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Antitumor Effects of Pan-RAF Inhibitor LY3009120 Against Lung Cancer Cells Harboring Oncogenic *BRAF* Mutation

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Abstract. *Background/Aim: The therapeutic strategy for patients with non-small-cell lung cancer (NSCLC) harboring the BRAF non-V600E mutation has not been established. LY3009120, a newly discovered pan-RAF inhibitor, has shown strong antitumor effects in cancers with various BRAF genotypes. This study investigated the antitumor effects of LY3009120 in NSCLC cells harboring the BRAF non-V600E mutation. Materials and Methods: We examined the antitumor effects of LY3009120 by MTS assay and flow cytometry. We analyzed the expression status of proteins by western blot. The mouse xenograft models were used for the in vivo experiments. Results: LY3009120 suppressed BRAF-related downstream pathway molecules and induced cleavage of poly ADP-ribose polymerase in all examined NSCLC cell lines. LY3009120 also inhibited in vivo tumor growth in NSCLC cells harboring the BRAF non-V600E mutation. Conclusion: LY3009120 is a potent therapeutic agent for patients with BRAF non-V600E mutant NSCLC.*

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Various molecular alterations, including driver mutations in oncogenes, are found in lung cancer cases (2). *BRAF* mutations occur in 0.5-3% of lung adenocarcinomas (3-5) and act as oncogenic drivers. About 30-50% of those mutations are *BRAF* V600E, and the remaining proportions are non-V600E mutations (6-9). Dabrafenib (BRAF inhibitor), when used in combination with trametinib (MEK inhibitor), has shown substantial antitumor effects in patients with non-small-cell lung cancer (NSCLC) harboring *BRAF* V600E mutations in clinical trials (10, 11). However, there are few reports of effective targeted therapy in patients with NSCLC harboring *BRAF* non-V600E mutations. Recent studies reveal that the *BRAF* V600E protein functions as a monomer, whereas most of the *BRAF* non-V600E mutant proteins function as dimers. The first-generation *BRAF* inhibitors, vemurafenib and dabrafenib, have been reported not to be effective inhibitors of BRAF dimers (12-15). LY3009120, a newly discovered pan-RAF inhibitor, showed strong antitumor effects in *BRAF*-mutant cancers, such as melanoma, colorectal, and pancreatic cancers in preclinical studies (16-19). LY3009120 has been reported to effectively inhibit active RAF dimers with minimal paradoxical activation (16, 20). These facts suggest that LY3009120 has a therapeutic potential not only for *BRAF* V600E-mutant NSCLC, but also other types of *BRAF*-mutant NSCLC. In this study, we evaluated the antitumor effects of LY3009120 in several types of *BRAF*-mutant NSCLC cells.

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Materials and Methods

Cell culture and reagents. The *BRAF*-mutant NSCLC cell lines H2087 (*BRAF* L597V), H1395 (*BRAF* G469A), H1666 (*BRAF* G466V), the *BRAF* mutant colon cancer cell line HT29 (*BRAF* V600E), and one normal human bronchial epithelial cell line (BEAS-2B) were purchased from the American Type Culture Collection (Manassas, VA, USA) and authenticated by short tandem repeat (STR) DNA analysis (BEX Co. Ltd, Tokyo, Japan). H2087 and H1395 were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum, and H1666,

HT29, and BEAS-2B were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37˚C in a humidified incubator under 5% CO_2 gas.

Plasmid constructs and transfection. Human cDNAs encoding full-length *BRAF* (wild-type and its variants, G469V and V600E) were inserted into the pIDT-SMART (C-TSC) vector, pCMViR-TSC (21). BEAS-2B cells were stably transfected with the mammalian expression vectors using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol.

Cell growth-inhibition assay. The degree of sensitivity to the drug was determined *via* a modified MTS assay. Briefly, cells (3×10^3 cells per well) were seeded on 96-well plates. After exposure to LY3009120 for 72 h (four-fold serial dilutions with a maximum concentration of 10 μM), 20 μl of CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was added to each well. The anti-proliferative activity of each drug is presented as the concentration of the drug required for 50% inhibition (IC_{50}).

Western blot analysis. All cell lines were cultured in 10-cm dishes for 24 h, then treated with dimethyl sulfoxide (DMSO) (Wako, Osaka, Japan) as a control or with 0.5 and 1 μM LY3009120 for 6 h. Subsequently, total cell lysates were extracted using a mixture of radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich), and a complete Mini Protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The primary antibodies used were against BRAF, phospho-BRAF, protein kinase B (AKT), phospho-AKT (Ser473), MEK, phospho-MEK, mitogen-activated protein kinase (MAPK), phospho-MAPK, poly ADP-ribose polymerase (PARP) (Cell Signaling Technology, Danvers, MA, USA), and β-actin (used as an internal control) (Merck Millipore, Burlington, MA, USA). The secondary antibodies were as follows: goat anti-mouse or anti-rabbit immunoglobulin G (IgG)-conjugated horseradish peroxidase (Cell Signaling Technology). Proteins were detected using the ECL Prime Western Blotting Detection System (GE Healthcare, Amersham, UK) and LAS-3000 (Fujifilm, Tokyo, Japan). The relative band intensities were assessed by densitometric analysis using the ImageJ software (National Institute of Health, Bethesda, MD, USA).

Cell cycle analysis. The cell cycle distribution was assessed using a propidium iodide staining-based assay (CycleTEST PLUS DNA reagent kit; BD Biosciences, Franklin Lakes, NJ, USA) and BD Accuri C6 flow cytometer (BD Biosciences). The cells were processed in accordance with the manufacturer's protocol, and cell cycle analysis was performed.

Tumor cell implantation experiments. The protocol was approved by the Institutional Animal Care and Use Committee, Okayama University (permission number: OKU-2018839). All experimental animal procedures were conducted in accordance with the recent legislation of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We subcutaneously injected 5×10^6 H2087 cells into the left flank of female BALB/c nude mice (5 weeks old; n=5 per group) purchased from Charles River Laboratories (Yokohama, Japan). Tumor volumes were calculated using the formula $V=1/2ab^2$ (*a*: the long diameter, *b*: the short diameter of the tumor). When the tumor volume exceeded approximately 150 mm^3, the mice were randomly allocated to 2 groups: a control group and a LY3009120 (20 mg/kg) group. The dose of LY3009120 was selected based on the results of previous reports (13-16). The drug was suspended in 0.5 w/v methyl cellulose and was orally administered 3 days per week and 2 times per day for 4 weeks. Body weights were measured three times per week and tumor volumes were measured at equal frequency using an electric caliper. After 4 weeks of treatment, the mice were euthanized, and the tumors were surgically removed.

Statistical analysis. All statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for the program R (The R Foundation for Statistical Computing, Vienna, Austria). The software is a modified version of R commander software (version 2.4-0) that contains additional statistical functions frequently used in biostatistics (22). Data from two groups were compared using a t-test. All tests were two-sided, and probability values <0.05 indicated statistically significant differences. The results are presented as the mean ± standard error (SE).

Results

BRAF mutations activate BRAF signaling, which is inhibited by LY3009120. To investigate whether *BRAF* alterations activate BRAF signaling pathway, normal bronchial epithelial cells (BEAS-2B) were stably transfected with vectors containing wild-type *BRAF* or two *BRAF* mutations (G469V and V600E) (Figure 1). We examined the sensitivity of BEAS-2B cells exogenously expressing wild-type or mutant BRAF to LY3009120. The transfected cells were treated with 0, 0.5, and 1 μM of LY3009120 for 6 h. LY3009120 strongly inhibited the phosphorylation of MEK and MAPK compared to untreated cells (Figure 2A). These results suggest that the *BRAF* mutation activated downstream signals, and LY3009120 treatment had an inhibitory effect on these signals, regardless of the *BRAF* mutation genotypes examined.

LY3009120 inhibits the growth of BRAF-mutant cancer cell lines. The antitumor activity of LY3009120 against *BRAF*-mutant cancer cell lines was examined. The cell lines used in this study were HT29, H2087, H1395, and H1666. The detailed *BRAF* mutation profiles of these four cell lines are presented in Table I. The proliferation of *BRAF*-mutant cancer cell lines was inhibited by LY3009120, and the IC_{50} values for LY3009120 ranged from 107 to 247 nM (Table I). LY3009120 demonstrated a potent antitumor effect on *BRAF*-mutant cancer cells, regardless of the mutation genotypes examined.

LY3009120 suppresses BRAF-related downstream pathway molecules in BRAF-mutant cancer cell lines. We investigated alterations in downstream pathway molecules, such as phosphorylated MEK and MAPK following LY3009120 treatment by western blot. Cells were treated with 0, 0.5, and

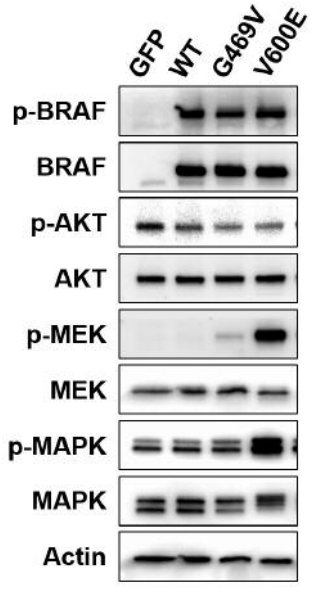

Figure 1. *Overexpression of wild-type or mutant BRAF activates BRAF signaling. BEAS-2B cells were stably transfected with GFP, WT BRAF, G469V, or V600E mutants. BEAS-2B cells exogenously overexpressing WT BRAF or mutants showed constitutive auto-phosphorylation of BRAF and activation of downstream signaling. Cells were cultured in media without fetal bovine serum, and the cell extracts were analyzed by Western blot analysis. GFP: Green fluorescent protein; WT: wild-type; p-: phosphorylated.*

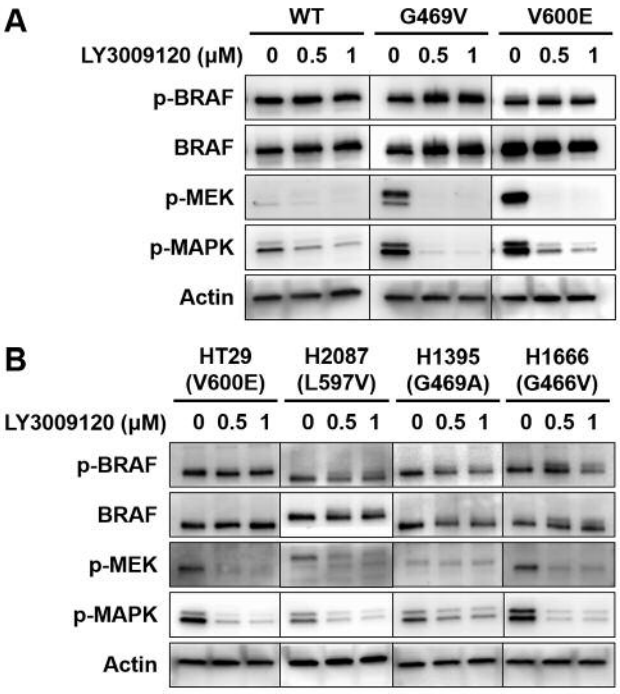

Figure 2. *LY3009120 suppresses BRAF-related downstream pathway molecules in BRAF-transfected cells (A) and BRAF-mutant cancer cells (B). Cells were treated with the indicated concentration of LY3009120 for 6 h and subjected to western blot analysis.*

Table I. *Characteristics and LY3009120 concentration required for 50% inhibition (IC_{50}) in cell lines.*

Cell line	Organ	Histologic subtype	*BRAF* mutation	IC_{50} of LY3009120 (nM)
HT29	Colon	AD	V600E	147.8
H2087	Lung	AD	L597V	107.4
H1395	Lung	AD	G469A	199.6
H1666	Lung	BA	G466V	247.1

AD: Adenocarcinoma; BA: bronchioloalveolar carcinoma.

1 μM of LY3009120 for 6 h. LY3009120 suppressed the phosphorylation of BRAF-related downstream pathway molecules MEK and MAPK in *BRAF* mutation cancer cells (Figure 2B).

LY3009120 induces cell cycle arrest and apoptosis in BRAF-mutant cancer cell lines. The effects of LY3009120 on the cell cycle and apoptosis in *BRAF*-mutant cancer cells (HT29, H2087, H1395, and H1666) were also examined to elucidate the mechanism underlying the growth inhibition. After 48 h treatment with 1 μM LY3009120, cells were analyzed by flow cytometry. To assess the cell cycle distribution, the sub-G1 fraction was excluded, and the percentage of cells in the G1 phase was measured (Figure 3A). LY3009120 treatment caused a significant increase in the number of cells in the G1 phase compared to untreated cells. Subsequently, after a 72-h treatment with 1 μM LY3009120, we evaluated the expression of cleaved PARP as an apoptosis marker by western blot. LY3009120 induced apoptosis in *BRAF*-mutant cancer cell lines (Figure 3B). These results suggest that LY3009120 inhibits proliferation *via* G1 arrest and apoptotic cell death in *BRAF*-mutant cancer cells (HT29, H2087, H1395, and H1666).

Antitumor effects of LY3009210 in a xenograft mouse model of BRAF-mutant lung cancer. Using the *in vitro* data, we

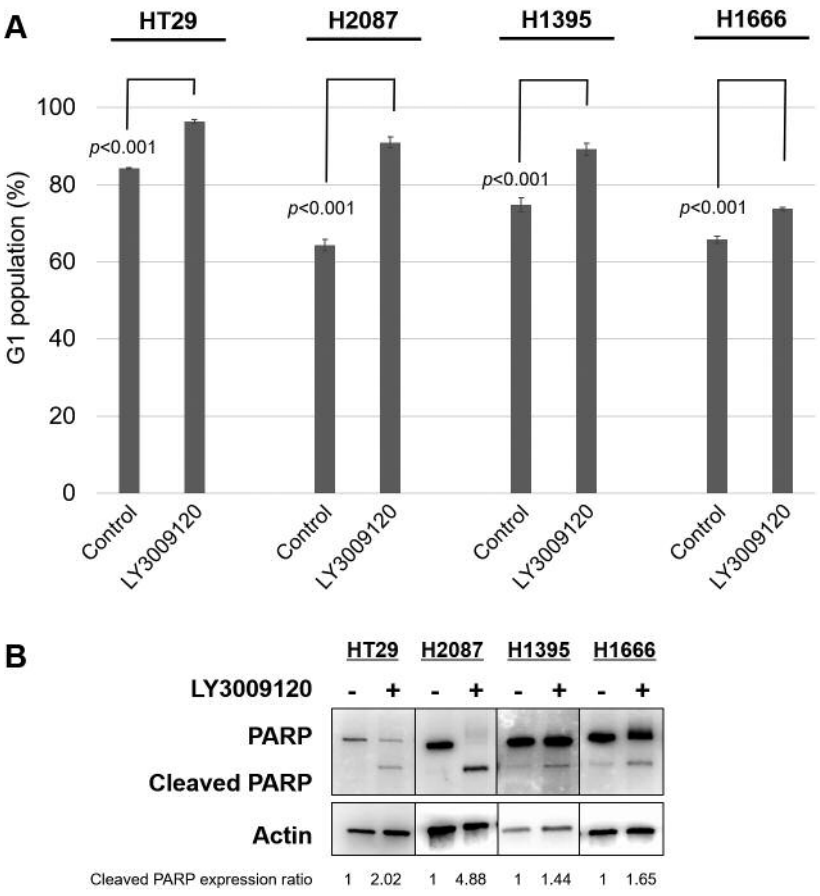

Figure 3. *LY3009120 induces cell cycle arrest and apoptosis in BRAF-mutant cancer cell lines. A: Cells were treated with 1μM LY3009120 for 48 h and then were subjected to cell cycle analysis using flow cytometry. The graph shows the percentage of cells in the G1 phase. B: Cells were treated with 1 μM LY3009120 for 72 h and subjected to western blot analysis. The expression of cleaved PARP, a marker for apoptosis (assessed by densitometric analysis using ImageJ software) was increased by treatment. PARP: poly adenosine diphosphate-ribose polymerase.*

investigated the antitumor effects of LY3009120 on the growth of the non-V600E *BRAF*-mutant lung cancer cell line H2087 *in vivo*. As shown in Figure 4A, the tumor growth was significantly inhibited in the LY3009120 treatment group compared to the vehicle control group at four weeks after initiation of treatment ($p<0.001$). Moreover, we did not observe significant body weight loss in the LY3009120 treatment group, suggesting a lack of severe adverse effects (Figure 4B).

Discussion

Several studies have demonstrated the *in vitro* and *in vivo* antitumor effects of LY3009120 against *BRAF*-mutant cancer cells, such as melanoma, colorectal cancer, pancreatic cancer, and thyroid cancer (16-19). In colorectal cancer, *BRAF* V600E mutation accounts for approximately 90% of such mutations (23). Vemurafenib had limited efficacy in *BRAF*-mutant colorectal cancer (24), although LY3009120 exhibited anti-proliferative effects in a preclinical model (17). Only few reports have discussed the effects of LY3009120 on NSCLC, and no reports have compared the efficacy of LY3009120 among several variants of *BRAF*-mutant NSCLC.

Recently, a large number of variants in oncogenic driver genes including *BRAF* variants have been identified by clinical tumor sequencing. In previous reports, about 30-50% of *BRAF* mutation is *BRAF* V600E, and the remaining proportions are non-V600E mutations in NSCLC (6-9). According to the publicly available database cBioPortal for Cancer Genomics, 41 cases (7.2%) had *BRAF* mutations among 566 cases of lung adenocarcinoma. In 9 cases (22%) the mutation was V600E, and 32 cases (78%) the mutations were non-V600E. Based on these data, overall survival was not significantly different between V600E and non-V600E mutations. Thus, further preclinical and clinical investigations

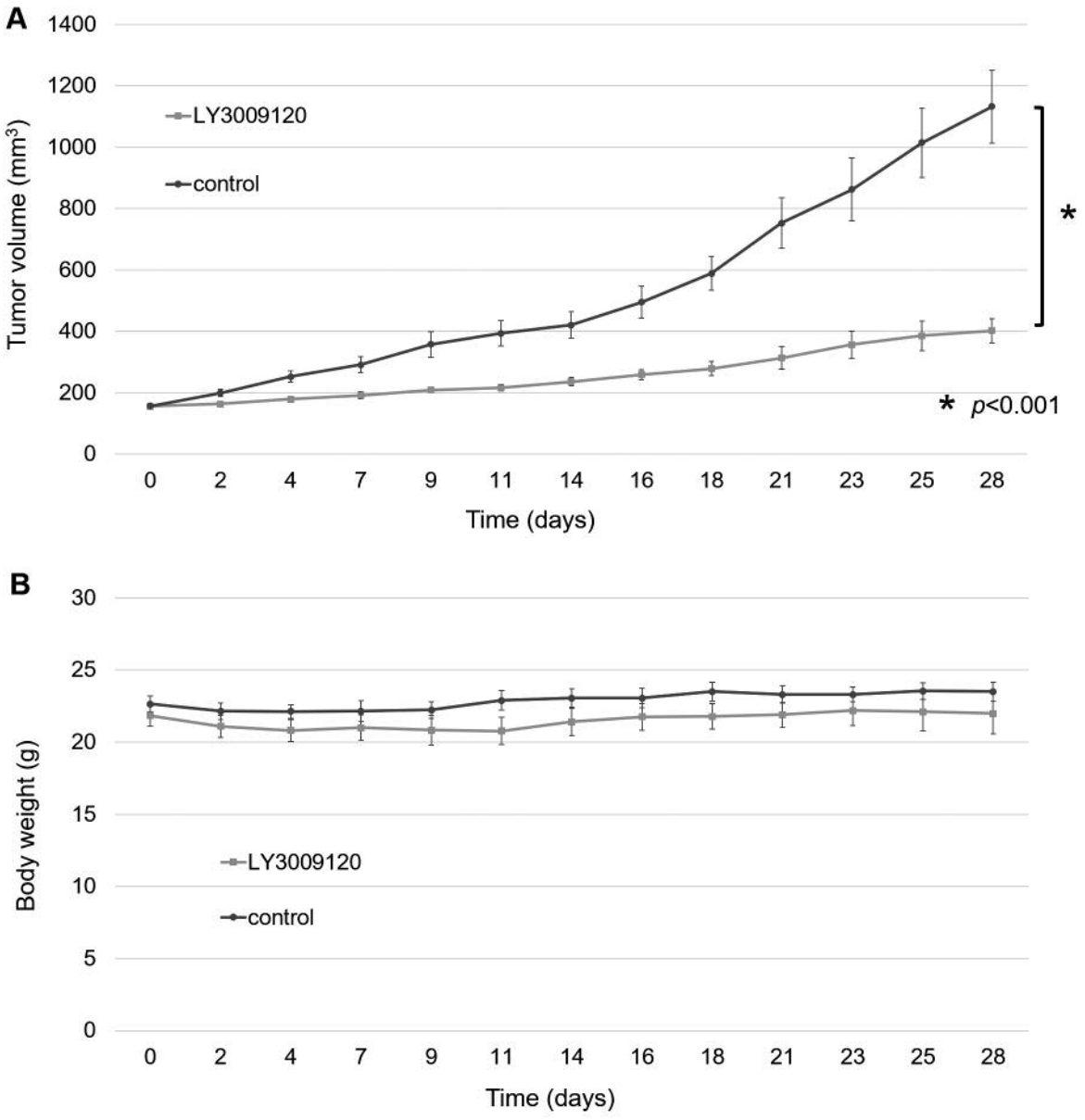

Figure 4. *LY3009120 inhibits the tumor growth in a xenograft mouse model of non-small-cell lung cancer cells that are harboring the BRAF non-V600E mutation (H2087). A: Tumor volumes were measured three times per week. *Significantly different at day 28 (p<0.001). B: Body weight was measured three times per week. Body weight was not significantly different at day 28 (p=0.359). Data are shown as the mean±SE (n=5).*

for the tumors harboring *BRAF* non-V600E mutations are required. In this study, we showed the potent antitumor effects of LY3009120 on three types of NSCLC cell lines harboring *BRAF* non-V600E mutations including L597V, G469A, and G466V, and one colon cancer cell line harboring the *BRAF* V600E mutation. The treatment with LY3009120 resulted in the suppression of BRAF-related downstream pathway molecules, cell cycle arrest, induction of PARP cleavage and cell apoptosis, which were believed to contribute to its efficacy. In previous reports, similar results were obtained in the case of melanoma and colorectal cancer (16, 17). Furthermore, in *BRAF*-transfected cells, the *BRAF* mutation activated downstream signals, and LY3009120 treatment had an inhibitory effect on these signals, regardless of the variants of *BRAF* mutation examined in our study. These results suggest that *BRAF* non-V600E mutations also act as oncogenic drivers in lung cancer, and that the *BRAF*-targeted therapy with LY3009120 could be effective,

regardless of the *BRAF* mutation genotypes. In our study, the antitumor activity of LY3009120 was also evaluated in a xenograft mouse model using a lung cancer cell line harboring the *BRAF* L597V mutation. One limitation of this study was that we examined only 5 genotypes of *BRAF* mutations and not all reported the *BRAF* mutants. There is a possibility that the antitumor effects of LY3009120 may be different according to the genotypes of *BRAF* mutations. Further investigation is mandatory to precisely determine the efficacy of LY3009120 based on the various *BRAF* mutations.

In conclusion, the antitumor effect of LY3009120 against lung cancer cells harboring *BRAF* non-V600E mutations was demonstrated both *in vitro* and *in vivo*. Our findings suggest that LY3009120 might be a promising therapeutic option for the treatment of *BRAF*-mutant NSCLC regardless of the variant of mutations.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Author's Contributions

Shunsaku Miyauchi performed majority of the experiments and wrote the article. Kazuhiko Shien designed the research and helped to write the article. Kentaro Nakata, Akihiro Miura, Yuta Takahashi, Eisuke Kurihara, and Yusuke Ogoshi contributed to *in vitro* experiments. Tatsuaki Takeda and Kota Araki participated in the animal experiments. Shuta Tomida performed the statistical analysis. Kei Namba, Ken Suzawa, Hiromasa Yamamoto, Mikio Okazaki, Junichi Soh, Masaomi Yamane, and Masakiyo Sakaguchi contributed to analysis and interpretation of data, and reviewed the article. Shinichi Toyooka supervised this study.

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