Effect of Simvastatin on Cetuximab Resistance in Human Colorectal Cancer With KRAS Mutations

Jeeyun Lee, Inkyoung Lee, Boram Han, Joon Oh Park, Jiryeon Jang, Chaehwa Park, Won Ki Kang

Manuscript received June 14, 2010; revised February 8, 2011; accepted February 10, 2011.

Correspondence to: Won Ki Kang, MD, PhD, Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Korea (e-mail: wkkang@skku.edu).

Monoclonal antibodies (mAbs) such as cetuximab and panitumumab that target the epidermal growth factor receptor (EGFR) have proven to be efficacious in terms of response rate and progression-free survival in combination with standard cytotoxic chemotherapy in metastatic colorectal cancer (CRC) (1–4). Recently, mutations in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), which occur in approximately 40% of CRC patients, have emerged as the major negative predictive factor for treatment response in patients receiving cetuximab (5,6). Currently, there is no effective treatment for metastatic CRC patients whose tumors harbor KRAS mutations and who have failed to respond to 5-fluorouracil combined with irinotecan or oxaliplatin chemotherapy (6). The benefit of adding cetuximab to first-line or second-line irinotecan-containing chemotherapy regimens has been addressed in two prospective trials—cetuximab combined with irinotecan in first-line therapy for metastatic CRC (CRYSTAL) (1) and oxaliplatin and cetuximab in first-line treatment of metastatic CRC (OPUS) (7). In the CRYSTAL trial, 1198 metastatic CRC patients were randomly assigned to first-line FOLFIRI (irinotecan, infusional 5-fluorouracil, and leucovorin) treatment with or without cetuximab. Patients with wild-type KRAS who received FOLFIRI treatment with cetuximab showed statistically significantly increased overall survival compared with patients who received FOLFIRI treatment without cetuximab (median overall survival, 23.5 vs 20.0 months; \( P = .009 \)) (8). In the randomized phase II OPUS trial, addition of cetuximab to FOLFOX (leucovorin, 5-fluorouracil, and oxaliplatin) as first-line treatment was associated with an increased objective response rate compared with FOLFOX chemotherapy alone (cetuximab + FOLFOX vs FOLFOX, 61% vs 37%; odds ratio = 2.54; \( P = .011 \)) in the KRAS wild-type subset of patients. Nevertheless, in the OPUS trial, there was no median overall survival benefit in cetuximab plus FOLFOX group compared with FOLFOX group (cetuximab + FOLFOX vs FOLFOX, 22.8 vs 18.5 months, hazard ratio = 0.855; \( P = .385 \)) (7,9). However, in patients with KRAS mutations, there was no survival benefit with the addition of cetuximab to FOLFIRI or FOLFOX...
treatment in both trials (1,7,9). KRAS mutations may appear in many codons, but mutations in codons 12 and 13 of exon 2 and codon 61 of exon 3 are more frequent than others, especially in CRC (10,11).

Cetuximab binds to the extracellular domain of EGFR, leading to the inhibition of its downstream signaling pathways, including the RAS-RAF-mitogen-activated protein kinase 1 (MAPK1) axis that is mainly involved in cell proliferation and the v-akt murine thymoma viral oncogene homolog 1 (Akt1) pathway that is mainly involved in cell survival and tumor invasion (12). It has been demonstrated that in the absence of KRAS mutations, resistance to cetuximab or panitumumab could be caused by alterations of other proteins of the RAS-RAF-MAPK1 pathway, such as v-ras murine sarcoma viral oncogene homolog B1 (BRAF) mutation (5,12). We postulated that modulation of BRAF activity, which is downstream of RAS, may overcome the resistance to cetuximab or panitumumab in KRAS mutant CRC.

Statins are widely used as lipid-lowering agents to lower cardiovascular risk with a favorable safety profile. Statins, such as lovastatin, simvastatin, pravastatin, and atorvastatin, are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, which lead to decreased production of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (13,14). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate are essential substrates for posttranslational modifications of RAS and ras homolog gene family, member A (RHOA), which play an important role in cellular proliferation (13–15). Based on the effect of statin on posttranscriptional modifications of RAS and RHOA, the antitumor effect of statins has been suggested in various cancer cell lines (16–18). Moreover, more recent studies using cancer gene signatures have systematically screened an array of drugs for potential antitumor effect and have discovered statins as potential novel targeted anticancer agents (19,20). Given the cardiovascular therapeutic dose of lovastatin is 1 mg kg\(^{-1}\) d\(^{-1}\), the equivalent dose of simvastatin in terms of potency is half of lovastatin, which is 0.5 mg kg\(^{-1}\) d\(^{-1}\) (21). Using this concentration, we have previously shown that simvastatin, at a concentration range of 0.1–0.2 µM, induced cell senescence or cytostatic effect of prostate cancer cells in vitro (17,22). In addition, we have shown the well tolerability and promising antitumor effect of a combination of 40 mg daily dose of simvastatin and standard FOLFIRI treatment in 49 metastatic CRC patients with disease control rate of 83.7% (95% confidence interval [CI] = 73.4% to 94.0%) (23). In this study, we hypothesized that simvastatin may overcome cetuximab resistance in KRAS mutant CRC cells by modulating the RAS-BRAF pathway. We investigated whether the antitumor activity of cetuximab can be potentiated by simvastatin in colon cancer cell lines with or without KRAS mutations. Functional and biochemical studies were performed in vitro to test the effect of cetuximab, simvastatin, and cetuximab plus simvastatin on cell proliferation and apoptosis. The effect of drugs was tested on tumor growth in mouse xenograft models. We also investigated whether simvastatin has an effect on RAS signaling and modulation of BRAF activity in KRAS wild-type and KRAS mutant cell lines.

**Materials and Methods**

**Cell Lines and Cell Culture**

Human CRC cells were purchased from American Type Culture Collection (Manassas, VA). We used eight known human CRC cell lines with documented KRAS mutations—LS13 (KRAS\(^{G12D}\)), LS174T (KRAS\(^{G12S}\)), DLD1 (KRAS\(^{G12D}\)), LoVo (KRAS\(^{G12V}\)), SW403 (KRAS\(^{G12V}\)), SW480 (KRAS\(^{G12V}\)), SNU175 (KRAS\(^{V600E}\)), and LS1034 (KRAS\(^{V600E}\)—and four KRAS wild-type (wt) CRC cells DiFi (KRAS\(^{wt}\) BRF\(^{wt}\)), SW48 (KRAS\(^{wt}\) BRAF\(^{wt}\)), HT29 (KRAS\(^{wt}\) BRAF\(^^{V600E}\)), and RKO (KRAS\(^{wt}\) BRAF\(^{V600E}\)). Most of the above-mentioned cell lines were purchased from American Type Culture Collection, except for the SNU175 cell line that was obtained from the Korean Cell Line Bank (Seoul, Korea), and the DiFi cell line that was generously provided by Dr. J. O. Park (Samsung Medical Center, Seoul, Korea). The genotypes were verified by direct sequencing (Supplementary Figure 1, available online). All cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 µU/mL penicillin, and 100 µg/mL streptomycin in a 5% CO atmosphere at 37°C. Medium and supplements were purchased from Invitrogen Corporation (Carlsbad, CA).

**Mutation Analysis**

DNA was extracted from CRC cell lines using QIAamp DNA Mini kit (Qiagen, Inc, Valencia, CA) according to the manufacturer’s
instructions. The polymerase chain reaction (PCR) was carried out in a total volume of 50 µL containing the following: 100 ng of DNA, 2.5 mM MgCl₂, 300 mM deoxynucleotide triphosphates, 10 pmol primers, and 2.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). PCR conditions were as follows: one cycle at 94°C for 10 minutes; 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; followed by one cycle at 72°C for 10 minutes. The following primers were used: for codon 600 in exon 15 of BRAF, forward, 5′-TCATAA TGGTTGCTCTGATAGGA-3′ and reverse, 5′-GGCCAAAAA TTTATCATGTTGA-3′; for codons 12 and 13 in exon 2 of KRAS, forward, 5′-GATGTTTGCTGACCCAGTAA-3′ and reverse, 5′- GAATGGTCCTGCACCAGTAA-3′; for codon 61 in exon 3 of KRAS, forward, 5′- TCAAGTCTTGTGCCCA TTTT-3′ and reverse, 5′-TGCATGGCATTAGCAAAAGAC-3′; and for codon 146 in exon 4 of KRAS, forward, 5′-TTGTGGGAG GTTGTGAAAGA-3′ and reverse, 5′-AGAAGCAATGCCCTCT CAAG-3′. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Inc) and sequenced with an ABI PRISM 3730 automated sequencer (Applied Biosystems). All mutated samples were confirmed twice by an independent PCR. Analysis of the sequencing data was carried out using the BLAST 2 sequences program (http://www.ncbi.nlm.nih.gov/blast/) by comparison against wild-type KRAS (NM_033360) and BRAF (NM_004333) sequences and by thorough examination of the generated chromatograms using the Sequencer program (GeneCodes, Ann Arbor, MI).

Antibodies
The following primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): mouse anti-human BRAF mAb (24), mouse anti-human B-cell CLL/lymphoma 2 (BCL2) mAb, rabbit anti-human BCL2-associated agonist of cell death (BAD) polyclonal antibody, mouse anti-human BCL2-associated X (BAX)
Figure 1. Effect of cetuximab and simvastatin on cell proliferation and colony formation of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutant and v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutant colorectal cancer (CRC) cells in vitro. A) Cell proliferation assay of eight KRAS mutant CRC cell lines treated with phosphate-buffered saline (PBS), 0.03–1.0 µM cetuximab, 0.2 µM simvastatin, or a combination of 0.03–1.0 µM cetuximab and 0.2 µM simvastatin for 3 days and stained with 0.4% trypan blue. Results of cell counts are expressed as percentage of cell proliferation using the control PBS as reference. P values are shown for cetuximab vs cetuximab + simvastatin. B) Cell proliferation assay of four KRAS wild-type cells (two BRAF wild-type and two BRAF V600E mutant) treated with control PBS or drugs and stained with trypan blue similarly as above. Results of cell counts are expressed as percentage of cell proliferation using the control PBS as reference. P values are shown for cetuximab vs cetuximab + simvastatin. C) Cell proliferation assay of KRAS mutant DLD1 and SW403 cells treated with a combination of SN38 (an irinotecan metabolite) and cetuximab or a combination of SN38, cetuximab, and simvastatin or treated with individual drugs alone for 3 days. Cells were stained with trypan blue as above. Results of cell counts are expressed as percentage of cell proliferation. P values are shown for SN38 + cetuximab vs SN38 + cetuximab + simvastatin. In (A–C), the results are means and 95% confidence intervals of two independent experiments performed in quadruplicate. D) Colony formation assay of KRAS and BRAF mutant cells. Cells were treated with 0.1 µM cetuximab and/or 0.2 µM simvastatin for 3 days and maintained at 37°C for 14 days, with fresh medium added after 7 days. Cells were stained with 0.1% crystal violet. P values are shown for cetuximab vs cetuximab + simvastatin. Results are means and 95% confidence intervals of two independent experiments performed in quadruplicate. All P values were calculated using two-sided Student t test.
mAb, rabbit anti-human BCL2-like 1 (BCL2L1, also known as BCL-X) polyclonal antibody, mouse anti-human KRAS mAb, mouse anti-human mitogen-activated protein kinase kinase 1 (MAP2K1) mAb, and rabbit anti-human tubulin, alpha 1a (TUBA1A) polyclonal antibody. The following rabbit polyclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA): anti-human AKT1, anti-human phosphorylated AKT1 (Ser473), anti-human BCL2-like 1 (BCL2L1), anti-human mitogen-activated protein kinase 1/2 (MAPK1/2), anti-human phospho-MAPK1/2 (Thr202/Tyr204) (also known as ERK1/2), and anti-human myeloid cell leukemia sequence 1 (MCL1). Rabbit anti-human phosphorylated mitogen-activated protein kinase kinase 1 (MAP2K1) (Ser218/222)/MAP2K2 (Ser222/226) (also known as MEK1/2) was obtained from Millipore Upstate Biotechnology (Bedford, MA). Mouse anti-human ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle (ATP5A1) (1:5000 dilution) was obtained from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP) conjugates of anti-rabbit and anti-mouse IgG (1:5000 dilution) were obtained from Zymed Laboratories (San Francisco, CA).

**Assays for Cell Proliferation and Colony Formation**

To measure cell growth in vitro, LS153, LS174T, DLD1, LoVo, SW403, SW480, SNU175, LS1034, DFi, SW48, HT29, and RKO cells were grown in RPMI-1640 medium with 10% FBS at 37°C. Cells were seeded (5 × 10^4 cells per well) in a 12-well plate (Nunc, Rochester, NY) and incubated at 37°C for 1 day (24 hours). Cells were then incubated with 0.03–1 µM cetuximab (Merck Serono, Geneva, Switzerland), 0.2 µM simvastatin (Calbiochem, San Diego, CA), a combination of 0.03 µM–1 µM cetuximab and 0.2 µM simvastatin, or phosphate-buffered saline (PBS; 1.54 mM KPO4 monobasic, 155.17 mM NaCl, 2.71 mM Na2PO4 dibasic, pH 7.2) (Invitrogen) in RPMI-1640 medium with 2% FBS at 37°C for an additional 3 days (72 hours). Cells were then treated with 0.25% trypsin–EDTA solution (2.5 g/L of trypsin and 0.38 g/L of EDTA) (Invitrogen), stained with 0.4% trypan blue solution (Sigma-Aldrich, Inc), and counted using a hemocytometer (Hauser Scientific, Horsham, PA). The results were expressed as percent cell proliferation, using the number of living cells incubated with PBS as a 100% reference. The half maximal inhibitory concentration (IC50) was defined as the concentration of a drug where the proliferation was reduced by 50% in a sigmoidal curve.

For anchorage-dependent colony formation, we used DLD1, SW403, HT29, and RKO cells. Briefly, 2 × 10^4 cells per well were seeded in six-well plates (Nunc) and treated with 0.1 µM cetuximab and/or 0.2 µM simvastatin for 3 days. Triplicate cultures of each cell type were maintained at 37°C for 14 days in an atmosphere of 5% CO2, with fresh medium being added after 7 days. Cells were stained with 0.1% (wt/vol) crystal violet. Colonies, defined as groups of cells containing a minimum of 50 cells, were counted under an inverted phase contrast microscope CKX31SF (Olympus Biosystems, Hamburg, Germany). The percent relative cell proliferation was expressed as (number of colonies from treated cells/number of colonies from controls) × 100. The assay was repeated three times with duplicate samples.

**Preparation of Cytosolic and Membrane Extracts**

LS174T, DLD1, SW403, HT29, and RKO were cultured in RPMI-1640 medium with 10% FBS at 37°C. Cells were seeded at the concentration of 1 × 10^5 cells per 100-mm dish (Nunc) and incubated at 37°C for 24 hours. Cells were then incubated with 0.2 µM simvastatin or PBS in RPMI-1640 medium with 2% FBS at 37°C for an additional 3 days. Cells were washed twice in ice-cold PBS and then scraped from the dishes in 1 mL PBS and transferred to 1.5-mL microtubes. Cells were centrifuged at 2000g for 5 minutes, the supernatant was discarded, and the cell pellet was allowed to swell and lyse after the addition of 300 µL hypotonic buffer (10 mM HEPES [pH 7.2], 5 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM EGTA, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM sodium orthovanadate). The lysates were incubated on ice for 30 minutes and then centrifuged at 10 000g at 4°C for 20 minutes. The supernatant was cytosolic extract. Pellets containing crude nuclei were resuspended in 100 µL of extraction buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate) and then incubated on ice for 30 minutes. The samples were then centrifuged at 15 000g at 4°C for 10 minutes to obtain supernatants containing membrane extracts. Membrane extracts were stored at −70°C, until use.

**Immunoblot Analysis**

DLD1, SW403, HT29, and RKO cells were cultured in RPMI-1640 medium with 10% FBS at 37°C. Cells were seeded at a concentration of 2 × 10^4 cells per 60-mm dish (Nunc) and incubated at 37°C for 1 day. Cells were then incubated with drugs (0.1 µM cetuximab, 0.2 µM simvastatin, a combination of 0.1 µM cetuximab and 0.2 µM simvastatin, or PBS) in RPMI-1640 medium with 2% FBS at 37°C for an additional 3 days. Cells were lysed in lysis buffer (20 mM HEPES [pH 7.4], 1% Triton X-100, 1 mM EDTA, 2% EGTA, 1 mM MgCl2, 150 mM NaCl, 10% glycerol, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 mM sodium orthovanadate). Cell lysates were also prepared from frozen xenograft tumors (three tumors per treatment group) grown in mice (described below), which were homogenized and lysed in glass homogenizers (Kontes Glass Co, Vineland, NJ) in ice-cold lysis buffer. Lysates were centrifuged at 15 000g at 4°C for 10 minutes. Protein concentrations in the lysates were measured using bovine serum albumin (Thermo Scientific, Rockford, IL) as the reference. Equal amount of proteins (20 µg per well) from the clarified lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane of 0.45-µm pore size (Upstate Biotechnology, Billerica, MA). After blocking the membranes with 5% nonfat dry milk at room temperature for 2 hours, membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-human BRAF mAb (1:1000 dilution), mouse anti-human BCL2 mAb (1:1000 dilution), rabbit anti-human BAD polyclonal Ab (1:1000 dilution), mouse anti-human BAX mAb (1:1000 dilution), rabbit anti-human BCL2L1 polyclonal Ab (1:1000 dilution), mouse anti-human KRAS mAb (1:1000 dilution), mouse anti-human MAP2K1 mAb (1:1000 dilution), rabbit anti-human AKT1, anti-human MAP2K1 mAb (1:1000 dilution), rabbit anti-human phosphorylated MAP2K1 mAb, and rabbit anti-human KRAS mAb. The membranes were then incubated with secondary antibody conjugates of anti-rabbit and anti-mouse IgG (1:5000 dilution) and incubated at 4°C for 1 hour. The membranes were washed three times in ice-cold PBS until the background was consistent. Proteins were visualized using ECL reagents (Thermo Scientific, Rockford, IL) and exposed to X-ray film (Kodak Biomax, Rochester, NY).
anti-human TUBA1A polyclonal Ab (1:2000 dilution), anti-human phosphorylated AKT1 (Ser473) Ab (1:1000 dilution), anti-human AKT1 Ab (1:1000 dilution), anti-human BCL2L11 Ab (1:1000 dilution), anti-human phosphorylated MAPK 1/2 Ab (1:2000 dilution), anti-human MCL1 Ab (1:1000 dilution), and anti-human phosphorylated MAP2K1 (Ser218/222)/MAP2K2 (Ser222/226) (1:2000 dilution).

After washing with Tris-buffered saline and Tween 20 (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20), membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000 dilution) for 1 hour, and bands were visualized by using ECL Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). To examine whether degradation of BRAF protein was mediated by the ubiquitin–proteasome pathway, DLD1, SW403, HT29, and RKO cells (1 x 10^6 cells per well) were seeded in a six-well plate and treated with 100 µg/mL cycloheximide, a protein synthesis inhibitor (Sigma-Aldrich), and 0.2 µM simvastatin. Immunoblots were performed using mouse anti-human BRAF mAb (1:1000 dilution) incubated for 2 hours and HRP-conjugated anti-mouse secondary antibodies (1:5000 dilution) for 1 hour, and BRAF protein levels were visualized as described above. BRAF levels in the immunoblots were quantified with densitometric analysis. Scanning densitometry was carried out with the Quantity One Program (Bio-Rad, Hercules, CA). BRAF levels were calculated as the ratio of the intensity of BRAF protein to that of TUBA1A. TUBA1A was used as the protein loading control. Densitometric analysis was based on two independent experiments.

**In Vitro BRAF Kinase Assay With Recombinant MAP2K1**

The BRAF kinase assay system from Millipore Upstate Biotechnology was used by following the manufacturer’s instructions, with modifications. The system contained recombinant active BRAF (product 14-530), recombinant inactive MAP2K1 as the substrate (product 14-420), assay dilution buffer I (ADBI, product 20-108), ATP (product 20-113), and anti-phosphorylated MAP2K1 (Ser218/222)/MAP2K2 (Ser222/226) antibody (product 07-461). The DLD1, SW403, HT29, and RKO cells were cultured in RPMI-1640 medium with 10% FBS at 37°C. Cells were seeded at the concentration of 1 x 10^5 cells per 60-mm dish and incubated at 37°C for 24 hours. Cells were then incubated with 0.2 µM simvastatin or PBS in RPMI-1640 medium with 2% FBS at 37°C for an additional 1 hour. Cells were lysed in lysis buffer (20 mM HEPES [pH 7.4], 1% Triton X-100, 1 mM EDTA, 2 mM EGTA, 1 mM MgCl₂, 150 mM NaCl, 10% glycerol, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate). Two hundred and fifty micrograms of protein was incubated with 2 µg of mouse anti-human BRAF mAb (24) and 20 µL of Protein A/G PLUS Agarose beads (Santa Cruz, CA) overnight. Immunoprecipitates were collected by centrifugation at 3000 g for 30 seconds and washed twice with lysis buffer and twice with assay dilution buffer I buffer. After washing the beads, they were incubated with 1 µg of recombinant inactive MAP2K1, 37.5 mM MgCl₂, and 250 µM ATP at 30°C for 30 minutes. After incubation, denaturing and reducing gel loading buffer (final concentration: 125 mM Tris–HCl [pH 6.8], 1% sodium dodecyl sulfate, 5% (vol/vol) glycerol, 0.8% dithiothreitol, and 0.05% bromophenol blue) was added and incubated at 95°C for 5 minutes and then at 4°C for 5 minutes. The resultant reaction product was detected by immunoblot analysis, following the manufacturer’s instructions. Two independent experiments were performed.

**Apoptosis Assay**

The Annexin V fluorescein isothiocyanate (FITC)–labeled Apoptosis Detection Kit I (Pharmingen, San Diego, CA) was used to detect and quantify apoptosis by flow cytometry according to the manufacturer’s instructions. Briefly, DLD1, SW403, HT29, and RKO cells (2 x 10^6 cells) were seeded in 60-mm dishes and cultured overnight in RPMI-1640 medium containing 10% FBS at 37°C. The next day, cells were treated with drugs (PBS, 0.1 µM cetuximab, 0.2 µM simvastatin, a combination of 0.1 µM cetuximab and 0.2 µM simvastatin) in RPMI-1640 medium with 2% FBS for 3 days. After treatment, the cells were harvested in ice-cold PBS and collected by centrifugation at 500 g for 10 minutes. Cells were then resuspended at a density of 1 x 10^6 cells per milliliter in 1× binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, and 2.5 mM CaCl₂) and stained simultaneously with FITC-labeled Annexin V (5 µL) and propidium iodide (10 µL) at room temperature for 15 minutes in the dark. Cells were analyzed using a fluorescence-activated cell sorter flow cytometer (Becton Dickinson, San Jose, CA), and the data were analyzed with CellQuest software.

**Plasmids**

BRAF^{V600E} mutant cDNA was isolated by PCR from a human colon cancer cell line RKO cDNA library by using the following primers: forward, 5′-GGCTTGAGGCCACCATGGGCAGCGTGGACTGGTGAGTGACAGGATCTT-3′ (PstI site included) and reverse, 5′-GGCGTGGACACTGGAGACAGGAAAGCCACCATATC-3′ (SalI site and a stop codon included). The amplified product was digested with PstI and SalI restriction enzymes (Stratagene, La Jolla, CA) and subcloned into the PstI and SalI sites of pCMV-Tag2 (Stratagene). The recombinant construct was verified by nucleotide sequencing. DLD1, HT29, RKO and SW403 cells (4 x 10^5 cells per 60-mm dishes) were transfected with 0.6 µg recombinant or control plasmid using Effectene transfection reagents (Qiagen, Inc.) according to the manufacturer’s instructions and were used for immunoblot analysis 48 hours after transfection.

**RNA Interference and Transfection**

DLD1, SW403, HT29, and RKO cells were transiently transfected with a small interfering RNA (siRNA) that silences expression of BRAF as previously described (25). Cells (4 x 10^5 cells per 60-mm dishes) were transfected with 20 nM siRNAs (Dharmacon, Lafayette, CO) using Effectene transfection reagents (Qiagen, Inc) according to the manufacturer’s instructions and were used for immunoblot analysis 48 hours after transfection. Sequences of the siRNAs used were control nontargeting siRNA (5′-UAG CGACUAACACAUCAA-3′) and BRAF-targeted siRNA (siBRAF) (5′-ACAGAGACCUCAGAGUUA-3′).

**Reverse Transcriptase (RT)–PCR**

DLD1, SW403, HT29, and RKO cells (2 x 10^6 cells) were seeded in 60-mm dishes and cultured overnight at 37°C in RPMI-1640...
A.

<table>
<thead>
<tr>
<th></th>
<th>KRAS mutant</th>
<th>BRAF mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS174T</td>
<td>RKO</td>
</tr>
<tr>
<td>cyto memb</td>
<td>cyto memb</td>
<td>cyto memb</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>Simvastatin</td>
<td>Simvastatin</td>
</tr>
<tr>
<td></td>
<td>cyto memb</td>
<td>cyto memb</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>Simvastatin</td>
<td>Simvastatin</td>
</tr>
<tr>
<td>LoVo (KRAS^G13V/BRAF^wt)</td>
<td>SW403 (KRAS^G12V/BRAF^wt)</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>simvastatin</td>
<td>control</td>
</tr>
<tr>
<td>0 4 8 24</td>
<td>0 4 8 24</td>
<td>0 4 8 24</td>
</tr>
<tr>
<td></td>
<td>BRAF</td>
<td>BRAF</td>
</tr>
<tr>
<td></td>
<td>TUBA1A</td>
<td>TUBA1A</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>LoVo (KRAS^G13V/BRAF^wt)</th>
<th>SW403 (KRAS^G12V/BRAF^wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>simvastatin</td>
<td>control</td>
</tr>
<tr>
<td>0 4 8 24</td>
<td>0 4 8 24</td>
<td>0 4 8 24</td>
</tr>
<tr>
<td>Density (BRAF/TUBA1A)</td>
<td>Density (BRAF/TUBA1A)</td>
<td></td>
</tr>
<tr>
<td>Time (h)</td>
<td>Time (h)</td>
<td></td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th></th>
<th>HT29 (KRAS^wt/BRAF^V600E)</th>
<th>RKO (KRAS^wt/BRAF^V600E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>simvastatin</td>
<td>control</td>
</tr>
<tr>
<td>0 4 8 24</td>
<td>0 4 8 24</td>
<td>0 4 8 24</td>
</tr>
<tr>
<td>Density (BRAF/TUBA1A)</td>
<td>Density (BRAF/TUBA1A)</td>
<td></td>
</tr>
<tr>
<td>Time (h)</td>
<td>Time (h)</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
medium containing 10% FBS. The next day, cells were treated with drugs (PBS, 0.1 µM cetuximab, 0.2 µM simvastatin, and a combination of 0.1 µM cetuximab and 0.2 µM simvastatin) in RPMI-1640 medium with 2% FBS for 2 or 3 days. Total cellular RNA was extracted using RNeasy mini kit (Qiagen, Inc) and treated with DNase I (Qiagen, Inc). One microgram of RNA was converted to cDNA using Omniscript RT kit (Qiagen, Inc). The primer sequences designed from the coding region of human BRAF cDNA are as follows: forward, 5'-CCCAGAGTGCTG TGCTGTTT-3', and reverse, 5'-TGAAGGAGACGGACTGG TGA-3'. The PCR conditions were as follows: 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by a final incubation at 72°C for 10 minutes.

**Xenograft Study**

Female BALB/c nude mice, 4–6 weeks old, were obtained from Orient Bio, Inc (Seongnam, Korea) or Jackson Laboratories (Bar Harbor, ME). Mice (n = 5 per cell line per treatment group) were implanted subcutaneously with DLD1 (3.0 × 10^6 cells), SW403 (1.0 × 10^7 cells), or HT29 (1.0 × 10^7 cells) in 100 µL volume using a 23-gauge needle. Each mouse received two subcutaneous injections in the bilateral flank for the development of two tumors (10 tumors per treatment group). Two weeks after implantation, mice (n = 5 mice per cell line per treatment group) were assigned into four groups—PBS only, cetuximab, simvastatin, or a combination of cetuximab and simvastatin. The mice were treated twice per week with intraperitoneal injection of 10 mg/kg cetuximab in PBS and/or once daily orally with 2 mg/kg simvastatin dissolved in PBS. Tumor diameters were serially measured with a digital caliper (Precision, Vitoria, Spain) every 2–3 days, and tumor volumes were calculated using the following formula: \( V = (L \times W^2)/2 \), where \( V \) = volume (in cubic millimeters), \( L \) = length (in millimeters), and \( W \) = width (in millimeters). The mice were killed by CO2 inhalation, and the tumors (three tumors per treatment group) were resected on day 15 and frozen in liquid nitrogen until later use for immunoblot analyses. Mice were handled at the institute’s (Samsung Medical Center, Seoul, Korea) animal facility, and all treatments were in accordance with the legal and institutional guidelines.

**Statistical Analysis**

The mean tumor volume in each mouse for each cell line was computed for growth curves (the mean tumor volume in each group = total volume from all mice per treatment group divided by number of mice). Statistical significance of the differences between treatment groups for cell growth, colony formation, apoptosis, and tumor volume was calculated using the Student t test. All P values less than .05 were considered to be statistically significant. All statistical tests were two-sided.

**Results**

**Antitumor Effect of Simvastatin and Cetuximab on KRAS Mutant CRC Cells In Vitro**

We screened a panel of CRC cell lines with varying genotypes by direct sequencing to confirm KRAS or BRAF mutations in these cells (Supplementary Figure 1, available online). Eight CRC cell lines had KRAS mutations—LS153 (KRAS\(^{G12D}\)), LS174T (KRAS\(^{G12D}\)), DLD1 (KRAS\(^{G12D}\)), LoVo (KRAS\(^{G12D}\)), SW403 (KRAS\(^{G12V}\), SW480 (KRAS\(^{G12V}\)), SNU175 (KRAS\(^{A146T}\)), and LS1034 (KRAS\(^{A146T}\)), and two of the four KRAS wild-type CRC cells had BRAF\(^{V600E}\) mutation—DiFi (KRAS\(^{BRAF^+}\)), SW48 (KRAS\(^{BRAF^+}\)), HT29 (KRAS\(^{BRAF^+}\)), and RKO (KRAS\(^{BRAF^+}\)). As demonstrated in many studies, KRAS mutation and BRAF\(^{V600E}\) mutation are mutually exclusive in CRC (12,26,27). Although the clinical importance needs to elucidated, there are several reports suggesting that concomitant KRAS and BRAF mutations may have a prognostic role in CRC patients (28,29).

We assessed whether addition of simvastatin potentiated the antitumor activity of cetuximab in the above-mentioned CRC cell lines. We tested a range of concentrations of cetuximab that can be used in combination with 0.2 µM simvastatin to effectively investigate the antitumor activity of the drugs in combination (17,22). Cells were exposed to 0.03–1.0 µM cetuximab, 0.2 µM simvastatin, or a combination of 0.03–1.0 µM cetuximab and 0.2 µM simvastatin for 3 days, and quantitative analysis of cell proliferation was done using the trypan blue staining method (Figure 1, A and B). Compared with cetuximab alone, the combination of cetuximab (at all concentrations) and simvastatin showed consistently and statistically significantly (\( P < .001 \)) reduced cell growth in all eight KRAS mutant cell lines (eg, DLD1, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 89.4% vs 58.9%, difference = 30.5%, 95% CI = 25.9% to 35.1%, \( P < .001 \); SW403, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 87.2% vs 62.8%, difference = 12.9%, 95% CI = 17.3% to 31.6%, \( P = .002 \)) (Figure 1, A). Besides cell lines harboring KRAS mutations at codons 12 and 13, uncommon KRAS mutations in cell lines SNU175 (KRAS\(^{A146T}\)) and LS1034 (KRAS\(^{A146T}\)) also showed similar antitumor effects of cetuximab and simvastatin combination (SNU175, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 83.9% vs...
Figure 3. Effect of simvastatin on cell proliferation, v-raf murine sarcoma viral oncogene homolog B1 (BRAF) activity, and signaling pathways downstream of BRAF in v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutant and BRAF mutant cells. A) BRAF\textsuperscript{V600E} was overexpressed in two KRAS mutant cells, DLD1 and SW403 by transient transfection to generate DLD1-BRAF\textsuperscript{V600E} and SW403-BRAF\textsuperscript{V600E} cells. Top panel: Cell proliferation assay of DLD1-BRAF\textsuperscript{V600E} and SW403-BRAF\textsuperscript{V600E} cells treated with 0.03–10 µM cetuximab for 3 days and stained with 0.4% trypan blue. Half maximal inhibitory concentration was assessed, and results of cell counts are expressed as percentage of cell proliferation using the control phosphate-buffered saline (PBS) as reference. Bottom panel: Cell proliferation assay of DLD1-BRAF\textsuperscript{V600E} and SW403-BRAF\textsuperscript{V600E} cells treated with BRAF-targeted siRNA (siBRAF) and with 0.03–10 µM cetuximab for 3 days. The graphs show the means and 95% confidence intervals of an experiment performed in triplicate.

B) Effect of simvastatin on BRAF enzymatic activity was assessed by an in vitro kinase assay using recombinant mitogen-activated protein kinase kinase 1 (MAP2K1) as a substrate. Two KRAS mutant (DLD1 and SW403) and two BRAF mutant (HT29 and RKO) cells were treated with 0.2 µM simvastatin for 1 hour and analyzed for MAP2K1 level by immunoblot assay. Control cells were treated with PBS. Two independent experiments were performed.

C) Effect of simvastatin on MAPK1 and v-akt murine thymoma viral oncogene homolog 1 (AKT1) signaling pathways in DLD1, SW403, HT29, and RKO cells. Cells were treated with 0.2 µM simvastatin for 1 hour, lysed, and analyzed for phosphorylated levels of MAPK1/2, MAP2K1, and AKT1 by immunoblotting. All control cells were treated with PBS.

57.8%, difference = 26.1%, 95% CI = 18.4% to 33.8%, \(P = .0007\); LS1034, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 95.2% vs 60.2%, difference = 35.0%, 95% CI = 24.2% to 45.7%, \(P = .0008\) (Figure 1, A). In cetuximab-sensitive DiFi and SW48 cells that have KRAS wild-type and BRAF wild-type genotypic profiles (Supplementary Figure 1, available online), the combination of 0.03–0.1 µM cetuximab and 0.2 µM simvastatin showed a greater inhibitory effect on cell growth compared with treatments with cetuximab or simvastatin as single agents (Figure 1, B). In contrary, in HT29 and RKO cells (KRAS wild-type and BRAF\textsuperscript{V600E} mutant), which are well characterized as refractory to cetuximab (5), the addition of 0.2 µM simvastatin did not effectively reverse cetuximab resistance (HT29, 0.5 µM cetuximab vs 0.5 µM cetuximab + 0.2 µM simvastatin, mean growth = 78.3% vs 82.5%, difference = -4.3%, 95% CI = -10.4% to 1.9%, \(P = .144\); RKO, 0.3 µM cetuximab vs 0.3 µM cetuximab + 0.2 µM simvastatin, mean growth = 94.4% vs 99.6%, difference = -5.3%, 95% CI = -10.9% to 0.4%, \(P = .076\) (Figure 1, B). We repeated the same set of experiments with a different lipid-lowering agent, lovastatin (22), and observed similar inhibitory effects on tumor cell viability of simvastatin in lovastatin-treated CRC cells (Supplementary Figure 2, available online).
Given the fact that cetuximab is combined with irinotecan in clinical practice to treat metastatic CRC patients (3,30), we added SN38, an irinotecan metabolite (31) to cetuximab and simvastatin and investigated the antitumor effect in vitro (Figure 1, C). The addition of simvastatin to cetuximab and SN38 combination in KRAS mutant cells demonstrated a greater inhibitory effect on cell growth compared with treatment of cells with SN38 and cetuximab combination for 3 days (SW403, 0.1 µM cetuximab + 0.3 nM SN38 vs 0.1 µM cetuximab + 0.3 nM SN38 + 0.2 µM simvastatin, mean growth = 50.0% vs 18.7%, difference = 31.4%, 95% CI = 19.1% to 43.6%, \(P = .002\)) (Figure 1, C).

Based on these results, we speculated that the addition of simvastatin to cetuximab or cetuximab and irinotecan combination may potentially enhance the antitumor effect in KRAS mutant colon cancer. Because anchorage-dependent colony formation assay in vitro remains one of the most effective tools to predict tumorigenic growth in vivo, we performed this assay to assess the antitumor activity of simvastatin. The addition of 0.2 µM simvastatin to 0.1 µM cetuximab showed a statistically significantly enhanced inhibition of tumor cell colony formation in KRAS mutant DLD1 and SW403 cells (DLD1, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 87.3% vs 62.2%, difference = 25.0%, 95% CI = 5.6% to 44.4%, \(P = .023\); SW403, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 89.2% vs 52.8%, difference = 36.4%, 95% CI = 20.3% to 52.5%, \(P = .003\)) (Figure 1, D, and Supplementary Figure 3, available online). However, cetuximab resistance was persistently observed in HT29 and RKO cells (KRAS\(^{wt}\), BRAF\(^{V600E}\)).
Figure 5. Effect of cetuximab and simvastatin combination on tumor growth in vivo. Effect of cetuximab and simvastatin was tested on v-Kras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutant tumors in vivo. A) BALB/c nude mice were injected subcutaneously in the bilateral flank (two injections per mouse) with DLD1 cells. Two weeks after the injection, mice (five mice and 10 tumors per treatment group) were assigned into four groups—phosphate-buffered saline (PBS) only, cetuximab, simvastatin, or a combination of cetuximab and simvastatin (cetuximab + simvastatin or cetuximab+s). The mice were treated twice per week with intraperitoneal injection of 10 mg/kg (continued)
of simvastatin (HT29, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 97.1% vs 95.4%, difference = 1.7%, 95% CI = 10.4% to 13.8%, P = .716; RKO, 0.1 µM cetuximab vs 0.1 µM cetuximab + 0.2 µM simvastatin, mean growth = 94.2% vs 85.5%, difference = 8.6%, 95% CI = −3.4% to 20.7%, P = .118) (Figure 1, D). These results showed that the addition of simvastatin to cetuximab reversed cetuximab resistance in KRAS mutant BRAF wild-type CRC cells but not in BRAF\(^{V600E}\)-mutant CRC cells. This suggested that simvastatin may sensitize cancer cells to cetuximab via modulation of BRAF activity.

**Effect of Simvastatin on BRAF Expression and Activity in KRAS Mutant CRC Cells In Vitro**

The regulation of BRAF by RAS has not been studied extensively, but it is reported that the association of RAS with downstream BRAF results in activation of BRAF (32). We first tested whether simvastatin had an effect on RAS and BRAF protein expression. Three KRAS mutant cell lines (LS174T, SW403, and DLD1) and two BRAF mutant cell lines (RKO and HT29) were treated with 0.2 µM simvastatin for 3 days, and KRAS and BRAF levels were analyzed in the cytosolic and membrane fractions by immunoblot assay (Figure 2, A). There was no alteration in the membranous KRAS level after treatment with simvastatin, regardless of KRAS or BRAF mutational status. However, cytoplasmic BRAF level decreased when KRAS mutant cells (LS174T, SW403, and DLD1) were treated with 0.2 µM simvastatin for 3 days. In contrast, there was no alteration in BRAF level in the BRAF\(^{V600E}\) mutant cells, RKO and HT29 (Figure 2, A). To investigate whether BRAF expression was regulated at transcriptional or post-transcriptional level, we treated DLD1, SW403, HT29, and RKO cells with a combination of 0.1–0.3 µM cetuximab and 0.2 µM simvastatin for 3 days and analyzed the level of BRAF mRNA by RT-PCR (Supplementary Figure 4, available online). We noted that there was no alteration in BRAF mRNA expression.

Next, we assessed any changes in the level of BRAF by first treating LoVo, SW403, HT29, and RKO cells with 0.2 µM simvastatin for 3 days and then with 100 µg/mL cycloheximide to inhibit protein synthesis (Figure 2, B and C). The turnover rate of BRAF was assessed by measuring the protein half-life at various time points after cycloheximide treatment. The level of BRAF declined more rapidly in simvastatin-treated (0.2 µM simvastatin for 3 days) than untreated cells (DLD1 control vs DLD1-BRAF\(^{V600E}\), IC\(_{50}\) = 1.892 [95% CI = 1.436 to 2.492 µM] vs not reached; SW403 control vs SW403-BRAF\(^{V600E}\), IC\(_{50}\) = 0.2869 [95% CI = 0.257 to 0.320 µM] vs not reached) (Figure 3, A, top panel). However, when DLD1 cells overexpressing BRAF\(^{V600E}\) were treated with siBRAF, cells became sensitive to cetuximab (DLD1-BRAF\(^{V600E}\), control vs siBRAF-treated, IC\(_{50}\) = 1.971 [95% CI = 1.432 to 2.713 µM] vs IC\(_{50}\) = 0.260 [95% CI = 0.188 to 0.358 µM]) (Figure 3, A, bottom panel). In a parallel experiment, we transiently overexpressed BRAF\(^{V600E}\) in HT29 and RKO cells (BRAF\(^{V600E}\) overexpression was confirmed by immunoblotting, data not shown) that express KRAS wild-type and BRAF\(^{V600E}\) naturally and observed similar results (Supplementary Figure 5, available online).

Next, we analyzed the effect of simvastatin on BRAF enzymatic activity by an in vitro kinase assay using recombinant MAP2K1 as the substrate. DLD1, SW403, HT29, and RKO cells were treated with 0.2 µM simvastatin for 1 hour and analyzed for MAP2K1 level by immunoblot assay (Figure 3, B). The level of phosphorylated MAP2K1 decreased substantially after 1 hour of treatment with 0.2 µM simvastatin. We also assessed whether simvastatin treatment affected the downstream targets of MAP2K1 and found that simvastatin treatment reduced the levels of phosphorylated MAPK1/2 and phosphorylated MAPK2 but did not alter the level of phosphorylated or total AKT1 (Figure 3, C).

**Effect of Simvastatin on Apoptosis**

Recently, BRAF has been shown to coordinately regulate two pro-apoptotic proteins—BAD and BCL2L11, which render tumor cells susceptible to apoptosis (25). Importantly, BRAF\(^{V600E}\) mutation was shown to increase resistance to apoptosis by promoting MAPK1-dependent phosphorylation of BCL2L11 and BAD (25,33). Based on our results in this study, we hypothesized that cetuximab in PBS and/or once daily orally with 2 mg/kg simvastatin. Tumor diameters were measured every 2–3 days with a digital caliper, and graphical representation of tumor volumes on different days after treatment is shown. The error bars represent the 95% confidence intervals of the mean volume. P values were calculated using two-sided Student t test. B) Representative DLD1 xenograft tumors resected on day 15 (four tumors per group) showing the difference in tumor volumes between individual drugs and combination drugs. C) Mice were injected subcutaneously in the bilateral flank (two injections per mouse) with SW403 cells, tumors were grown and treated with drugs, and tumor volumes were analyzed, similar to (A). D) Representative SW403 xenograft tumors resected on day 23 (five tumors per group). E) Effect of cetuximab and simvastatin on v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutant xenograft tumors. Injection with HT29 cells, tumor growth, treatment with drugs, and analysis of tumor volumes were similar to (A) and (C). F) Representative HT29 xenograft tumors resected on day 15 (n = 5 mice per group). G) Tumors were analyzed for pro-apoptotic (BAD and BCL2L11) and anti-apoptotic (BCL2L1) proteins by immunoblot assay. BRAF levels were also checked. TUBA1A was used as the protein loading control.
Simvastatin may suppress BRAF expression in KRAS mutant CRC cells and subsequently sensitize KRAS mutant cells to cetuximab via inducing BCL2L11 and BAD. The induction of BCL2L11 and BAD proteins is a well-recognized process for tumor cell apoptosis (34,35). Therefore, we analyzed the levels of BAD and BCL2L11 in KRAS mutant DLD1 and SW403 cells and observed that the levels were substantially decreased in these cells (Figure 4, A). When cells were treated with a combination of 0.1 µM cetuximab and 0.2 µM simvastatin for 3 days, the protein levels of BCL2L11 and BAD substantially increased in DLD1 and SW403 cells, but there was no increase in HT29 and RKO cells (Figure 4, A, left panel) (33). There were no changes in the levels of proapoptotic BAX, or antiapoptotic BCL2, BCL2L1 or MCL1 proteins in KRAS mutant or BRAFV600E mutant cells treated with cetuximab and simvastatin combination. In concordance with these experiments, BCL2L11 and BAD levels were not changed in BRAFV600E-overexpressed HT29 or RKO cells when treated with 0.1 µM cetuximab and 0.2 µM simvastatin for 3 days (Supplementary Figure 6, available online). Furthermore, the induction of apoptosis of DLD1 and SW403 cells was confirmed using Annexin V staining (Figure 4, B). Taken together, our results indicated that simvastatin reduced BRAF enzymatic activity in KRAS mutant, but not BRAF mutant, CRC cells.

**Effect of Simvastatin and Cetuximab on Growth of KRAS Mutant CRC Tumors In Vivo**

To determine whether the enhanced antitumor action of the drug combination was also observed in vivo, we implanted DLD1 or SW403 tumors in mice and assigned to the following four groups (n = 5 mice per cell line per treatment group)—untreated control, cetuximab, simvastatin, or a combination of cetuximab and simvastatin. The combination of cetuximab and simvastatin showed a statistically significantly reduced tumor volume compared with cetuximab alone in KRAS mutant DLD1 (Figure 5, A and B) and SW403 (Figure 5, C and D) xenografts (for DLD1 tumors, cetuximab vs cetuximab + simvastatin, mean tumor volume on day 15 = 49.4 vs 20.2 cm³, mean difference = 29.2 cm³, 95% CI = 19.7 to 38.5, P = <.001). In contrast, there was no statistically significant antitumor activity with simvastatin, cetuximab, or cetuximab and simvastatin combination in BRAF mutant xenograft tumors (Figure 5, E and F). Next, xenograft tumors were extirpated and protein expression was analyzed from three tumors per cell line per treatment group. Two proapoptotic proteins, BCL2L11 and BAD, were markedly induced on treatment with simvastatin and cetuximab combination (Figure 5, G). Hence, we observed that the addition of 0.2 µM simvastatin to cetuximab could also overcome cetuximab resistance in KRAS mutant CRC tumors in vivo.

**Discussion**

In this study, we demonstrated that the addition of 0.2 µM simvastatin to cetuximab statistically significantly enhanced antitumor activity in KRAS mutant but not in BRAF mutant CRC cells. Simvastatin could overcome cetuximab resistance via modulation of BRAF activity and induced apoptosis by inducing the proapoptotic proteins BCL2L11 and BAD. In a mouse model, the growth of KRAS mutant xenograft tumors was statistically significantly inhibited when simvastatin was added to cetuximab during treatment; however, the antitumor effect was not observed in KRAS wild-type BRAFV600E mutant xenograft tumors. To our knowledge, this is the first report to show the potential role of simvastatin as an anticancer drug that may overcome cetuximab resistance in KRAS mutant CRC cells.

Several studies have reported the antitumor activity of statins via inhibiting cellular proliferation or inducing apoptosis of various cancer cells (16,17,36–38). However, most studies used high concentrations of statin ranging from 2 to 100 µM in vitro to demonstrate an antitumor effect (36,37,39). Of note, to reach serum concentrations of 2–20 µM, simvastatin has to be administered at a dose of 20–200 mg kg⁻¹ d⁻¹, a dose not feasible for human use. We have previously reported that high-dose lovastatin monotherapy produced a high toxicity profile with a low response rate in metastatic gastric cancer patients, which precluded lovastatin from further clinical use as an antitumor drug. Our current study is important because we demonstrated antitumor effect of simvastatin using a dose level that is equivalent to cardiovascular therapeutically useful dose level in humans.

Recently, the potential beneficial effect of statins beyond lipid-lowering effect has been underscored in several epidemiology studies (40–42). One of the studies, the Molecular Epidemiology of Colorectal Cancer (MECC) study (42), demonstrated that the use of statins for more than 5 years was associated with a 47% relative reduction in the risk of CRC after adjustment for other known risk factors such as age, sex, ethnic group, hypercholesterolemia, history of CRC in a first-degree relative, and level of vegetable consumption. Although the results from these studies support the hypothesis that statins may reduce the risk of cancer, overall results from observational studies still remain elusive.

Recently, BAD and BCL2L11 were shown to be major BRAF-responsive proteins regulating apoptosis in melanoma cells (33). One previous study showed that BRAF-MAP2K1 signaling regulated the phosphorylation of BAD and decreased the mRNA expression of BAD (25). Additionally, BRAF-MAP2K1 signaling regulated the expression of BCL2L11, mainly through control of its protein turnover (25). In concordance, the induction of simvastatin in our study included BAD and BCL2L11 proteins in KRAS mutant, but not in BRAF mutant, CRC cells. Based on our experiments, simvastatin modulated BRAF protein stability in KRAS mutant but not in BRAF mutant cells and reduced phosphorylated MAPK1 and phosphorylated MAP2K1 protein levels implicating the MAPK1-MAP2K1 axis in the activity of simvastatin.

Our study has a few limitations. The mice were not randomly assigned to the different treatment groups or the researchers blinded to treatments, per Animals in Research Reporting In Vivo Experiments guidelines (43). However, we attempted to assign mice with approximately similar mean tumor volumes to each treatment group. In addition, the mechanism of action for simvastatin in modulating BRAF protein needs to be defined in future studies. Although based on the fact that simvastatin did not reverse cetuximab resistance in BRAF mutant CRC cells, we hypothesized and demonstrated in this study that simvastatin decreased BRAF protein stability and BRAF enzymatic activity, the precise mechanism responsible for the effect of simvastatin need to be elucidated in future studies.
The results of this study indicate that the addition of simvastatin at a dose (40–80 mg once daily) used in patients with cardiovascular disease may overcome cetuximab resistance in KRAS mutant CRC via modulating BRAF protein stability and inducing apoptosis via BCL2L11 and BAD proteins. We have previously demonstrated that the combination of simvastatin to the standard FOLFIRI regimen was safe with no additional toxicity profile (23). Based on these preclinical and clinical studies, we are currently planning a phase II study (clinicaltrials.gov registration number NCT01281761) to evaluate the effect of addition of simvastatin to cetuximab and irinotecan combination in metastatic CRC patients with KRAS mutations who have failed standard chemotherapy, including oxaliplatin- and irinotecan-based combination chemotherapy.

References


**Funding**

Merck Serono and Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (0412-CR01-0704-0001 to WKK); Samsung Biomedical Research Institute (SBRI C-B-0220 to JL and SBRI C-B-0-201 to WKK).

**Notes**

J. Lee and I. Lee contributed equally to this work.

We thank Dr Soonmyung Paik for helpful discussions and critical review of the article. The authors are solely responsible for the design of the study, the analysis and interpretation of the data, the writing of the article, and the decision to submit the article for publication.

**Affiliations of authors:** Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea (JL, BH, JOP, JJ, WKK); Biomedical Research Institute, Samsung Medical Center, Seoul, Korea (CP, IL).