

# Synergistic effects of vemurafenib and fingolimod (FTY720) in vemurafenib-resistant melanoma cell lines

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**Abstract.** Vemurafenib, a selective inhibitor of mutated BRAF, is used to treat late-stage melanoma. However, resistance to vemurafenib is urgently required as it can have fatal consequences. Fingolimod (FTY720), a sphingosine-1-phosphate receptor modulator, has been used for the treatment of several malignant neoplasms in clinical trials. The present study investigated the effects of FTY720 and vemurafenib combination treatment on cell death induction, and defined the molecular mechanisms in vemurafenib-resistant melanoma cells. The combination treatment with FTY720 and vemurafenib reduced cell viability, and the expression of apoptosis-associated cleaved poly (adenosine diphosphate-ribose) polymerase (PARP) was increased when compared with treatment with vemurafenib alone in WM-115 cells, a vemurafenib-resistant human melanoma cell line. In addition, the protein expression of phosphorylated extracellular signal-related kinase (ERK) in WM-115 cells was decreased by this combination treatment. Vemurafenib-resistant SK-Mel-28 cells (R-SK-Mel) were established by culturing SK-Mel-28 cells, which are the most sensitive to vemurafenib, in the presence of vemurafenib. Similar to WM-115 cells, the viability of R-SK-Mel cells was reduced and the expression of cleaved PARP was increased by the combination treatment with FTY720 and vemurafenib. In addition, the expression of phosphorylated ERK and Akt was also reduced by this treatment. These results suggested that FTY720 and vemurafenib synergistically induced cell death by downregulating proliferation and survival signalling pathways in vemurafenib-resistant melanoma cells.

## Introduction

Numerous anticancer drugs have been used for cancer chemotherapy, but safer and more effective molecular targeted

anticancer agents are needed. In the last decade, immune checkpoint inhibitors and target therapies have been important and effective therapies for many intractable malignant neoplasms including progressive malignant melanoma. In 2002, it was demonstrated that oncogenic *BRAF* mutations, predominantly at codon 600, are in approximately 70% of cutaneous melanomas (1). The most common *BRAF* mutations are V600E or V600K (2,3). These mutations lead to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway and increased extracellular signal-related kinase (ERK) activation, which drive the growth and differentiation of malignant cells (4). *BRAF* inhibitors, such as vemurafenib or dabrafenib, efficiently inhibit ERK activation and tumour proliferation, which rapidly respond after the onset of therapies (5,6). However, unfortunately, responses to *BRAF* inhibitors are short-lived, with evidence of disease progression within 6-8 months after the beginning of therapy (7). To reduce resistance to *BRAF* inhibitors, several combinatorial treatments using mitogen-activated protein kinase kinase (MEK) inhibitors (8) or phosphoinositide 3-kinase (PI3K) inhibitors (9) have proven effective. In particular, the MEK inhibitor, trametinib, has been clinically used with dabrafenib as a combination therapy. However, more effective methods to treat *BRAF* inhibitor-resistant melanomas are needed.

Sphingolipids are the main components of lipid rafts and have crucial functions as signalling molecules. Sphingosine 1-phosphate (S1P) and ceramide regulate proliferation and apoptosis (10-13). In response to various stimuli, ceramide mediates cell death and apoptosis, whereas S1P abrogates apoptosis and mediates cell proliferation and migration (14). Sphingosine kinase (SK) is the key enzyme responsible for converting sphingosine to S1P. Signalling pathways via the S1P receptor contribute to cancer cell survival and proliferation (15), apoptosis reduction (16), and oncogenic transformation (17). Various cancer cells have high levels of SK1 expression/activity, resulting in their enhanced resistance to anticancer agents such as anthracyclines, doxorubicin, and camptothecin (18,19). Thus, SK1 may play an important role in the development and proliferation of cancers such as melanoma (20-22). Fingolimod (FTY720), an immune-suppressive drug developed by chemical modification of myriocin and a metabolite of the fungus *Isaria sinclairii* (23,24), is phosphorylated by SK1 and SK2 (23). Phosphorylated FTY720 (FTY720-P) is a structural analogue of S1P and binds to four S1P receptor subtypes (S1P<sub>1</sub>, S1P<sub>3</sub>, S1P<sub>4</sub>, and S1P<sub>5</sub>) (25).

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However, persistent activation of  $\text{SIP}_1$  by FTY720-P causes its internalization and degradation, thereby acting as a functional antagonist in lymphocytes (26). FTY720 also induces apoptosis in melanoma (27,28), liver cancer (16), and breast cancer (29) by direct inhibition of SK1 (29). FTY720 has predominantly been used for the treatment of multiple sclerosis (30). Recent *in vitro* and *in vivo* studies have shown that it is cytotoxic and efficiently reduced the viability of ovarian (31), breast (32) and prostate (33) cancer cells.

We previously reported that combination treatment with cisplatin and FTY720 has synergistic effects on apoptosis induction in cisplatin-resistant melanoma cells (34). Therefore, in this study we investigated the combined effects of FTY720 and vemurafenib in established vemurafenib-resistant melanoma cells, as well as the underlying molecular mechanisms.

## Materials and methods

**Chemical reagents.** FTY720 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Vemurafenib, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), and Eagle's minimal essential medium (EMEM) were purchased from Wako Pure Chemical Industries (Osaka, Japan). RPMI-1640, non-essential amino acids (NEAA), and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Rabbit polyclonal antibodies against SK1, p53, cleaved poly (adenosine diphosphate-ribose) polymerase (PARP), PI3K, phosphorylated PI3K (p-PI3K), Akt, p-Akt, MEK, p-MEK, ERK, p-ERK, S6 kinase (S6 K), mammalian target of rapamycin (mTOR), p-mTOR, epidermal growth factor receptor (EGFR), and p-EGFR were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Cell lines and cell viability measurement.** Human melanoma SK-Mel-28 cells were obtained from JCRB Cell Bank (Osaka, Japan), and human melanoma cell lines (A375, A2058, WM115) were purchased from the European Collection of Cell Cultures. SK-Mel-28 and WM115 cells were cultured in RPMI-1640 containing 10% foetal bovine serum (FBS) and 0.1% tyrosine. A375 cells were cultured in DMEM containing 10% FBS. A2058 cells were grown in EMEM containing 1% NEAA and 10% FBS. To assess viability, melanoma cells were plated at a density of  $5 \times 10^3$  cells/well in 96-well plates, and after 24 h vemurafenib and/or  $3 \mu\text{M}$  FTY720 were added to the medium. Viability was analysed after 24, 48, 72 and 96 h using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, where viable cells with active metabolism, but not dead cells, convert MTT into the corresponding purple-coloured formazan. We found the most effective time to be 72 h on vemurafenib in melanoma cells. When the determination of 50% inhibitory concentrations ( $\text{IC}_{50}$ ) for vemurafenib was performed in a 96-well plate, vemurafenib was added to the cells at concentrations from 1 to  $15 \mu\text{M}$  for 72 h in the presence or absence of  $3 \mu\text{M}$  FTY720. Cells treated with DMSO only were used as a control, and  $\text{IC}_{50}$  values were calculated.

To prepare vemurafenib-resistant cells, SK-Mel-28 cells were repeatedly exposed to high doses of vemurafenib over a

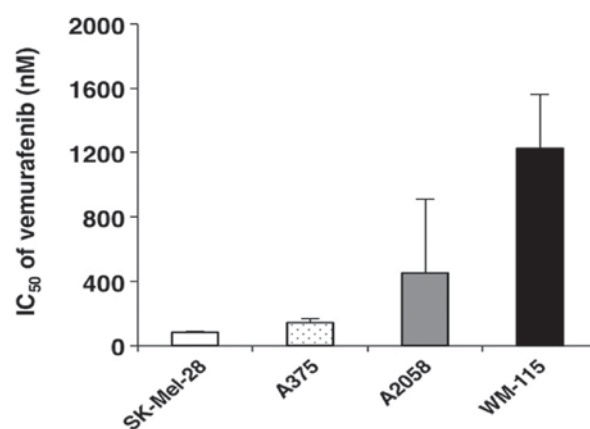


Figure 1. Comparison of the sensitivity to vemurafenib in various melanoma cells. Four melanoma cell lines ( $1 \times 10^4$  cells/well) were incubated with various concentrations of vemurafenib for 72 h, and MTT assays were performed. Each condition was measured in triplicate, and  $\text{IC}_{50}$  values were calculated. The data are expressed as the mean  $\pm$  standard deviation of three independent experiments.  $\text{IC}_{50}$ , 50% inhibitory concentrations; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

period of 10 months as described by Stordal *et al* (35). When the cell cultures had undergone approximately five doublings in the presence of  $10 \mu\text{M}$  vemurafenib, the proliferating cells were collected and transferred to drug-free culture conditions for recovery and MTT assay was then performed. The treatment was repeated at doses of  $10 \mu\text{M}$  vemurafenib for 10 months and the most resistant clone, R-SK-Mel, was selected. R-SK-Mel cells were grown in DMEM medium supplemented with  $10 \mu\text{M}$  vemurafenib, and before each experiment, the resistant cells were cultured in the absence of the target drug for 24 h.

**Western blot analysis.** Cells were lysed by sonication in RIPA buffer [50 mM Tris-HCL buffer (pH 7.6), 150 mM NaCl, 1% (w/v) NP-40, 0.5% (w/v) sodium deoxycholate, protease inhibitor cocktail, and 0.1% (w/v) sodium dodecyl sulfate (SDS)]. Total cell lysates ( $5 \mu\text{g}$  protein) were separated by electrophoresis on 7.5-10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% BSA. Expression of each protein was measured by western blotting with each antibody. Anti- $\beta$ -actin antibody was used as the loading control. After several washes, bound antibodies were detected using the ECL western blotting detection system (Luminescent Image Analyzer LAS-4000; Fujifilm, Tokyo, Japan). Protein band density was determined with a densitometer (Multi gauge, version 3.1; Fujifilm).

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. Microsoft Excel (Microsoft Corp., Redmond, WA, USA) was used to calculate the correlation index. The statistical significance was analysed by either Student's *t*-test or one-way factorial analysis of variance with Fisher's protected least significant difference post hoc test for multiple comparisons, using EZR version 1.35 software (Saitama Medical Center, Jichi Medical University, Saitama, Japan).  $P < 0.05$  was considered to indicate a statistically significant difference.

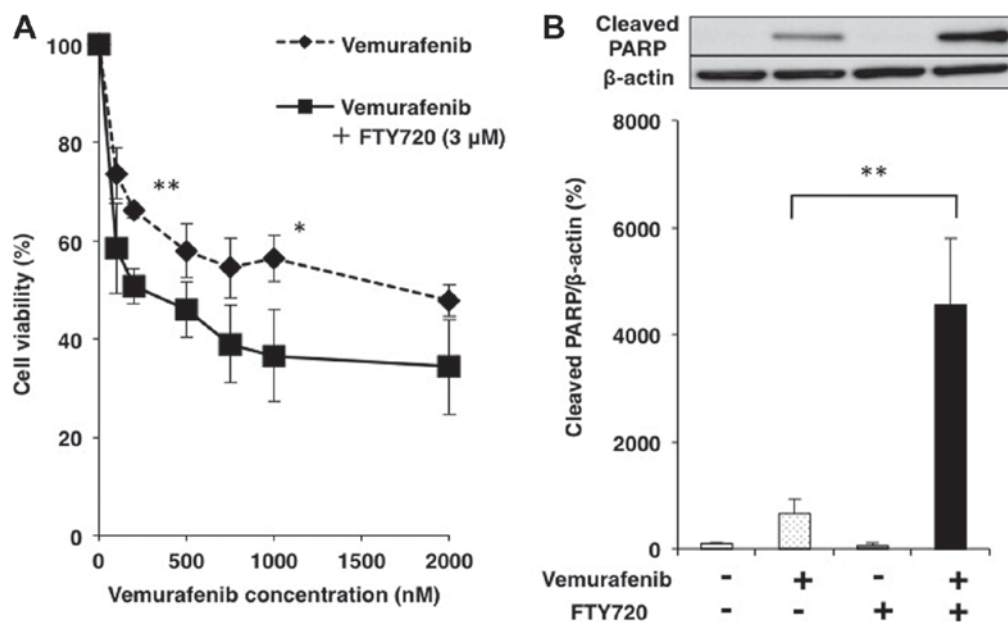


Figure 2. (A) The effects of FTY720 on the viability of WM-115 cells. WM-115 cells ( $1 \times 10^4$  cells/well) were incubated with various concentrations of vemurafenib in the presence or absence of  $3 \mu\text{M}$  FTY720 for 72 h, then MTT assays were performed. Each condition was measured in triplicate, and the data are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  vs. vemurafenib+FTY720 at the corresponding concentration. (B) The effects of FTY720 on the apoptosis of WM-115 cells. WM-115 cells were treated with  $3 \mu\text{M}$  FTY720 and  $2 \mu\text{M}$  vemurafenib, alone or in combination for 48 h. Cell lysates were subjected to western blot analysis with an antibody against cleaved PARP, and the band intensity was measured. The results are expressed as a percentage increase relative to the untreated controls. The data are expressed as the mean  $\pm$  standard deviation. \*\* $P < 0.01$ , as indicated. FTY720, fingolimod; PARP, poly (adenosine diphosphate-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

## Results

### *Sensitivity of various melanoma cells to vemurafenib.*

To examine the sensitivity of four melanoma cell lines (SK-Mel-28, A375, A2058 and WM-115) to vemurafenib, cell viability was measured after a 72 h incubation with vemurafenib. The  $\text{IC}_{50}$  for vemurafenib was highest in WM-115 cells and lowest in SK-Mel-28 cells (Fig. 1). The relative  $\text{IC}_{50}$  values for vemurafenib in SK-Mel-28, A375, A2058 and WM-115 cells were  $82 \pm 9.19$ ,  $145 \pm 24.7$ ,  $452 \pm 46.1$  and  $1227 \pm 336$ , respectively. Therefore, WM-115 and SK-Mel-28 cells were used in subsequent experiments as vemurafenib-resistant and vemurafenib-sensitive cell lines, respectively.

### *Synergistic effects of FTY 720 and vemurafenib combination treatment on apoptosis in vemurafenib-resistant cells.*

To evaluate the effects of FTY720 on WM-115 cell viability, we compared the viability of cells treated with vemurafenib alone and the combination of vemurafenib and FTY720. The  $\text{IC}_{50}$  of treatment with vemurafenib alone was reduced by approximately 75% in the presence of  $3 \mu\text{M}$  FTY720 (Fig. 2A). FTY720 alone at  $3 \mu\text{M}$  had no significant effects on viability (data not shown). These results suggest that the addition of FTY720 to vemurafenib significantly reduced viability compared to treatment with vemurafenib alone in resistant WM-115 cells. The effects of FTY720 and vemurafenib on the apoptosis of WM-115 cells were examined using western blotting to measure the amount of PARP protein degradation, a marker of apoptosis. In WM-115 cells, cleaved PARP protein was expressed by  $2 \mu\text{M}$  vemurafenib alone, but not by  $3 \mu\text{M}$  FTY720 alone (Fig. 2B). However, the combination treatment with  $2 \mu\text{M}$  vemurafenib and  $3 \mu\text{M}$  FTY720 induced a synergistic increase in PARP

degradation (Fig. 2B). These results showed that the addition of FTY720 to vemurafenib enhanced apoptosis compared with vemurafenib alone in WM-115 cells.

*Changes in the expression of proteins in cell signaling pathways and SK1 by combination treatment with vemurafenib and FTY720 in vemurafenib-resistant cells.* The effects of combination treatment with  $2 \mu\text{M}$  vemurafenib and  $3 \mu\text{M}$  FTY720 on MEK and ERK phosphorylation in WM-115 were evaluated by western blot analysis. The ratio of phosphorylated to total MEK (p-MEK/t-MEK) was decreased by combination treatment, but the difference was not significant (Fig. 3A). In contrast, the ratio of phosphorylated to total ERK (p-ERK/t-ERK) was remarkably decreased by combination treatment compared with vemurafenib alone ( $P < 0.01$ ; Fig. 3B). We investigated the changes in the ratio of phosphorylated to total Akt (p-Akt/t-Akt) after the treatment with  $2 \mu\text{M}$  vemurafenib,  $3 \mu\text{M}$  FTY720 or both agents in WM-115 cells.

The treatment with  $2 \mu\text{M}$  vemurafenib or  $3 \mu\text{M}$  FTY720 alone induced no significant changes in Akt protein expression (Fig. 4A). Conversely, the treatment with combined FTY720 and vemurafenib caused a synergistic decrease in t-Akt protein expression (Fig. 4A), whereas there were no significant changes in p-Akt/t-Akt (Fig. 4B).

The effects of the combination treatment on SK1 with  $2 \mu\text{M}$  vemurafenib and  $3 \mu\text{M}$  FTY720 was evaluated by western blotting. The expression of SK1 was significantly decreased with combination treatment compared with vemurafenib treatment alone (Fig. 4C).

*Establishing vemurafenib-resistant R-SK-Mel cells and evaluating changes in the expression of cell signaling*

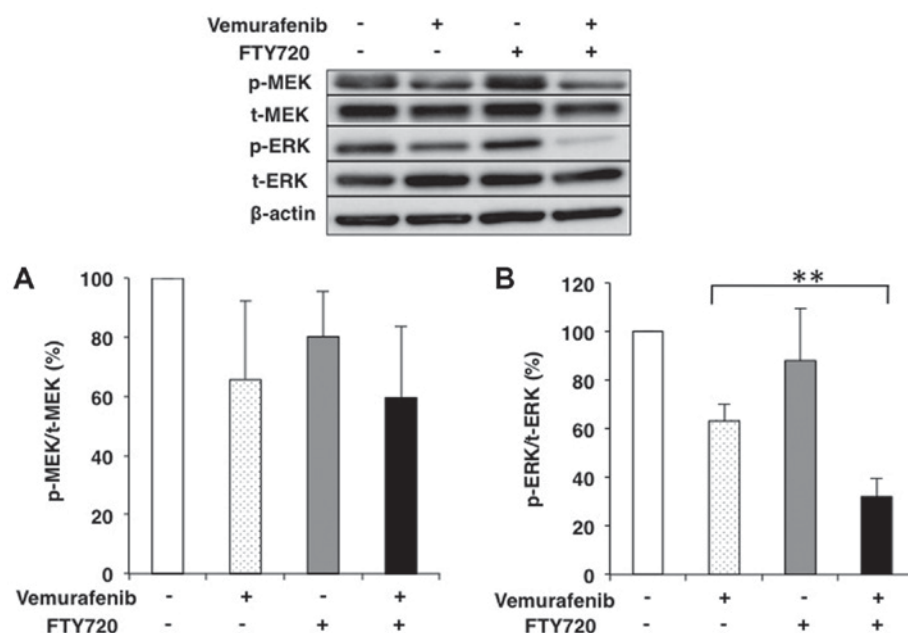


Figure 3. The effects of FTY720 and vemurafenib on the expression of (A) MEK and (B) ERK in WM-115 cells. WM-115 cells were treated with 3  $\mu$ M FTY720 and 2  $\mu$ M vemurafenib, alone or in combination for 48 h. Cell lysates were subjected to western blot analysis with antibodies against the total (t) and phosphorylated (p) forms of (A) MEK and (B) ERK. The ratios of phosphorylated to total MEK and ERK were calculated.  $\beta$ -actin was used as the loading control. The results are expressed as the ratios of treated to untreated cells. Each condition was examined in triplicate, and the data are expressed as the means  $\pm$  standard deviation. \*\* $P$ <0.01, as indicated. FTY720, fingolimod; MEK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; p-, phosphorylated; t-, total.

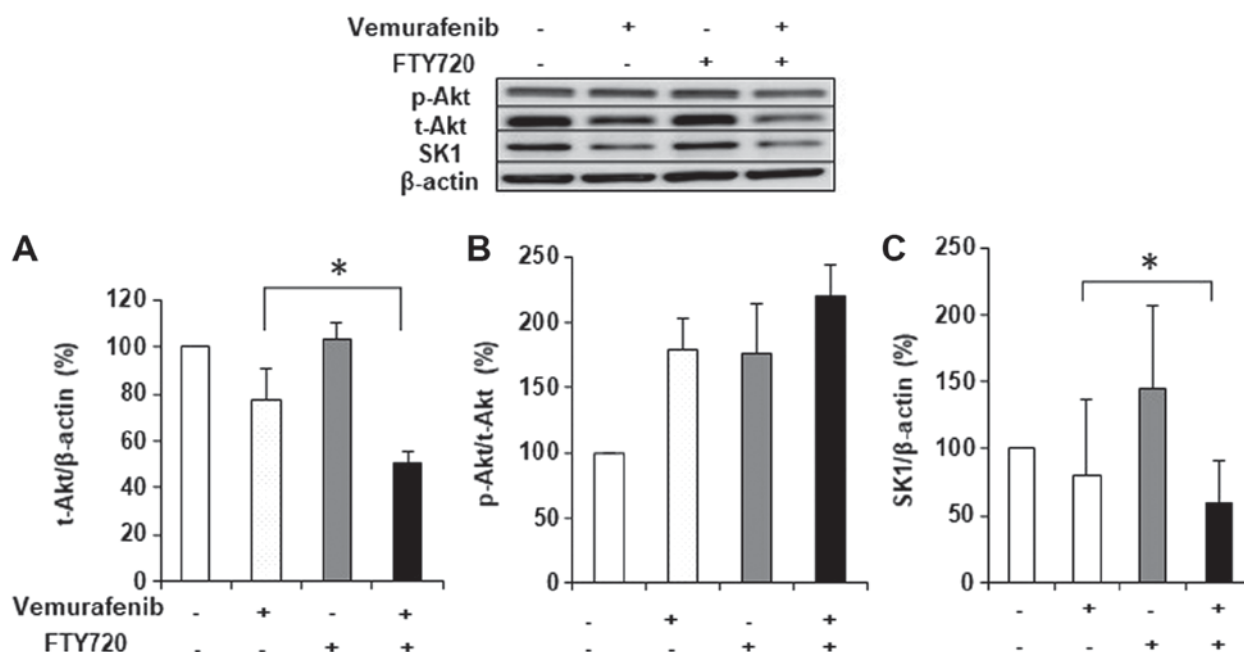


Figure 4. Effects of FTY720 and vemurafenib on the protein expression of (A and B) Akt and (C) SK1 in WM-115 cells. WM-115 cells were treated with 3  $\mu$ M of FTY720 and 2  $\mu$ M vemurafenib, alone or in combination, for 48 h, and cell lysates were subjected to western blot analysis with antibodies against the total forms of (A) Akt and (C) SK1, and against the total (t) and phosphorylated (p) forms of (B) Akt.  $\beta$ -actin was used as the loading control. The results are expressed as ratios of treated to untreated cells. Each condition was examined in triplicate, and data are expressed as the means  $\pm$  standard deviation. \* $P$ <0.05, as indicated. FTY720, fingolimod; Akt, protein kinase B; SK1, sphingosine kinase 1; p-, phosphorylated; t-, total.

proteins in these cells. To confirm the role of signalling proteins in the sensitivity to vemurafenib and FTY720, we attempted preparation of the vemurafenib-resistant cell line by treating the original vemurafenib-sensitive SK-Mel-28 cells with a high concentration of vemurafenib as described

in the Materials and Methods. Consequently, we succeeded in establishing the vemurafenib-resistant cell line R-SK-Mel, which can proliferate even in the presence of 10  $\mu$ M vemurafenib. As shown in Fig. 5A, the  $IC_{50}$  of vemurafenib in R-SK-Mel cells was 50-fold higher than that in the parent



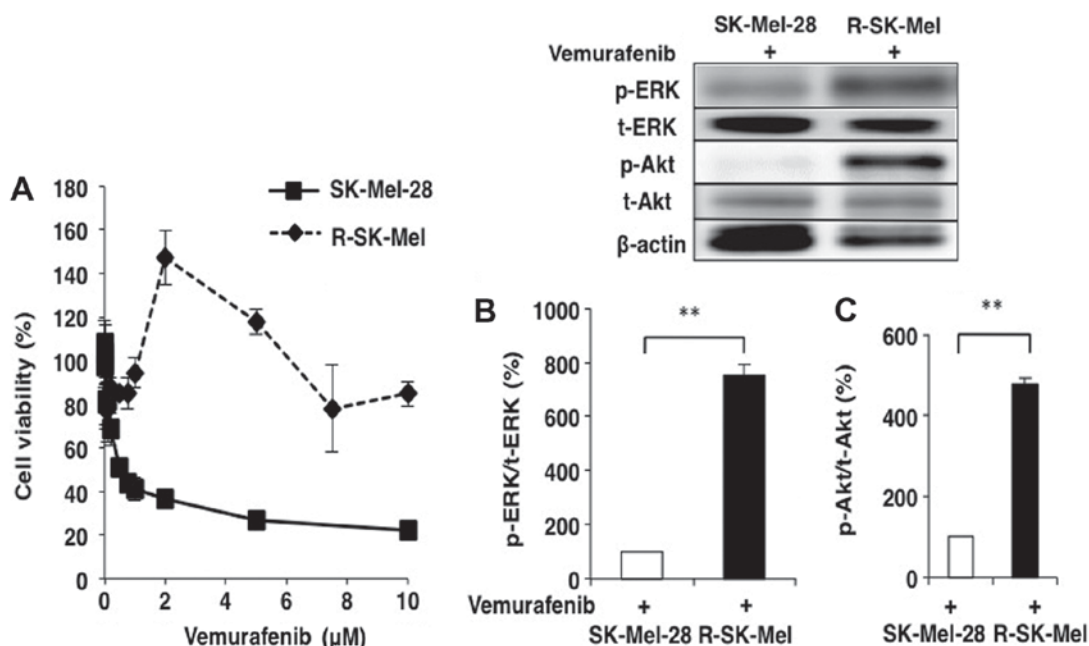


Figure 5. (A) The effects of vemurafenib on the viability of SK-Mel-28 and R-SK-Mel cells. SK-Mel-28 and R-SK-Mel cells ( $1 \times 10^4$  cells/well) were incubated with various concentrations of vemurafenib for 72 h, then MTT assays were performed. Each condition was measured in triplicate, and the data are expressed as the mean  $\pm$  standard deviation of three independent experiments. Comparisons of the expression of (B) ERK and (C) Akt in SK-Mel-28 and R-SK-Mel cells. SK-Mel-28 and R-SK-Mel cells were treated with 2  $\mu$ M vemurafenib for 48 h. Cell lysates were subjected to western blot analysis with antibodies against total (t) and phosphorylated (p) forms of (B) ERK and (C) Akt. The ratios of phosphorylated to total ERK and Akt were calculated.  $\beta$ -actin was used as the loading control. The results are expressed as a percentage increase relative to the ratio of SK-Mel-28 cells. Each condition was examined in triplicate, and the data are expressed as the means  $\pm$  standard deviation. \*\* $P < 0.01$ , as indicated. R-SK-Mel, SK-Mel-28 resistant; ERK, extracellular signal-related kinase; Akt, protein kinase B; p-, phosphorylated; t-, total.

cell line. Changes in the expression of cell signaling proteins was examined between the parent and resistant cells. The expression levels of p-ERK/t-ERK and p-Akt/t-Akt were remarkably increased in R-SK-Mel cells compared with those in parent SK-Mel-28 cells treated with 2  $\mu$ M vemurafenib (Fig. 5B and C).

**Synergistic effects of FTY 720 and/or vemurafenib combination treatment on cell viability and apoptosis in R-SK-Mel cells.** We used MTT assays to compare the cell viability of SK-Mel-28 and R-SK-Mel cells treated with FTY720. The  $IC_{50}$  of vemurafenib in R-SK-Mel cells was remarkably reduced by treatment with FTY720 compared with that in SK-Mel-28 cells (Fig. 6A). The effects of FTY720 and vemurafenib combination treatment on R-SK-Mel cell apoptosis were evaluated using western blotting to measure the expression of cleaved PARP. In R-SK-Mel cells, only combination treatment with 10  $\mu$ M vemurafenib and 10  $\mu$ M FTY720 induced a synergistic increase in PARP degradation (Fig. 6B).

**Changes in the expression of p-ERK/t-ERK and p-Akt/t-Akt by combination treatment with vemurafenib and FTY720 in R-SK-Mel cells.** Changes in the expression of proteins in the MAPK and PI3K-Akt signalling pathways upon combination treatment with 10  $\mu$ M vemurafenib and 10  $\mu$ M FTY720 in R-SK-Mel cells were investigated. The expression levels of p-ERK/t-ERK and p-Akt/t-Akt were remarkably decreased by combination treatment (Fig. 7A and B). However, p-MEK/t-MEK expression did not significantly change with this combination treatment (Fig. 7C).

## Discussion

The MAPK and PI3K-Akt pathways are major pathways underlying cancer development and progression (36), and the latter pathway is particularly important for cell survival in melanoma (37). Several studies have previously described the mechanism of resistance to BRAF inhibitors, and noted that acquisition of the activated *NRAS* mutation (38) leads to reactivation of the MAPK pathway. Furthermore, there have been many reports on the mechanisms underlying resistance to BRAF inhibitors such as activation of receptor tyrosine kinase (39), increased expression of mutated BRAF kinase, increased expression of Cancer Osaka Thyroid (40), acquisition of *MAP2K1* mutations (41) and loss of *NFI* (42).

In this study, we demonstrated that the melanoma cell line WM-115 was most resistant to the BRAF inhibitor (vemurafenib), and exhibited much higher expression levels of p-ERK compared with vemurafenib-sensitive cells (data not shown). Furthermore, when changes in the expression of cell signaling molecules in R-SK-Mel cells were compared with the parent cells, we found that the levels of p-ERK and p-AKT were remarkably increased in R-SK-Mel cells, suggesting that both the MAPK and PI3K-Akt pathways were enhanced in the vemurafenib resistant-melanoma cells.

We also examined the effects of FTY720 and vemurafenib combination treatment on the resistant cells. This treatment strongly reduced cell viability, and also induced a synergistic increase in cleaved PARP in the vemurafenib-resistant WM-115 and R-SK-Mel cells. These results suggested that the addition of FTY720 to vemurafenib was effective in enhancing apoptosis

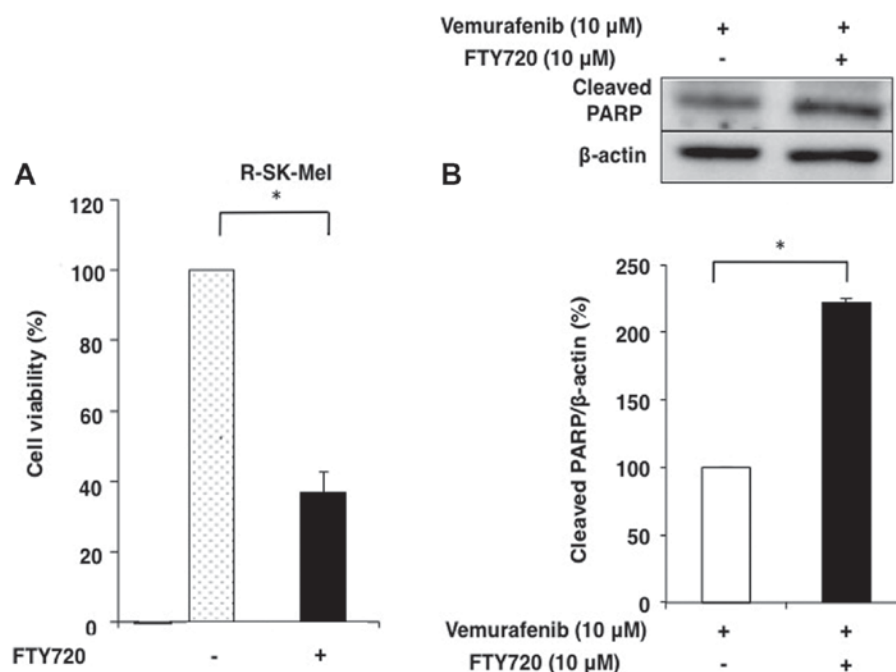


Figure 6. (A) The effects of FTY720 on the viability of R-SK-Mel cells. R-SK-Mel cells ( $1 \times 10^4$  cells/well) were incubated without or with 10  $\mu$ M of FTY720 for 72 h, then MTT assays were performed. Each condition was measured in triplicate, and the data are expressed as the mean  $\pm$  standard deviation of three independent experiments. (B) The effects of FTY720 on the apoptosis of vemurafenib-resistant R-SK-Mel cells. Vemurafenib-resistant R-SK-Mel cells were treated with 10  $\mu$ M vemurafenib with or without 10  $\mu$ M FTY720. Cell lysates were subjected to western blot analysis with antibodies against cleaved PARP, and the band intensity was measured.  $\beta$ -actin was used as the loading control. The results are expressed as a percentage increase relative to the treatment without FTY720. The data are expressed as the means  $\pm$  standard deviation. \* $P < 0.05$ , as indicated. FTY720, fingolimod; R-SK-Mel, SK-Mel-28 resistant; PARP, poly (adenosine diphosphate-ribose) polymerase.

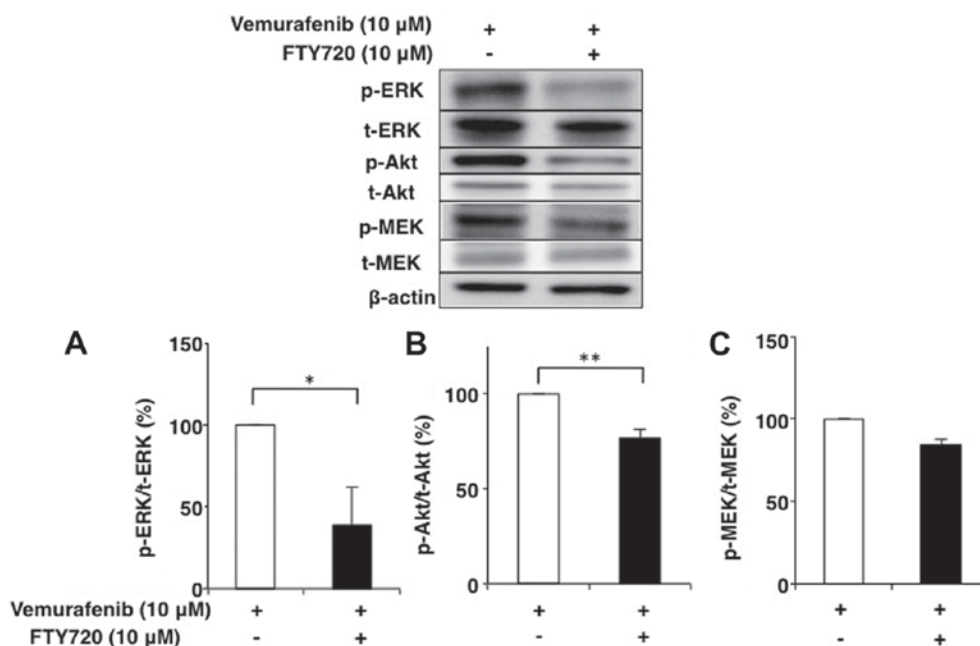


Figure 7. Effects of FTY720 on the expression of (A) ERK, (B) Akt and (C) MEK in vemurafenib-resistant R-SK-Mel cells. Vemurafenib-resistant R-SK-Mel cells were treated with 10  $\mu$ M vemurafenib with or without 10  $\mu$ M FTY720 for 48 h. Cell lysates were subjected to western blot analysis with antibodies against total (t) and phosphorylated (p) forms of (A) ERK, (B) Akt and (C) MEK. The ratios of phosphorylated to total ERK, Akt and MEK were calculated.  $\beta$ -actin was used as the loading control. The results are expressed as a percentage relative to the treatment without FTY720. The data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$  and \*\* $P < 0.01$ , as indicated. FTY720, fingolimod; R-SK-Mel, SK-Mel-28 resistant; MEK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; Akt, protein kinase B; p-, phosphorylated; t-, total.

compared with vemurafenib treatment alone. Furthermore, we also examined the change in protein expression in cell signaling

pathway components by this combination treatment in the resistant cells. In the MAPK pathway, p-ERK/t-ERK, and t-Akt

and SK1 were remarkably decreased by this treatment, whereas there was no significant change in p-MEK/t-MEK. Moreover, this combination treatment induced remarkable decreases in the levels of p-ERK/t-ERK and also p-Akt/t-Akt in R-SK-Mel cells.

We previously reported that the combination treatment with cisplatin and FTY720 had synergistic effects on apoptosis induction in cisplatin-resistant melanoma cells by SK1 degradation, possibly due to downregulation of the PI3K/Akt/mTOR pathway via the S1P receptor and reduced EGFR expression (34). In addition, FTY720 mediates many anticancer effects through inactivation of the PI3K/Akt pathway mediated via a variety of mechanisms including the inhibition of PI3K, increased PTEN expression, activation of protein phosphatase 2A activity and SK1 inhibition (43). In accordance with these studies, our results strongly suggest that the combination treatment with FTY720 and vemurafenib induces apoptosis by downregulating both PI3K/Akt and MAPK pathways in melanoma cells.

Recently, several cases of malignant melanoma have been documented in patients with multiple sclerosis who were being treated with FTY720 (44-46), and a relationship between treatment by FTY720 and occurrence of melanoma has been suggested. However, since the exact mechanism of the relationship has not been elucidated, we believe that further studies are necessary. Furthermore, we strongly suggest that FTY720 is most effective as combination treatment with anticancer drugs rather than as treatment by itself in clinical applications.

This combination treatment in vemurafenib-resistant melanoma cells did not induce significant changes in p-MEK/t-MEK expression. A recent study demonstrated that another pathway is activated by BRAF mutation, which directly activates ERK through Abl and Arg activation, but not through MEK (47). The authors also indicated that Abl/Arg cooperates with a parallel, compensatory signalling pathway (PTEN loss/Akt activation) to promote melanoma growth and survival. Based on this report, FTY720 likely plays a major role in inhibiting Akt activity, which consequently enhances cell apoptosis. The latest therapy for patients affected by BRAF mutated melanoma is a combination therapy of BRAF inhibitor and MEK inhibitor. Our data suggest that more effective pharmacodynamic actions can be obtained by using FTY720, which may block signalling pathways via ERK, but not via MEK, as well as the Akt pathway.

The results of this study suggest that FTY720 may be an effective agent for enhancing antineoplastic effects and apoptosis, and thus, decreasing resistance to vemurafenib in patients being treated with this agent.

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#### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

TT, HK, YB and MS conceived and designed the study. TT, NA and YB performed the experiments. TT, YB and MS wrote the paper. All authors have read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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