For sotorasib, G13D, A59S, A59T, R68M, Y96D and Y96S were highly resistant (RI > 100), whereas Y96D, Y96S and Q99L were highly resistant for adagrasib (Fig. 3C). Thus, secondary Y96D and Y96S mutations would cause cross resistance to both sotorasib and adagrasib. Nevertheless, several secondary mutations had differential sensitivities to sotorasib and adagrasib. V8E, G13D, A59S, A59T, and R68M, which were resistant to sotorasib, remained sensitive to adagrasib, whereas Q99L, which was resistant to adagrasib, was sensitive to sotorasib.

**Validation of KRAS Secondary Mutations That Were Detected in Sotorasib- or Adagrasib-Resistant Cells**

To confirm that the observed resistance was due to the emergence of the secondary KRAS mutations and not owing to other unidentified mechanisms, we introduced KRAS G12C plus either of the seven secondary mutations (G13D, A59S, A59T, R68M, Y96D, Y96S, or Q99L) with RI greater than 100 (Fig. 3C) into the Ba/F3 cells. All Ba/F3

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**Figure 3.** Growth inhibition analysis of sotorasib- or adagrasib-resistant clones generated through ENU mutagenesis using KRAS G12C Ba/F3 cells. (A) Growth inhibition curves of Ba/F3 cells harboring KRAS G12C plus secondary mutations derived through ENU mutagenesis and treated with the indicated concentrations of sotorasib for 72 hours. The results are revealed as the mean values of three individual experiments. Error bars indicate the SD. (B) Growth inhibition curves of Ba/F3 cells harboring KRAS G12C plus secondary mutations derived through ENU mutagenesis and treated with the indicated concentrations of adagrasib for 72 hours. (C) Summary of the RI values of sotorasib- and adagrasib-resistant clones generated through ENU mutagenesis. The RI value was calculated with the following formula: IC50 of each resistant clone/IC50 of parental Ba/F3 cell with KRAS G12C. Green indicates low resistance, corresponding to an RI value less than 10. Yellow indicates moderate resistance, corresponding to an RI value higher than 10 but lower than 100. Red indicates high resistance, corresponding to an RI value higher than 100. ENU, N-ethyl-N-nitrosourea; IC50, half maximal (50%) inhibitory concentration; RI, resistance index.
cells harboring these mutations proliferated independently of IL-3 (Supplementary Fig. 3).

We then conducted growth inhibition assays using these Ba/F3 (Fig. 4A and B). The sensitivity of each of these reconstructed Ba/F3 cells to KRAS<sup>G12C</sup> inhibitors was comparable to that of clones established with ENU mutagenesis (Fig. 4C and Supplementary Tables 4 and 5). Western blotting revealed that the levels of phosphorylation of ERK and S6 in cells carrying the G12C plus Y96D and G12C plus Y96S mutations were not suppressed by either sotorasib or adagrasib treatment (Fig. 4D), whereas carrying the G12C plus G13D and A59S were suppressed by adagrasib, and those carrying the G12C plus Q99L were suppressed by sotorasib (Supplementary Fig. 4).

**Figure 4.** Generation of Ba/F3 cells harboring KRAS<sup>G12C</sup> plus secondary mutations that caused high resistance to sotorasib or adagrasib generated by the ENU mutagenesis screening. (A, B) Growth inhibition assay of Ba/F3 cells harboring KRAS<sup>G12C</sup> plus secondary mutations and treated with the indicated concentrations of sotorasib (A) or adagrasib (B) for 72 hours. The results are revealed as the mean values of three individual experiments. Error bars indicate the SD. (C) Summary of the RI values of Ba/F3 cells harboring KRAS<sup>G12C</sup> plus secondary mutations. The RI value was calculated with the following formula: IC<sub>50</sub> of each reconstructed Ba/F3 cell/IC<sub>50</sub> of parental Ba/F3 cells with KRAS<sup>G12C</sup>. Green indicates low resistance, corresponding to an RI value less than 10. Yellow indicates moderate resistance, corresponding to an RI value higher than 10 but lower than 100. Red indicates high resistance, corresponding to an RI value higher than 100. (D) Western blotting of Ba/F3 cells harboring G12C plus Y96D and Ba/F3 cells harboring G12C plus Y96S treated with DMSO, 10<sup>–</sup>100 nM sotorasib or adagrasib for 6 hours. (E, F) Growth inhibition assay of H358 cells with KRAS<sup>G12C</sup> plus secondary mutations treated with the indicated concentrations of sotorasib (E) or adagrasib (F) for 72 hours. The results are revealed as the mean values of three individual experiments. Error bars indicate the SD. IC<sub>50</sub>, half maximal (50%) inhibitory concentration; pERK, phospho-ERK; pS6, phospho-S6; RI, resistance index; tERK, total ERK; tS6, total S6.
We also retrovirally introduced KRAS G12C plus Y96D, G12C plus Y96S, and G12C plus A59S into the NCI-H358 cells that originally harbored KRAS G12C mutation. In the growth inhibition assay, H358 cells harboring G12C plus Y96D or G12C plus Y96S had approximately 30 times higher IC50 values compared with parental H358 cells (Fig. 4E and F and Supplementary Table 6). H358 cells harboring G12C plus A59S were less resistant to both KRASG12C inhibitors than H358 cells harboring G12C plus Y96D or G12C plus Y96S, consistent with the Ba/F3 cell experiments.

Strategies to Overcome Resistance to KRAS G12C Inhibitors by Secondary Mutations

As described previously, some of the resistant mutations, such as G13D, A59S, A59T, R68M, and Q99L, could be overcome by switching from sotorasib to adagrasib or vice versa. The most problematic secondary mutations may be Y96D and Y96S mutations that emerged after treatment with high concentrations of sotorasib or adagrasib, and thus they were highly resistant to both agents (Fig. 4C).

We then asked whether BI-3406, a novel SOS1 inhibitor, or TNO 155, a novel SHP2 inhibitor, could overcome resistance. Treatment of Ba/F3 cells harboring G12C plus either Y96D or Y96S with BI-3406 resulted in IC50 of 38.0 nM and 25.7 nM, respectively, compared with 3326 nM and 1465 nM with sotorasib alone (Fig. 5A and Supplementary Table 7). After 72 hours of treatment with BI-3406, the pERK level, but not pS6 level, was suppressed by Western blotting (Fig. 5B). On the other hand, IC50 values of TNO 155 in Ba/F3 cells harboring G12C plus secondary mutations were higher than 1000 nM (Supplementary Fig. 5A, Supplementary Table 7). In G12C plus A59S mutant Ba/F3 cells, neither BI-3406 nor TNO155 was able to inhibit cell proliferation (IC50 of 4850 nM and 3050 nM, respectively) (Fig. 5A and Supplementary Fig. 5A and Supplementary Table 7). As synergism of KRASG12C inhibitor plus either an SOS1 inhibitor or an SHP2 inhibitor was reported previously, we evaluated combination of KRASG12C inhibitors plus BI-3406 or TNO 155 in Ba/F3 carrying the KRAS G12C plus Y96D and G12C plus Y96S. As revealed in Supplementary Fig. 5B to E and Supplementary Table 8, we observed that addition of BI-3406 or TNO

Figure 5. BI-3406, a novel SOS1 inhibitor, plus trametinib revealed activity against an in vitro model with concurrent G12C and secondary Y96D or Y96S mutations. (A) Growth inhibition assay of Ba/F3 cells harboring KRAS G12C plus secondary A59S, Y96D, or Y96S mutation treated with the indicated concentrations of BI-3406 for 72 hours. The results are revealed as the mean values of three individual experiments. Error bars indicate the SD. (B) Western blotting of Ba/F3 cells harboring KRAS G12C plus Y96D and G12C plus Y96S mutations treated with DMSO or 10-1000 nM BI-3406 for 6 hours. (C, D) 3D growth inhibition assay of H358 cells with KRAS G12C plus secondary A59S, Y96D, or Y96S mutation treated with the indicated concentrations of BI-3406 (C) or trametinib plus 1 µM BI-3406 (D) for 72 hours. 3D, three-dimensional; pERK, phospho-ERK; pS6, phospho-S6; tERK, total ERK; tS6, total S6.
155 did not restore the sensitivities to KRAS\textsuperscript{G12C} inhibitor-acquired resistance Ba/F3 models of KRAS G12C with Y96D or G12C with Y96S secondary mutation at the 72-hour time point using 10% FBS in medium.

We also performed 2D- and 3D-growth inhibition assays using H358 cells expressing G12C plus Y96D or G12C plus Y96S. In both 2D and 3D assays, BI-3406 monotherapy modestly inhibited growth of H358 parental cells consistent with a previous report\textsuperscript{26} and the growth of H358 cells with G12C plus Y96D or G12C plus Y96S (Fig. 5\textit{C} and Supplementary Fig. 6 and Supplementary Table 9). Nevertheless, H358 cells harboring G12C plus Y96D or G12C plus Y96S were sensitive to BI-3406 plus trametinib combination, and their sensitivities were comparable with parental H358 cells (Fig. 5\textit{D} and Supplementary Table 10).

**Discussion**

In this study, we searched for secondary KRAS mutations that would confer resistance to the KRAS G12C inhibitors, sotorasib or adagrasib, using Ba/F3 models subjected to ENU mutagenesis. This method was efficient in generating resistant clones with secondary mutations, although it is artificial and there is a preference for G:C to A:T or A:T to G:C transitions and A:T to T:A transitions,\textsuperscript{36} and this was the case in our experiment (Supplementary Fig. 7). There is a concern as to whether mutations identified in this way are clinically relevant. Nevertheless, the secondary mutations identified in this assay are consistent with the acquired resistance mutations found in clinical setting after treatment failures of EGFR-, ALK-, and MET-tyrosine kinase inhibitors.\textsuperscript{28,29,37} Recently, Tanaka et al.\textsuperscript{38} reported that an NSCLC patient with KRAS G12C who developed acquired resistance to adagrasib harbored 10 mutations in KRAS, NRAS, BRAF and MAP2K1. Of three KRAS secondary mutations (G12V, G13D, and Y96D) described in their report,\textsuperscript{38} we identified two (G13D and Y96D) in this study. Furthermore, Awad et al.\textsuperscript{39} have recently reported 11 secondary KRAS mutations (G12R, G12V, G12W, G13D, Q61H, R68S, H95D, H95Q, H95R, and Y96C) together with mutations in other MAP kinase pathway genes in 12 patients with NSCLC or colorectal cancer harboring KRAS G12C treated with adagrasib. Of these mutations, we identified G13D and R68S. We also found secondary mutations occurring at Q61 and Y96. In addition, the fact that recurrent KRAS G13D, A59S, A59T, Q61L, and R68S mutations in various types of human cancer are present in the COSMIC database (https://cancer.sanger.ac.uk/cosmic) further substantiates the validity and clinical and biological relevance of our experiments.

We identified various secondary mutations of the KRAS gene. The sites of the secondary mutations were dependent on drugs and their concentrations. Of 52 secondary mutations that conferred resistance to sotorasib, KRAS codons G13, A59, and R68 were common sites (>20%) for the secondary mutations, whereas of 72 resistant mutations for adagrasib, codons R68, Y96, and Q99 were common. In the case of adagrasib, Y96D was the only secondary mutation at higher concentrations of the drug (>200 nM) and was never detected at lower concentrations. Instead, Q99L was only detected at 50 or 100 nM of adagrasib. In the case of sotorasib, such preference depending on the concentration of the drug was less clear. Nevertheless, G13D was only detected at 200 nM of sotorasib.

A crystal model of GDP-KRAS\textsuperscript{G12C} binding with sotorasib (PDB 6OIM) or adagrasib (PDB 6UT0) revealed that G13, A59, Q61, R68, Y96, and Q99 face the drug-binding pocket (Fig. 6\textit{A}). Therefore, these mutations are anticipated to structurally impair covalent binding. During the development of sotorasib, structural improvements in the quinazoline scaffold to leverage the cryptic pocket formed by H95, Y96, and Q99 in the α3-helix of the GDP-KRAS G12C protein produced potent pharmaceutical properties.\textsuperscript{18} Pantsar\textsuperscript{40} recently reported that Y96 of KRAS\textsuperscript{G12C} forms π-π-stacking, a noncovalent intermolecular interaction between aromatic rings; in addition, R68 formed cation-π interactions, which are derived from an electrostatic interaction between a cation and adjacent electron-rich compounds, with the azaquinazoline base of sotorasib in a molecular dynamics simulation of the KRAS\textsuperscript{G12C}-sotorasib complex. In addition, we found that Y96 is located at the entrance of the hydrophobic pocket, where sotorasib and adagrasib bind with the KRAS G12C protein. This finding suggests that secondary mutations in Y96 might have similar effects to the so-called solvent front resistance mutations, such as the G1202R and D1203N in the ALK or the G2032R and D2033N in the ROS1 (Supplementary Fig. 8).\textsuperscript{41,42}

Detection of the secondary resistance mutations at A59 or Q61, which are known to disrupt intrinsic GTPase activity, is in line with the fact that intact GTPase activity is required for KRAS (G12C) inhibition by inactive state-selective drugs, such as sotorasib or adagrasib.\textsuperscript{43} In contrast, G13D is characterized by its more than 10-fold rapid GDP-GTP exchange kinetics compared with other KRAS mutations.\textsuperscript{44} Thus, G13D would result in strong reduction of GDP-bound inactive KRAS, which is necessary for G12C inhibitors to bind, leading to resistance.

As has been found, the spectrum of the secondary mutations was significantly different between sotorasib and adagrasib. For example, G13D, A59S, A59T, and R68M conferred resistance to sotorasib but remained sensitive to adagrasib. Alternately, Q99L secondary mutation was resistant to adagrasib but was sensitive to...
Nevertheless, Y96D and Y96S secondary mutations were shared with the two KRAS\textsuperscript{G12C} inhibitors as resistant mutations, necessitating other strategies for overcoming. We found that a novel SOS1 inhibitor, BI-3406, that inhibits SOS1 Y884-KRAS R73 interaction with or without trametinib had potent activity in this situation.\textsuperscript{26} We assume that the Y96D and Y96S secondary mutations did not affect BI-3406 activity against mutant KRAS proteins because Y96 is not a part of SOS1 interaction surface of the KRAS protein (Supplementary Fig. 9).\textsuperscript{45}

On the basis of these findings, we propose the choice of the second-line drug(s) depending on the secondary mutations that emerge on acquiring resistance (Fig. 6B).

Figure 6. Structural analysis of secondary KRAS mutations affecting the interaction between KRAS\textsuperscript{G12C} and sotorasib or adagrasib and treatment strategy to overcome the resistance owing to secondary KRAS mutations. (A) Amino acids affected by select KRAS-resistant secondary mutations are indicated in the crystal structure of KRAS\textsuperscript{G12C} covalently bound with sotorasib (PDB 6OIM) and adagrasib (PDB 6UTO). Sotorasib forms \(\pi-\pi\)-stacking with Y96 and a cation-\(\pi\) interaction with R68M. A59, Q61, R68, M72, and Q99 are also located around the drug-binding site. This figure was prepared using PyMOL. (B) Possible treatment strategies on the basis of our experiments to overcome the acquired resistance caused by secondary KRAS mutations. GDP, guanosine diphosphate.
KRAS\textsuperscript{G12C} inhibitors with pan-KRAS (e.g., SOS1 inhibitor and SHP2 inhibitor) or RTK inhibitors (cetuximab and afatinib) upstream and a MEK inhibitor downstream are being studied by several pharmaceutical companies in phase 1 clinical trials with the aim of delaying the appearance of resistance and achieving a durable response (NCT04185883, NCT04330664, and NCT03785249). This study reveals that resistance mediated by some secondary KRAS mutations might be overcome in a second-line treatment using a SOS1 inhibitor plus MEK inhibitor combination. We expect that our in vitro model will also help strategize the development of such newer options.

**CRediT Authorship Contribution Statement**


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**Supplementary Data**

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at https://doi.org/10.1016/j.jtho.2021.04.015.

**References**


