Efficacious Combination Drug Treatment for Colorectal Cancer That Overcomes Resistance to KRAS G12C Inhibitors

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ABSTRACT

Recent advances in combinatorial chemistry led to the discovery of inhibitors targeting the KRAS G12C-mutant protein. However, efficacy of its monotherapy on colorectal cancer is limited. Thus, effective combination drugs should be explored for applicable patients with colorectal cancer to fully benefit from the KRAS G12C inhibitor treatment. Here we used a patient-derived colorectal cancer stem cell (PD–CRC-SC) spheroid culture and showed that three-drug combination of inhibitors against KRAS G12C, EGFR, and FGFR synergistically suppressed the growth of colorectal cancer cells carrying the KRAS G12C mutation. Likewise, a combination of KRAS G12C and SHP2 inhibitors was also effective. Importantly, activation of the PI3K/AKT pathway in heregulin-responsive colorectal cancer cells canceled out the effect of KRAS G12C inhibition, which was largely overcome by PI3K inhibitors. These results reveal that evaluating efficacy of combination therapies with PD–CRC-SC spheroids can be a promising strategy to find the best regimen for patients with colorectal cancer.

Introduction

Colorectal cancer is one of the most common and lethal cancers in the world, accounting for approximately 1.8 million new cases and approximately 900,000 deaths per year (1). Although the prognosis of patients with colorectal cancer has steadily improved during past decades due to the development of cytotoxic and molecular-targeted drugs, the 5-year survival rate of stage IV patients still remains less than 20% (2).

Activating mutations in the *KRAS* gene are the key driver of most lethal cancers such as pancreatic (86%), colorectal (41%), and lung (32%) cancers (3). The mutant KRAS protein constitutively activates several downstream effector pathways, including the MAPK and PI3K pathways (4). Although direct inhibition of KRAS activity was a long-standing objective for more than 3 decades of research, no such targeted therapies were discovered. Thus, the KRAS mutants were thought "undruggable" due to their high affinity to GTP and lack of accessible drug-binding pockets (5). However, a paradigm of KRAS-targeted therapy has evolved through the development of allele-specific covalent inhibitors for KRAS G12C. These inhibitors bind to the mutant cysteine residue and occupy the switch II region that is present only in the inactive GDP-bound form of KRAS G12C, which prevents its reactivation through nucleotide exchange and traps the oncoprotein in the inactive state (6).

The natural occurrence of KRAS G12C mutation is approximately 13% in lung cancer and 3% in colorectal cancer (7). In recent years, several KRAS G12C inhibitors have entered clinical trials for the treatment of these cancers (8). Sotorasib (AMG-510), the first inhibitor in clinical development, showed promising antitumor activity in patients with KRAS G12C-mutant solid tumors in a phase I clinical trial (9). Adagrasib (MRTX849) also showed objective responses in patients with lung and colorectal adenocarcinomas (10). However, antitumor activity of sotorasib monotherapy is modest as reported previously for colorectal cancer (11). Furthermore, the response rates to these drugs in patients with colorectal cancer are lower than those in patients with lung cancer (9).

An adaptive response of cancer cells to KRAS G12C inhibitors was reported to limit the efficacy of treatment, in which the initial inhibition of oncoprotein signaling was followed by reaccumulation of active KRAS and reactivation of ERK signaling (12). These results suggest that compensatory activation of receptor tyrosine kinases (RTKs) and SOS1/2 is responsible for the adaptive resistance to KRAS G12C inhibitor treatment (13). To overcome this problem, several preclinical studies for combination therapy have been conducted. So that upstream or parallel signaling is blocked simultaneously, the following combinations are efficatious: an SHP2 inhibitor or a PI3K inhibitor with a KRAS G12C inhibitor for lung cancer and an EGFR inhibitor with a KRAS G12C inhibitor for colorectal cancer (8, 14).

Recently, patient-derived xenografts and organoids have emerged as promising tools for drug development and personalized diagnosis (15). We previously reported an efficient method for culturing patient-derived colorectal cancer "tumor-initiating" or stem cell (PD–CRC-SC) spheroids at an efficient rate (> 90%; refs. 16, 17). We also developed ectopic xenografts by injecting immunocompromised mice with these spheroids (patient-derived spheroid xenografts; PDSX), and demonstrated that the cancer chemosensitivity in PDSX mice precisely reflected that of each patient (18). Using these models, we recently showed that combination treatment with EGFR and FGFR inhibitors efficiently suppressed the growth of PD–CRC-SC carrying wild-type *RAS*/*RAF* genes, which led us to hypothesize that concomitant inhibition of EGFR and FGFR might be an

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effective strategy to overcome adaptive resistance to the KRAS G12C inhibitors (19).

In the current study, we showed that three KRAS G12C inhibitors, sotorasib (AMG-510), adagrasib, and ARS1620 significantly suppressed the growth of PD–CRC-SC spheroids that carried the KRAS G12C mutation. We also demonstrated that combination treatment of these spheroids with either EGFR/FGFR inhibitors or the SHP2 inhibitor resulted in more efficient suppression of cancer stem cell proliferation, likely by blocking the signaling through wild-type RAS proteins, namely, HRAS and/or NRAS as previously reported by others (20). In addition, we found that activation of PI3K/AKT pathway by an ERBB3 agonist, heregulin-β1, could induce resistance to the KRAS G12C inhibitor (See Supplementary Fig. S1).

Materials and Methods

Human samples

Human colorectal cancer samples were obtained from patients who underwent resection operations at the Department of Surgery, Kyoto University Hospital (Kyoto, Japan) from 2014 to 2017. The study protocol was approved by the Ethics Committee of Kyoto University (Kyoto, Japan), and written informed consents were obtained from all patients (approval No. R0915 and R0857, FY2015–2022).

PD–CRC-SC spheroid culture

The procedures were described previously for establishing primary cancer stem cell spheroids and normal epithelial stem cell spheroids from tumor specimens (16, 21, 22). In short, collected tumor samples were transferred from the operation room to the laboratory in ice-cold washing medium (DMEM/F12 containing 10% bovine calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin). Fragments of excised tumor were minced and digested by collagenase type I (Thermo Fisher Scientific). Then, epithelial cells were collected and suspended in Matrigel (Corning) and cultured in the cancer medium (16) with or without 50 ng/mL EGF (PeproTech) and 100 ng/mL basic FGF (PeproTech).

Immunoblotting analysis

Spheroids were treated with DMSO or drugs in DMSO for 2 hours, harvested and lysed as described previously. Aliquots (5 μg protein each) were subjected to SDS-PAGE, electrotransfer, and immunoblotting with the following antibodies: for pEGF receptor Tyr1067, EGF receptor, pSHP-2 Tyr580, SHP-2, pMEK1/2 Ser217/221, MEK1/2, pERK1/2 Thr202/Tyr204, and ERK1/2 (1:1,000 dilution), all from Cell Signaling Technology, and for ACTB (1:20,000 dilution) from Sigma-Aldrich.

RAS-GTP pulldown assay

RAS-GTP pulldown assay was performed according to the manufacturer's protocol (Cytoskelton No. BK008). Pulldown and total lysates were subjected to Western blotting analysis as described above. The kit provided primary antibody for pan-RAS detection (1:1,000 dilution).

Phospho-RTK antibody array

The PathScan RTK Signaling Antibody Array Kit (Cell Signaling Technology, No. 7982) was performed by the chemiluminescent sandwich ELISA format to assess the phosphorylation form of 28 RTKs and 11 signaling nodes per protocol. Slide images were analyzed with a conventional imaging device (Gel Doc XR+, Bio-Rad).

Chemicals

Sotorasib (AMG510, KRAS G12C inhibitor; Medkoo Biosciences), adagrasib (KRAS G12C inhibitor; Chemgood), ARS1620 (KRAS G12C inhibitor; MedChemExpress), RMC4550 (SHP2 inhibitor; Selleck), erdafitinib (Erda; JNJ42756493, pan-FGFR1–4 inhibitor; Active Biochem), and erlotinib (EGFR inhibitor; Chem Scene) were dissolved and diluted in DMSO (23–25). Cetuximab (Cmab; Erbitux, anti-EGFR antibody, Merck) was diluted in PBS.

Luminescence-based drug-dosing tests in spheroid culture

Luciferase-expressing spheroids were cultured using 96-well "white" cell culture plates (Corning; 3 μL/well). The luminescence scoring system was described previously (16). Luminescence was determined on days 1 and 4, and the effects of drug dosing were evaluated by computation of growth effect index (GEI; defined as the growth rate of a treated group relative to that of its solvent-only control) in each paired assay. The cut-off GEI between "responsive" and "nonresponsive" was assigned at 0.7 (70%).

Preparation of stem cell spheroid DNA samples

For genomic DNA preparation, spheroids in Matrigel were suspended in Cell Recovery Solution (Corning) and collected in 1.5-mL tubes. Matrigel was digested at 4°C with rotation for 30 minutes. Spheroids were centrifuged for 5 minutes and washed with PBS twice at 4°C. DNA was purified using DNeasy Blood & Tissue Kit (Qiagen).

Mutation analysis

Targeted next-generation sequencing of cancer-related genes was performed by Macrogen. In brief, amplicon libraries were prepared using Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific) and sequenced with the Ion Proton sequencer (Thermo Fisher Scientific). The sequencing data were processed using Ion Torrent Suite Software v5.0.4 (Thermo Fisher Scientific), and variants against the hg19 human genome reference were called using Torrent Variant Caller v5.0.4 (Thermo Fisher Scientific).

Filtration of variant gene sequences

The called variants were annotated using ANNOVAR software (26) and filtered to select nonsynonymous, frameshift, and splicing mutations with >20% frequency. Then, the polymorphic alleles were removed by referring to two databases, Human Genetic Variation Database and 1KJPN (27, 28). Erroneous mutations were also eliminated by surveying their coverage tracks with Integrative Genomics Viewer software v2.3 (Broad Institute, Cambridge, MA).

PDSX

Four- to 5-week-old female nude (BALB/c-*nu*) mice were purchased from Charles River. All animal experiments were conducted according to the protocol approved by the Animal Care and Use Committee of Kyoto University: "Chemosensitivity studies of gastrointestinal cancers using patient-derived tumor xenografts" [Approval No. 14546, 15091, 16047, 16654, 17086, 18080, and 19601 (2014–2022)].

Drug-sensitivity tests in mice

PDSX mice were prepared as reported previously (19). The mice in each dosing group were given sotorasib and erdafitinib orally and/or with cetuximab intraperitoneally at clinically relevant doses for 21 days. For sotorasib treatment, mice were dosed orally with MediGel Sucralose (ClearH_2O) containing the drug at 30 mg/mL. For erdafitinib treatment, mice were dosed orally with MediGel Sucralose (ClearH_2O) containing the drug at 10 mg/mL, as reported

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previously (18, 19). For cetuximab treatment, mice were injected intraperitoneally with 250 μg of the drug per injection twice a week for 3 weeks, which was calculated according to the following formula: mouse dose (mg/kg) = human dose (mg/kg) × 37 (hKm)/3 (mKm) where Km indicates human (h) or mouse (m) body surface coefficient. The relative tumor volume was estimated by calibration to the initial tumor volume on day 0. To evaluate the drug dosing effects, the ratio of tumor volume on day 21 to that on day 0 was compared among the four groups.

Histologic examinations

Paraffin-embedded tissues of PDSXs were prepared according to standard procedures. Sections were stained with hematoxylin and eosin (H&E) or immunostained for Ki67 with an anti-human Ki67 antibody (1:1,000 dilution) from Agilent.

Statistical analysis

Data were analyzed using one-way or two-way ANOVA followed by Tukey post-test, or paired *t* test using GraphPad Prism version 8 (GraphPad software, Inc).

Data availability

The data generated in this study are available within the article and its Supplementary Figures and Table.

Results

KRAS G12C inhibitors suppress the *in vitro* growth of PD–CRC-SC spheroids carrying the target mutation

We assessed the mutational status of *KRAS* in 170 colorectal cancer spheroid lines derived from patients of Kyoto University Hospital, and identified four lines (2.3%) carrying the G12C heterozygous mutation (Supplementary Table S1). Only one (HC100T) of the four lines was omitted from drug treatment due to its slow growth rate. A KRAS G12C inhibitor sotorasib (AMG510) substantially suppressed the growth of a spheroid line (HC42T; **Fig. 1A**). Two other lines (HC17T, HC71T) also behaved in a similar manner.

Accordingly, we then quantified the effects of KRAS G12C inhibitors by luminescence-based growth monitoring. Namely, we introduced a luciferase gene into spheroid lines and performed drug-dosing tests with sotorasib, adagrasib (29), and ARS1620 (30). To quantify the growth inhibitory effects, we determined luminescence from spheroids with and without drug treatment and calculated their GEIs (the relative growth rates of treated spheroids to those of solvent-only control). All three inhibitors hindered the growth of these three KRAS G12C-mutant colorectal cancer spheroid lines (HC17T, HC42T, HC71T) in a dose-dependent manner (**Fig. 1B**). Drug sensitivity to sotorasib was similar with all three KRAS G12C-mutant colorectal cancer spheroid lines (**Fig. 1B**). The IC_{50} values of sotorasib and adagrasib were in the range of 85 to 100 nmol/L, whereas those of ARS1620 were approximately 1,560 nmol/L (**Fig. 1C**). As anticipated, these inhibitors failed to suppress the growth of normal colonic epithelial stem cell (NCE-SC) spheroids (**Fig. 1B**). Sotorasib also showed no effects on two CRC-SC spheroid lines carrying wild-type RAS/RAF protein and on KRAS G12V or BRAF V600E mutants (Supplementary Fig. S2A).

Synergistic growth suppression by simultaneous inhibition of KRAS G12C and EGFR/FGFR

The efficacy of KRAS G12C inhibitors was insufficient to completely halt the proliferation of KRAS G12C-mutant CRC-SC spheroids (40%–60% decrease in GEI; **Fig. 1B**).

EGFR and FGFR have been activated after treatment with sotorasib as detected by phospho-RTK antibody array and Western blotting (Supplementary Fig. S3A and S3B).

Thus, we speculated that the wild-type RAS proteins of different isoforms still transmitted some signals to the MAP kinase cascade in the presence of KRAS G12C inhibitors as reported previously (20). We have recently reported that simultaneous blockade of EGFR and FGFR synergistically inhibits the growth of CRC-SC spheroids carrying wild-type RAS/RAF proteins (19). The result suggests that the three-drug combination of KRAS G12C, EGFR, and FGFR inhibitors may be more efficacious than single-drug treatment on KRAS G12C-mutant CRC-SC spheroids.

To test this hypothesis, we treated KRAS G12C-mutant CRC-SC spheroids with sotorasib, combination of erlotinib (EGFR inhibitor) and erdafitinib (FGFR inhibitor), or combination of all three inhibitors. As anticipated, growth inhibition was strengthened in the presence of three inhibitors (70%–80% decrease in GEI) compared to treatment with sotorasib alone or combination of erlotinib/erdafitinib (**Fig. 2A**). Furthermore, the three-drug combination treatment was significantly more effective than two-drug treatments, sotorasib/erlotinib or sotorasib/erdafitinib (Supplementary Fig. S2B).

To investigate the downstream signaling affected by sotorasib, erlotinib, and erdafitinib in CRC-SC spheroids, we determined the levels of expression and phosphorylation of key signaling proteins in the MAPK cascade by Western immunoblotting. Cells were treated for 2 hours to observe the initial impact of sotorasib because occupancy of KRAS G12C by sotorasib was maximal at that timing (6). The phosphorylation levels of ERK were decreased by the inhibitor treatments in three-dimensional (3D) culture, which was in agreement with the inhibition of spheroid growth (Supplementary Fig. S4C). GTP-bound RAS is susceptible to hydrolysis by RAS-GAPs during and after cell lysis, resulting in Ras inactivation. Western blotting of CRC-SC spheroids in 3D culture requires more than 30 minutes during cell lysis. Thus, we assessed Western blotting in two-dimensional (2D) culture for detecting the RAS-GTP. To this end, spheroid cells were transferred to the cell culture dish without Matrigel (i.e., 2D culture) and treated with the inhibitors for 2 hours. The three-drug combination efficiently reduced GTP loading of RAS proteins in HC17T and HC42T, whereas HC71T showed weaker growth suppression, suggesting a quick feedback mechanism in response to the inhibitor treatment (**Fig. 2B**). There was no significant difference between the 3D and 2D cultures in phosphorylation levels of ERK and AKT (Supplementary Fig. S4C; **Fig. 2B**). Phosphorylation was inhibited in the Src homology 2 (SH2) domain-containing phosphatase 2 (SHP2) when cells were treated with a combination of erlotinib and erdafitinib but not with sotorasib alone, consistent with the interpretation that the signaling through the wild-type RAS isoforms remained active (**Fig. 2B**).

SHP2 regulates cell survival and proliferation primarily through activation of the RAS-ERK signaling pathway. It has been previously reported that an SHP2 inhibitor is effective in human cancer cells with RTK/RAS pathway mutations that are dependent upon RAS guanine-nucleotide cycling, including cells with BRAF class 3 mutations, loss-of-function NF1 mutations, and certain KRAS G12 mutation (e.g., KRAS G12C; ref. 25). Thus, we hypothesized that an SHP2 inhibitor might be one of efficacious alternatives to the combination of EGFR and FGFR inhibitors. Practically, an SHP2 inhibitor RMC4550 suppressed the growth of CRC-SC spheroid lines carrying either wild-type RAS/RAF proteins (HC1T and HC6T) or the KRAS G12C-mutant protein (HC17T, HC42T), whereas it did not affect three spheroid lines carrying the BRAF V600E (HC13T), KRAS G12V (HC5T), or KRAS G12C (HC71T) mutations (Supplementary Fig. S2B and S2C). The

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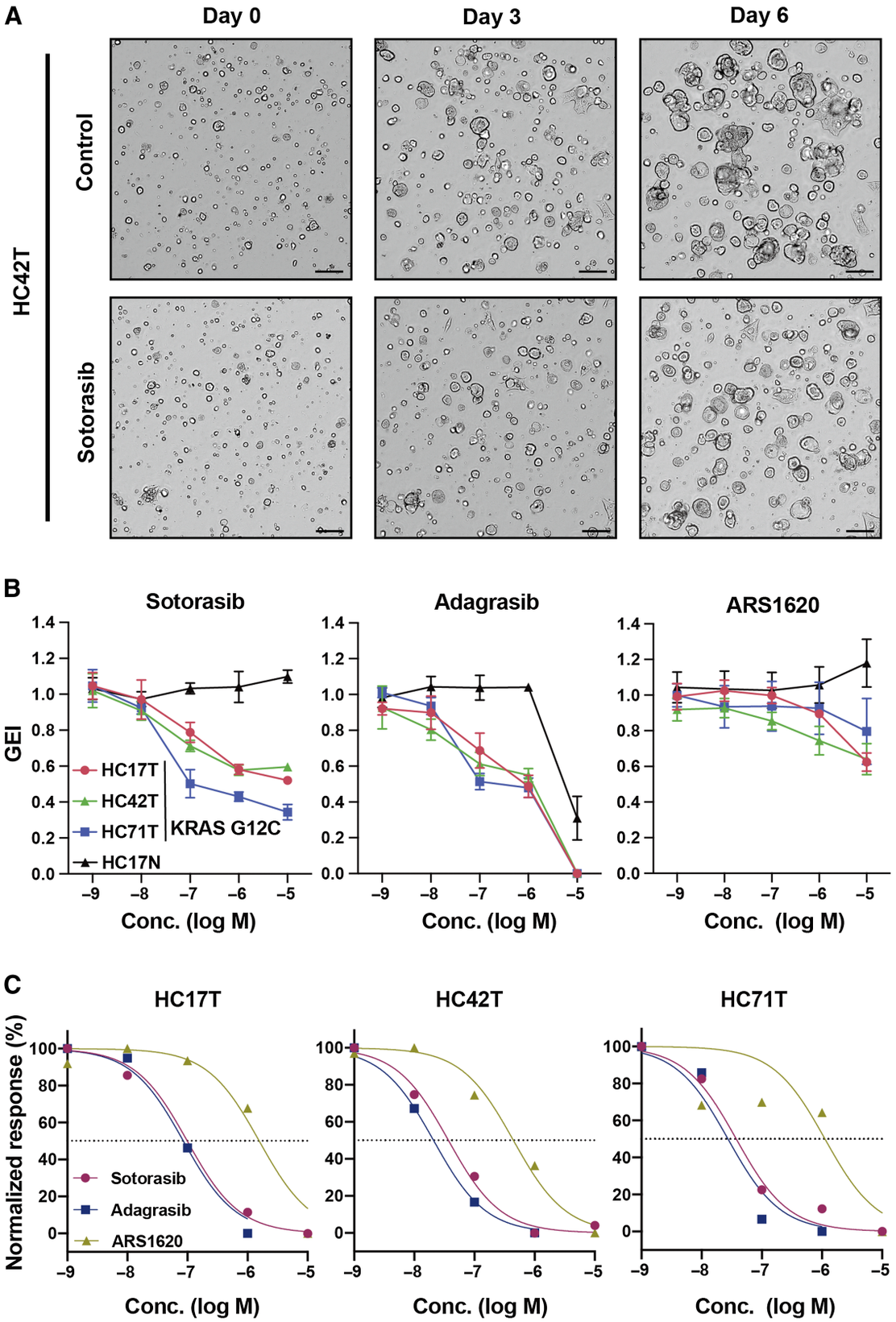

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mean GEI at 1 μmol/L was 58.5% ± 5.5% in four sensitive lines, whereas it was 82.0% ± 4.2% in three insensitive lines. We then tested the combined effects of sotorasib and RMC4550 on the three KRAS G12C-mutant lines. As anticipated, growth inhibition was significantly strengthened in the presence of both inhibitors compared with either inhibitor alone (**Fig. 2C**).

Three-drug combination treatment with sotorasib, erdafitinib, and cetuximab reduces the size of PDSX tumors at clinically relevant doses

To test the clinical feasibility of the combination treatment with sotorasib and FGFR/EGFR inhibitors *in vivo*, we prepared PDSX tumors in immunocompromised nude mice transplanted with a spheroid line HC17T that efficiently responded *in vitro* not only to sotorasib singly but also to the three-combination treatments (**Fig. 2A**). We adopted cetuximab rather than erlotinib in the PDSX dosing experiments because it has been used clinically for colorectal cancer treatment. We divided the PDSX mice into four treatment groups: control (solvent only), sotorasib, combination of cetuximab and erdafitinib, and three-drug combination. In HC17T PDSX mice, sotorasib monotherapy as well as combination of cetuximab/erdafitinib reduced the tumor volume with statistically significant differences from no-drug control tumors (**Fig. 3A**). Notably, the combination of three drugs inhibited cancer stem cell proliferation most efficaciously (**Fig. 3A**; Supplementary Fig. S4B). The mean relative tumor size in the three-combination therapy group was much lower than that in the erda/cmab group (0.19 vs. 0.53), although the statistical difference appeared somewhat weak in the relative tumor size between the erda/cmab and the three-combination therapy group ($P = 0.08$). There was no significant difference in the body weight of the mice during either treatment (Supplementary Fig. S4A).

Although no discernible changes were found in the histopathologic features of PDSX tumors at the end of treatment for 3 weeks (**Fig. 3B**), the proportion of Ki67-positive cells in tumor sections was reduced to approximately 40% of no-drug control in the sotorasib or cetuximab/erdafitinib therapy group and to <10% in the three-combination therapy group (**Fig. 3C**; Supplementary Fig. S4C), which was consistent with the degrees of tumor growth suppression.

A PI3K inhibitor enhances the effect of EGFR/FGFR-inhibitor combination treatment by reducing the growth of PD–CRC-SC spheroids

Several lines of evidence indicate that active RAS protein can induce activation of PI3K/AKT signaling (31, 32). If so, inhibition of KRAS G12C may reduce AKT signaling. Yet, sotorasib did not affect phosphorylation of AKT in KRAS G12C-mutant colorectal cancer spheroids (**Fig. 2B**). To investigate whether activation of PI3K/AKT signaling weakened the tumor inhibitory effect of KRAS G12C inhibitors, we employed heregulin-β1 (neulegulin-β1), a ligand of ERBB3 and potent activator of PI3K (33). As expected, heregulin-β1 induced AKT phosphorylation in all three KRAS G12C-mutant spheroid lines, although its basal levels varied (**Fig. 4A**). Namely, HC17T and HC42T that carried PIK3CA activating mutations (C420R and H1047R, respectively) had higher phosphorylation levels of AKT than that in HC71T (PIK3CA wild-type).

On the other hand, heregulin-β1 did not affect MEK/ERK signaling (**Fig. 4A**), suggesting little cross-talk between these two signaling pathways in three CRC-SC spheroid lines. The growth rate of HC71T was increased more than 50% by heregulin-β1, whereas those of the other two lines (HC17T and HC42T) were comparable with control (Supplementary Fig. S3). Interestingly, the growth inhibitory effect of sotorasib on HC71T was offset by addition of heregulin-β1 (**Fig. 4B**). These results suggested that simultaneous blockade of MEK/ERK and PI3K/AKT pathways could be an efficacious chemotherapy for a subset of the KRAS G12C-mutant colorectal cancer.

To test this hypothesis further, we next investigated dose–response effects of a PI3K inhibitor alpelisib on the heregulin-β1–treated CRC-SC spheroids *in vitro*. Alpelisib suppressed the growth of two spheroid lines (HC42 and HC71) in a dose-dependent manner but not that of HC17T (**Fig. 4C**). We treated HC17T additionally with another PI3K inhibitor Pictilisib as well as an AKT inhibitor ipatasertib. However, neither showed any inhibitory effects on cancer stem cell proliferation, suggesting that more downstream signaling loop is activated rather than at the single node of PI3K (Supplementary Fig. S5B).

Interestingly, the combination treatment with sotorasib, RMC4550, and alperisib inhibited the growth of three KRAS G12C-mutant lines more efficiently than with sotorasib and RMC4550 (**Fig. 4D**). Alpelisib substantially inhibited AKT phosphorylation in HC42T and HC71T, but not in HC17T (**Fig. 4E**), suggesting that a mutant PIK3CA C420R was refractory to alpelisib. We also applied Pictilisib or ipatasertib instead of alpelisib to HC17T cultures, but there were little add-on effects in the three-drug combination therapy (Supplementary Fig. S5C). These results indicate that blocking both MEK/ERK and PI3K/AKT signaling pathways can be more efficacious than that of either one in the treatment of colorectal cancer with KRAS G12C.

Discussion

While the efficacy of KRAS G12C inhibitor monotherapy for patients with colorectal cancer is more limited than for lung cancer, the mechanism is not understood well (9). In this study, we searched for effective drug combinations and key signals involved in suppressing proliferation of KRAS G12C-mutant colorectal cancer cells.

KRAS G12C inhibitors alone showed only moderate inhibition of the cancer stem cell proliferation *in vitro* (**Fig. 1B** and **C**), suggesting that the wild-type RAS proteins of different isoforms made a significant contribution to the MAPK signaling in KRAS G12C-mutant colorectal cancer cells. Among these inhibitors, sotorasib and adagrasib were more potent than ARS1620, which was likely due to the

Figure 1.

Effects of KRAS G12C inhibitors on the *in vitro* growth of PD–CRC-SC spheroids. **A,** Representative microscopic images of cancer spheroids (HC42T) harboring heterozygous KRAS G12C mutation cultured in the presence or absence of 1 μmol/L sotorasib for 6 days. Magnification bar, 200 μm. **B,** Dose-dependent effects of KRAS G12C inhibitors, sotorasib (left), adagrasib (center), and ARS1620 (right), on the growth of three PD–CRC-SC spheroid lines (HC17T, HC42T, and HC71T) carrying heterozygous KRAS G12C mutation and an NCE-SC spheroid line (HC17N). Luciferase-expressing spheroid cells were cultured for 3 days with the indicated concentrations of drugs, and their relative growth rates (GEI) were calculated from the photon counts. Shown are the mean GEI ± SD in three independent experiments. Note that adagrasib showed nonspecific toxicity at 10^{-5} mol/L (10 μmol/L). **C,** Computer-fitted dose–response curves of normalized mean value of three KRAS G12C inhibitors on the growth of three colorectal cancer spheroid lines, HC17T (left), HC42T (center), and HC71T (right). The response in Fig. 1C was obtained by normalization to run between 0% and 100%. The adagrasib data at the concentration of 10^{-5} mol/L (10 μmol/L) were excluded from the computation of the best-fit dose–response curves because of their nonspecific nature of the toxicity.

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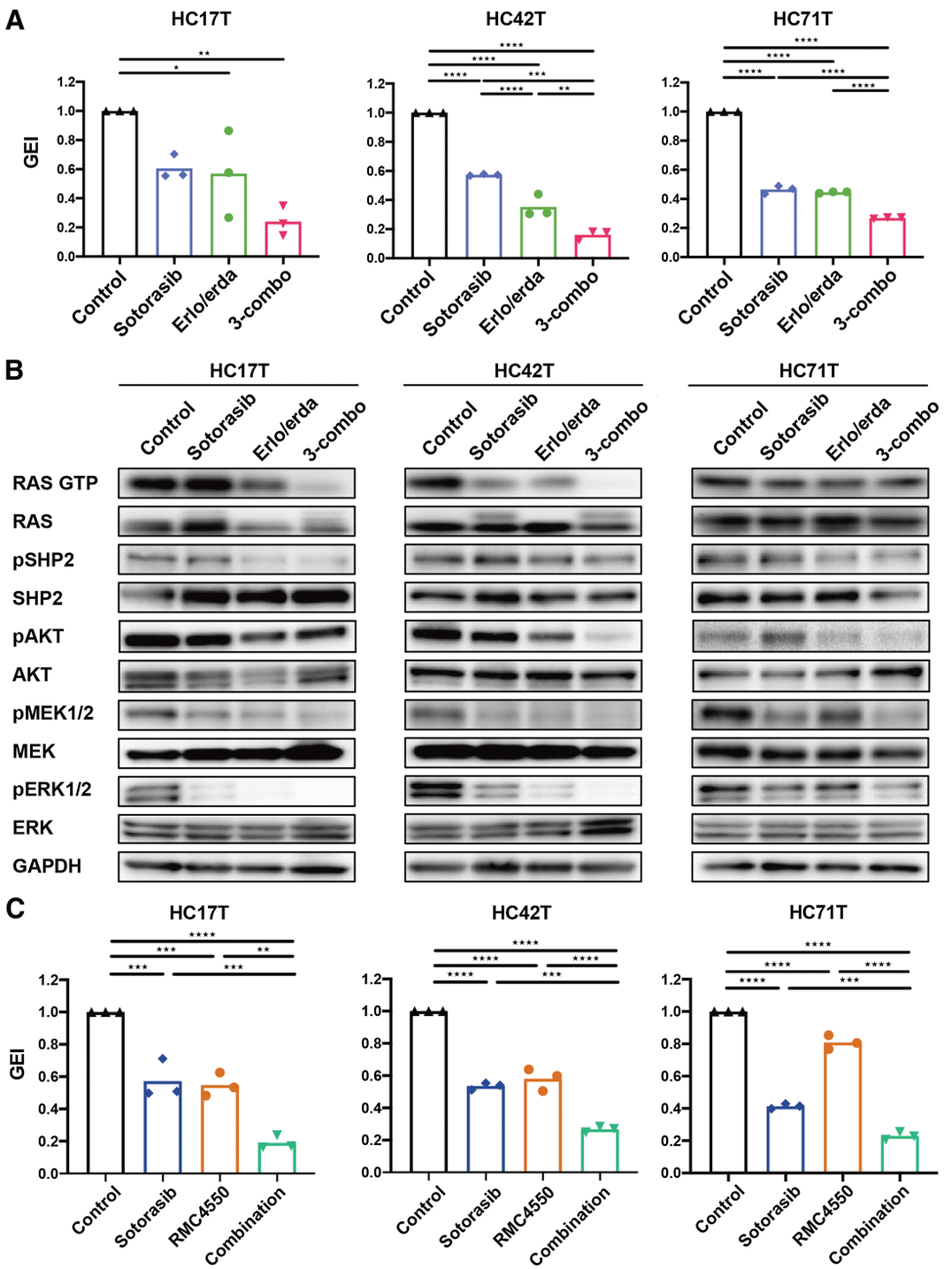

Figure 2.

Synergistic effects of simultaneous inhibition of upstream signaling together with KRAS G12C on the growth of PD–CRC-SC spheroids. **A,** Inhibitory effects of sotorasib (1 μmol/L), combination of erlotinib (1 μmol/L) and erdafitinib (100 nmol/L; erlo/erda), and a combination of all three compounds (3-combo) on the spheroid lines harboring the KRAS G12C mutation (HC17T, left; HC42T, center; and HC71T, right). Ordinate shows GEI with the mean values in three independent experiments. The *P* values are shown: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; or ****, $P < 0.0001$ in one-way ANOVA followed by Tukey post-test. **B,** Immunoblotting analysis of the above spheroid lines for downstream effector proteins EGFR and FGFR. Spheroid cells attached to the cell culture plate were cultured with DMSO (control), sotorasib alone, combination of erlotinib and erdafitinib (Erlo/Erda), or combination of all three compounds (3-combo) for 2 hours. A pulldown assay was performed to detect GTP-bound RAS (RAS-GTP). Antibodies were used to detect protein; RAS (RAS), SHP phosphorylated at Tyr580 (pSHP), SHP (SHP), AKT phosphorylated at Ser473 (pAKT), AKT (AKT), MEK phosphorylated at Ser217/221 (pMEK1/2), MEK (MEK), ERK phosphorylated at Thr202/Tyr204 (pERK1/2), ERK (ERK), and GAPDH (GAPDH) as a loading control. **C,** The inhibitory effects of sotorasib (1 μmol/L), RMC4550 (1 μmol/L), and their combination on three spheroid lines harboring KRAS G12C mutation (HC17T, left; HC42T, center; and HC71T, right). Plotted are the GEI with the mean values in three independent experiments. The *P* values are shown: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; or ****, $P < 0.0001$ in one-way ANOVA followed by Tukey post-test.

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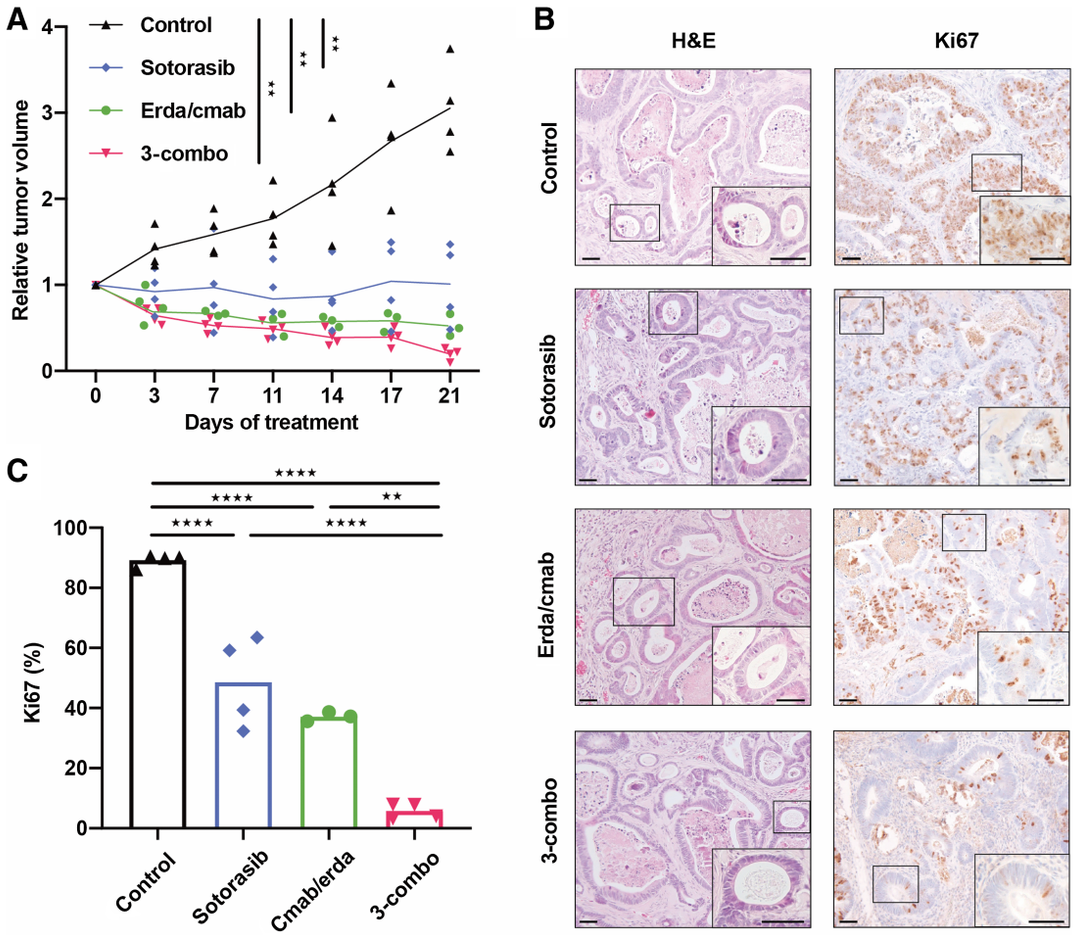

Figure 3.

Drug-sensitivity tests with sotorasib alone, combination of erdafitinib (erda) and cetuximab (cmab), and a combination of all three drugs (3-combo) against PDSX tumor growth in mice. **A,** Changes in the subcutaneous tumor relative volumes of CRC-SC spheroid xenografts during drug treatment with sotorasib alone (30 mg/kg/day), combination of erdafitinib (10 mg/kg/day; erda) and cetuximab (250 μg/twice a week; cmab), or a combination of all three drugs (3-combo). The *P* values are shown: **, $P < 0.01$ in two-way ANOVA followed by Tukey post-test. **B,** Histologic sections of xenograft tumors derived from a colorectal cancer spheroid line HC17T on day 21. Sections were stained with H&E (left) or immunostained with a proliferation marker, Ki67 (right). Magnification bar, 200 μm. **C,** The fraction of proliferating cells (%) monitored by Ki67 expression for each treatment group. The mean values are shown as bars with raw datapoints in symbols of various shapes. The *P* values are shown: **, $P < 0.01$; ***, $P < 0.001$; or ****, $P < 0.0001$ in one-way ANOVA followed by Tukey post-test.

difference in their binding affinities to the S-II pocket (6). These results led us to seek additional compounds that were capable of suppressing MAPK signaling more efficiently by means of combination treatments.

Previously, we showed that a combination of FGFR and EGFR inhibitors was effective against *RAS*/*RAF* wild-type CRC-SC both in culture and xenografts (19). Because wild-type RAS isoforms distinct from the mutated KRAS can promote oncogenesis (20), we speculated that the combination of inhibitors against KRAS G12C, FGFR, and EGFR might suppress the proliferation of G12C-mutant colorectal cancer cells efficiently by blocking the signal transduction through both mutant and wild-type RAS isoform proteins. As expected, the growth suppression by the three-combination therapy was achieved not only in SC spheroids but also in PDSX tumors (**Figs. 2** and **3**). Consistently, the combination treatment reduced the levels of RAS-GTP and phosphorylated MEK/ERK more potently than their mono-treatments (**Fig. 2B**). Likewise, we showed that an SHP2 inhibitor, RMC4550, was also effective in combination with sotorasib. Because SHP2 mediates signaling from RTK to RAS/MAPK, it is considered to be one of the most promising drugs for solid tumors carrying KRAS mutations (34). Taken together, the current results provide insights into treatment for G12C-mutant colorectal cancer that is affected at multiple effectors of the RAS-MAPK pathway.

Several lines of evidence have indicated that the PI3K/AKT pathway plays important roles in the progression of colorectal and lung cancers (32, 33, 35). However, sotorasib did not inhibit AKT phosphorylation in any of our three KRAS G12C-mutant colorectal cancer spheroid lines (**Fig. 2B**), suggesting little involvement of RAS to PI3K/AKT signaling in this cellular context. On the other hand, recent studies have shown that heregulin (neuregulin), a potent activator of the PI3K/AKT pathway, induced drug resistance in several types of cancer through its binding to the ERBB3/ERBB4 dimer (33). Consistently, heregulin attenuated the therapeutic effect of sotorasib on G12C-mutant colorectal cancer spheroids (**Fig. 4B**). The current results suggest a possibility that choosing PI3K inhibitors is an efficacious option for the combination therapy of G12C-mutant

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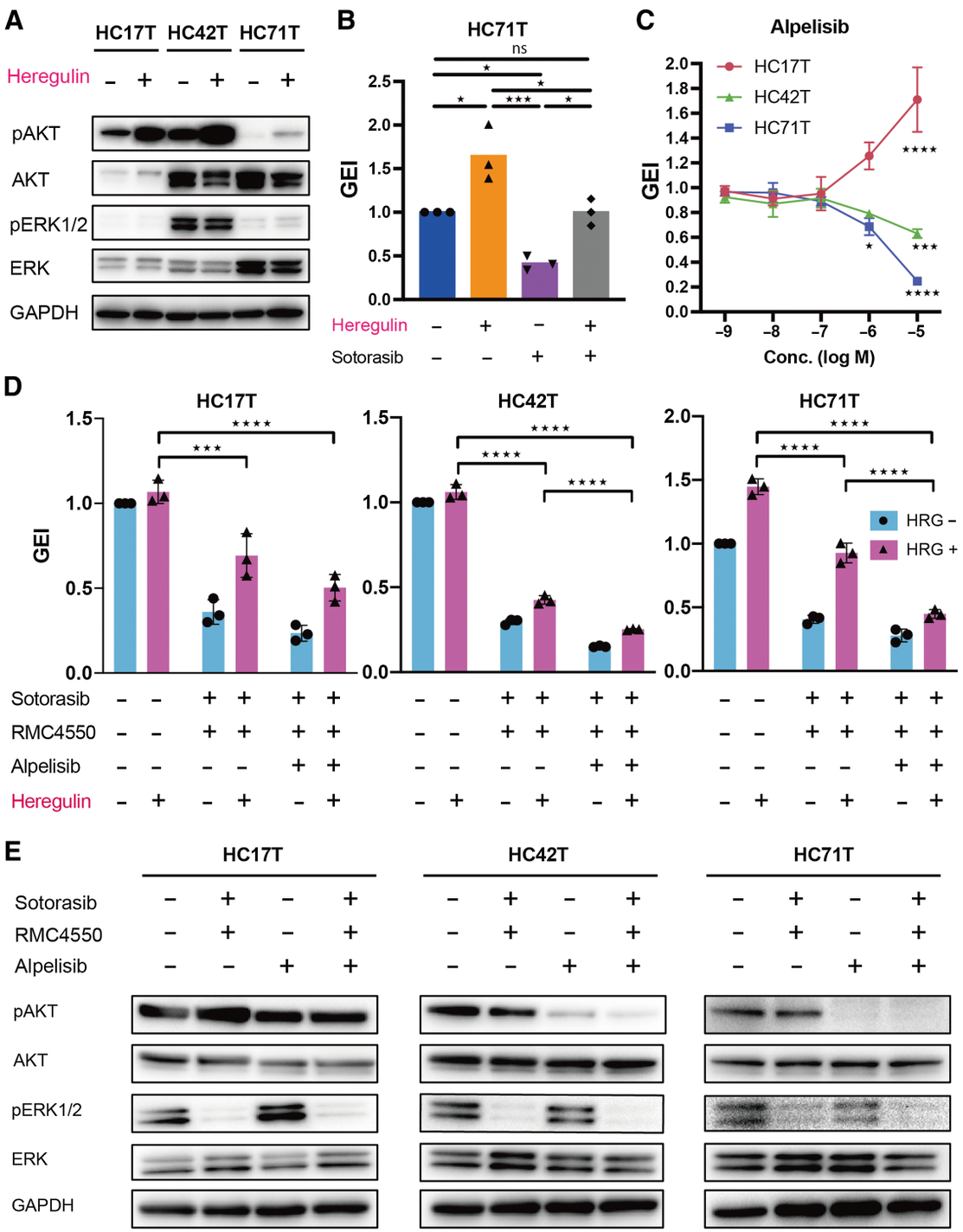

Figure 4.

Synergistic effects of MEK/ERK and PI3K/AKT pathway inhibition on the growth of KRAS G12C-mutant PD–CRC-SC spheroids. **A,** Immunoblotting analysis of three spheroid lines for effector proteins in PI3K/AKT and MAPK signaling pathways. Spheroid cells were cultured in the presence or absence of heregulin-β1 (10 ng/mL) for 2 hours. Antibodies were used to detect AKT phosphorylated at Ser473 (pAKT), AKT protein (AKT), ERK phosphorylated at Thr202/Tyr204 (pERK1/2), ERK protein (ERK), and GAPDH protein (GAPDH) as a loading control. **B,** The inhibitory effects of sotorasib (1 μmol/L) in the presence or absence of heregulin-β1 on a spheroid line (HC71T). Ordinate shows GEI with the mean values in three independent experiments. **C,** Dose-dependent effects of alpelisib on the growth of three PD–CRC-SC spheroid lines (HC17T, HC42T, and HC71T). Shown are the mean GEI ± SD in three independent experiments. **D,** Inhibitory effects of combination treatment with sotorasib (1 μmol/L), RMC4550 (1 μmol/L), and alpelisib (1 μmol/L) on three spheroid lines (HC17T, left; HC42T, center; and HC71T, right). Plotted are the GEI with the mean values in three independent experiments. The *P* values in **B**, **C**, and **D** are shown. ns, not statistically significant; or *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; or ****, $P < 0.0001$; in one-way ANOVA followed by Tukey post-test. **E,** Western Immunoblotting analysis of the above three spheroid lines for effector proteins in the PI3K/AKT and MAPK signaling pathways. Spheroid cells were cultured in the presence of DMSO (control), combination of sotorasib and RMC4550, alpelisib alone, and combination of all three compounds dissolved in DMSO for 2 hours.

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colorectal cancer because a combination of sotorasib, RMC4550, and PI3K inhibitor alperisib effectively suppressed the growth of heregulin-treated colorectal cancer spheroids (**Fig. 4D**), and because cancer-associated fibroblasts can be the source of heregulin in the tumor microenvironment (36).

In conclusion, we elucidated one of the mechanisms of resistance to KRAS G12C inhibitors using patient-derived CRC-SC spheroids. Because the key therapeutic targets in the growth-signaling pathways can vary depending on the cancer cell–intrinsic context and microenvironment, drug-sensitivity tests using PD–CRC-SC spheroids help find the efficacious drug combinations for personalized treatments of G12C-mutant colorectal cancer (See Supplementary Fig. S1).

Authors' Disclosures

H. Miyoshi reports grants from the Japan Society for the Promotion of Science during the conduct of the study; grants from Kyo Diagnostics K.K., SCREEN Holdings Co., Ltd., and Nippi, Incorporated outside the submitted work. In addition, H. Miyoshi has a patent for "Cancer spheroid production method and method for selecting colon cancer patients," pending to Kyo Diagnostics K.K. F. Kakizaki reports grants from the Japan Society for the Promotion of Science during the conduct of the study. M.M. Taketo reports grants from Japan Science and Technology Agency, Japan Agency for Medical Research and Development, Kyoto University Hospital, Japan Society for the Promotion of Science, and Japan Society for the Promotion of Science during the conduct of the study. In addition, M.M. Taketo has a patent pending to Kyo Diagnostics K.K. No disclosures were reported by the other authors.

Authors' Contributions

H. Matsubara: Conceptualization, data curation, formal analysis, validation, investigation, writing–original draft. **H. Miyoshi:** Resources, formal analysis, supervision, investigation, methodology. **F. Kakizaki:** Resources, supervision. **T. Morimoto:** Resources. **K. Kawada:** Resources. **T. Yamamoto:** Conceptualization, resources. **K. Obama:** Resources. **Y. Sakai:** Resources. **M.M. Taketo:** Conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, project administration.

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