Antitumor Activity of the Selective Pan-RAF Inhibitor TAK-632 in BRAF Inhibitor-Resistant Melanoma

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Abstract

The mitogen-activated protein kinase (MAPK) pathway is particularly important for the survival and proliferation of melanoma cells. Somatic mutations in BRAF and NRAS are frequently observed in melanoma. Recently, the BRAF inhibitors vemurafenib and dabrafenib have emerged as promising agents for the treatment of melanoma patients with BRAF-activating mutations. However, as BRAF inhibitors induce RAF paradoxical activation via RAF dimerization in BRAF wild-type cells, rapid emergence of acquired resistance and secondary skin tumors as well as presence of few effective treatment options for melanoma bearing wild-type BRAF (including NRAS-mutant melanoma) are clinical concerns. Here, we demonstrate that the selective pan-RAF inhibitor TAK-632 suppresses RAF activity in BRAF wild-type cells with minimal RAF paradoxical activation. Our analysis using RNAi and TAK-632 in preclinical models reveals that the MAPK pathway of NRAS-mutated melanoma cells is highly dependent on RAF. We also show that TAK-632 induces RAF dimerization but inhibits the kinase activity of the RAF dimer, probably because of its slow dissociation from RAF. As a result, TAK-632 demonstrates potent antiproliferative effects both on NRAS-mutated melanoma cells and BRAF-mutated melanoma cells with acquired resistance to BRAF inhibitors through NRAS mutation or BRAF truncation. Furthermore, we demonstrate that the combination of TAK-632 and the MAPK kinase (MEK) inhibitor TAK-733 exhibits synergistic antiproliferative effects on these cells. Our findings characterize the unique features of TAK-632 as a pan-RAF inhibitor and provide rationale for its further investigation in NRAS-mutated melanoma and a subset of BRAF-mutated melanomas refractory to BRAF inhibitors. Cancer Res; 73(23): 7043–55. ©2013 AACR.

Introduction

Melanoma is one of the deadliest and most aggressive forms of skin cancer, arising from the malignant transformation of pigment-producing cells, melanocytes (1). The mitogen-activated protein kinase (MAPK) pathway is particularly important for the survival and proliferation of melanoma cells (2,3). Since oncogenic mutations frequently occur in components of the MAPK pathway in melanoma (BRAF, NRAS, and KRAS in approximately 45%, 18%, and 2%, respectively; ref. 4), research and development targeting the MAPK pathway has been extensively performed (2, 5, 6). Recently, it has been reported that the BRAF inhibitors vemurafenib (Roche/Plexxikon) and dabrafenib (GlaxoSmithKline) showed high response rates and improved overall survival in melanomas with BRAF-activating mutations (7–12). Thus, the rationale for targeting BRAF to treat BRAF-mutant melanomas is strongly demonstrated. However, several reports have shown that BRAF inhibitors activate RAF in BRAF wild-type cells by inducing RAF dimer formation (13–15). Since the paradoxical activation of RAF requires cooperation with RAS, it does not occur in BRAF-mutated melanoma cells in which RAF activates downstream components in a RAS-independent manner (16, 17). Therefore, while vemurafenib showed comparable inhibitory activity to all RAF isoforms in in vitro kinase assays (7), it suppressed cellular RAF activity exclusively in BRAF-mutant cells (18, 19). Such a RAF inhibitor is categorized as a "BRAF inhibitor," which demonstrates preferential inhibition of the MAPK pathway and proliferation of BRAF-mutated melanoma cells. On the other hand, a lack of such a treatment option for melanomas with wild-type BRAF remains an unresolved clinical issue. A subset of these tumors contains activating mutations in NRAS, implying the relevance of the MAPK pathway. These observations warrant the development of a small-molecule inhibitor that suppresses RAF activity without inducing RAF paradoxical activation for the treatment of NRAS-mutated melanomas (here referred to as "pan-RAF inhibitor"). Although the response of BRAF inhibitors to BRAF-mutant melanomas is apparent, rapid development of acquired
resistance has been observed (11, 12). Many resistance mechanisms have been identified to date (16, 20–27), and most involve reactivation of the MAPK pathway, which suggests that tumor survival and growth remains dependent on the MAPK pathway. However, as many resistance mechanisms (e.g., RAS mutation, CRAF overexpression, and BRAF truncation: refs. 16, 21, 25) involve RAF dimer signaling and thus the potential for RAF paradoxical activation by BRAF inhibitors, a pan-RAF inhibitor may be useful to develop a new therapeutic option in these resistant settings.

In this preclinical study, we describe the biologic characterization of TAK-632 (28) as a potent and selective pan-RAF inhibitor that overcomes paradoxical RAF activation. We used both genetic and chemical approaches to investigate the dependence of NRAS-mutated melanoma and BRAF inhibitor-resistant BRAF mutant melanoma cells on RAF. In addition, we explored the potential of TAK-632 as a monotherapy and in combination with a MEK inhibitor to robustly suppress MAPK pathway activation in melanoma cells.

Materials and Methods

Cell lines and culture

Human fibroblast Cell System-Fb (CsFb) cells were obtained from Cell Systems Corporation, GAK cells from the Health Science Research Resources Bank, HMV-II cells from European Collection of Cell Cultures, and other cell lines from American Type Culture Collection. The cell lines were cultured at 37°C with 5% CO₂ in the recommended medium supplemented with 10% FBS (Invitrogen). The cell lines were authenticated by the cell banks with short-tandem repeat profiling and used within 2 months after resuscitation. A375 stable cells (AcGFP-mock, -NRAS<sup>G12K</sup>, and -ΔN-BRAF cells) were established by virus transduction and cultured in the presence of 1 μg/mL puromycin.

Reagents

TAK-632 (28) and TAK-733 (29) were synthesized by Takeda Pharmaceutical Company Limited. Vemurafenib was prepared according to a published method (7).

Transfection, immunoprecipitation, and Western blot analysis

Cells were transfected with plasmids or siRNAs using Lipofectamine 2000 or RNAi MAX reagent (Invitrogen), respectively. Details of immunoprecipitation and Western blot analysis are described in the Supplementary Methods.

Cell viability assay

Cell viability was assessed (3 replicates) using the Sulforhodamine B assay or by the CellTiter-Glo luminescent cell viability assay (Promega). The concentrations of chemical compounds that produced 50% growth inhibition (GI₅₀) were calculated using PCP software (SAS Japan). The combination index (CI) was calculated using CalcuSyn software (Biosoft). CI < 0.9, CI = 0.9–1.1, and CI > 1.1 indicate synergism, additive effect, and antagonism, respectively.

Kinase assay

Immunoprecipitated BRAF or CRAF was incubated with recombinant inactive MEK (K97R) (Millipore) at 30°C for 30 minutes in kinase reaction buffer containing ATP/Mg<sup>2+</sup>. Additional details are provided in the Supplementary Methods.

Animal study

A solid dispersion (SD) formulated compound was dissolved in distilled water, and the resultant suspension was orally administered to xenograft-implanted nude mice. All the animals were dosed with vehicle (SD powder formulated in water) or compound suspension by oral gavages. Additional details are provided in the Supplementary Methods.

Results

TAK-632 suppresses the MAPK pathway without robust RAF paradoxical activation in multiple cell lines

To screen for pan-RAF inhibitors, we evaluated MAPK pathway regulation in BRAF-mutant melanoma cells and fibroblast cells. Since we observed that the MAPK pathway of fibroblast cells was activated by BRAF inhibitors via RAF paradoxical activation, we explored RAF inhibitors that suppressed the MAPK pathway in BRAF-mutant melanoma cells but did not activate that in fibroblast cells. Some of our (5,6)-fused bicyclic class inhibitors achieved these criteria. Eventually, among the screened RAF inhibitors, TAK-632 was selected as a candidate for further development (28). Potent and selective inhibition of RAF by TAK-632 was confirmed by a panel of in vitro kinase assays (IC₅₀ of BRAF = 8.3 nmol/L, CRAF = 1.4 nmol/L, BRAF-V600E = 2.4 nmol/L; ref. 28). We also confirmed RAF kinase inhibition by vemurafenib (IC₅₀ of BRAF = 64 nmol/L, CRAF = 90 nmol/L, BRAF-V600E = 43 nmol/L, comparable to the reported IC₅₀ values; ref. 7) and used vemurafenib as a representative BRAF inhibitor in following studies.

Here, to formally characterize its cellular activity, we initially compared the effect of TAK-632 with vemurafenib on the MAPK pathway in BRAF mutant A375 cells, which have demonstrated sensitivity to BRAF inhibitors (27). Consistent with the inhibitory activities to purified RAF, TAK-632 exhibited relatively high inhibitory activities on MEK and ERK phosphorylation compared with vemurafenib, although the difference was not significant (Supplementary Fig. S1A: IC₅₀ of TAK-632 for pERK = 25 nmol/L and IC₅₀ of vemurafenib for pERK = 75 nmol/L). To investigate the effect of TAK-632 on BRAF wild-type cells in detail, we tested multiple cell lines with wild-type RAS/RAF, cell lines with KRAS mutations, and NRAS-mutated melanoma cell lines. We found that TAK-632 had a biphasic effect on the MAPK pathway: MEK and ERK phosphorylation was modestly induced at low TAK-632 concentrations but inhibited at higher concentrations in BRAF wild-type cells (Fig. 1A). Although many ATP-competitive RAF inhibitors increase MEK and ERK phosphorylation in cells with wild-type RAF (15), interestingly, paradoxical activation by TAK-632 appeared to be weak and occurred at low concentrations. Notably, this weak paradoxical activation occurred in a similar pattern in the cells; however, the MAPK pathway of NRAS-
mutated melanoma cells was suppressed by TAK-632 at lower concentrations than that of other cells (Fig. 1A). In contrast, as reported previously, vemurafenib significantly induced RAF paradoxical activation in NRAS-mutated melanoma cells in a biphasic manner (Supplementary Fig. S1B; ref. 18). We also compared the effect of TAK-632 on the MAPK signaling with the potent MEK inhibitor TAK-733 (29). Western blot analysis revealed that the inhibitory activity to ERK phosphorylation of TAK-733 in A375 cells was comparable to that in SK-MEL-2 cells (Supplementary Fig. S1C). Meanwhile, the inhibition of ERK phosphorylation by TAK-632 was relatively weak in SK-MEL-2 cells compared to the inhibition in A375 cells (Fig. 1A and Supplementary Fig. S1A), suggesting that the minimal RAF paradoxical activation by TAK-632 reduces its inhibitory activity at low concentrations. Thus, our data indicate that TAK-632 displays potent inhibition of MAPK pathway in NRAS-mutated melanoma cells with minimal RAF paradoxical activation, although the inhibitory activity was relatively weak compared to that in BRAF-mutated melanoma cells.

To investigate the relative sensitivity to TAK-632 in NRAS-mutated melanoma cells, we performed knockdown analysis using siRNA to each RAF isoform. We found that ARAF depletion by siRNA did not affect MEK and ERK phosphorylation but BRAF or CRAF depletion decreased it in multiple NRAS-mutated melanoma cells (Fig. 1B). Furthermore, simultaneous BRAF and CRAF depletion suppressed MEK and ERK phosphorylation more robustly than each siRNA alone (Fig. 1B). The data suggest that BRAF and CRAF but not ARAF dominantly regulate the MAPK pathway in NRAS-mutated melanoma cells.

RAF dimer formation mediates RAF paradoxical activation by TAK-632

BRAF inhibitors reportedly induce RAF paradoxical activation through RAF dimer formation (13–15). Therefore, we
investigated whether RAF dimer formation is responsible for paradoxical activation by TAK-632. When SK-MEL-2 cells were treated with TAK-632, the inhibitor induced the formation of BRAF–CRAF dimers, detectable in coprecipitation experiments, in a concentration-dependent manner (Fig. 2A, top). We also observed an increase in BRAF–CRAF dimers in vemurafenib-treated cells, albeit at higher concentrations than those in TAK-632 (Fig. 2A, top). Such modest RAF dimerization was also observed in PLX4720, a precursor analog of vemurafenib (14, 30, 31). With regard to the effects of TAK-632 and vemurafenib on RAF dimerization, similar results were obtained in KRAS-mutant A549 cells (Fig. 2B, top). Interestingly, the level of MEK and ERK phosphorylation induced by RAF inhibitors was the maximum at lower concentrations than those required to induce maximal RAF dimer formation (Fig. 2A and B). The data support the model that the paradoxical activation of the MAPK pathway is mediated by RAF dimers in which one protomer is occupied by the RAF inhibitor and another is not (13, 15). At higher concentrations of RAF inhibitors, more RAF dimers are formed; however, both protomers tend to be occupied by inhibitors, resulting in effective inhibition of kinase activity (Supplementary Fig. S2).

To demonstrate the significance of RAF in the paradoxical activation of the MAPK pathway, we examined the effect of TAK-632 in RAF-depleted cells. The paradoxical activation induced by TAK-632 and vemurafenib was suppressed by CRAF depletion in SK-MEL-2 cells (Fig. 2C). Similar data was obtained in KRAS-mutant cells (data not shown). In these experiments, we used the most appropriate concentration of each inhibitor that induced the paradoxical activation of the MAPK pathway (TAK-632, 10 nmol/L and vemurafenib, 1,000 nmol/L; Fig. 1A and Supplementary Fig. S1B). These results are consistent with previous reports using BRAF inhibitors (13–15), indicating that CRAF is particularly required for RAF paradoxical activation induced by TAK-632.

Although we have shown that TAK-632 induced modest RAF paradoxical activation and RAF dimerization, a direct link between them remains to be determined. To clarify whether TAK-632–mediated paradoxical activation was attributed to RAF dimerization, we prepared plasmids encoding the dimer interface mutants E586K-BRAF and E478K-CRAF, which dimerize easily (13, 32), and evaluated the contribution of RAF dimer formation to paradoxical RAF activation. Unlike the biphasic effects observed in control cells, the paradoxical activation of the

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Figure 2. Formation of RAF dimer mediates RAF paradoxical activation by TAK-632. A and B, SK-MEL-2 and A549 cells were treated with TAK-632 and vemurafenib at the indicated concentrations for 2 hours, respectively. Immunoprecipitated proteins (IP) and cell lysates were analyzed by Western blot analysis. C, SK-MEL-2 cells were transfected with siRNA as indicated. Forty-eight hours after transfection, cells were treated with TAK-632 or vemurafenib (Vem) at the indicated concentrations. Cell lysates were analyzed by Western blot analysis. Individual panels with dividing lines are combined from a single electrophoresis gel. D, HCT-116 cells were transfected with plasmids as indicated. Twenty-four hours after transfection, cells were treated with TAK-632 at indicated concentrations for 2 hours. Cell lysates were analyzed by Western blot analysis. Individual panels with dividing lines are combined from a single electrophoresis gel. Numbers in a rectangle represent densitometric analysis of phospho-MEK and phospho-ERK, normalized to DMSO-treated control.
MAPK pathway was not detected in response to TAK-632 treatment of cells coexpressing E586K-BRAF and E478K-CRAF (Fig. 2D). Therefore, dimerization of RAF appears to be associated with minimal activation of the MAPK pathway by TAK-632.

Inhibitor off-rate affects RAF dimer activation/ inhibition

To directly determine the modulation of cellular RAF kinase activity by RAF inhibitors, we performed immunoprecipitation–kinase assays. Consistent with the effect on MEK or ERK phosphorylation observed in SK-MEL-2 cell lysates (Fig. 2A, bottom), TAK-632 slightly activated kinase activity detected in CRAF or BRAF immunoprecipitates at low concentrations and suppressed these kinase activities at higher concentrations (Fig. 3A and Supplementary Fig. S3A). On the other hand, vemurafenib increased RAF activities in a concentration-dependent manner (Fig. 3A and Supplementary Fig. S3A), which was different from the effects on MEK and ERK phosphorylation in cell lysates (Fig. 2A, bottom). Similar observations have been reported for other BRAF inhibitors (13). Taken together, RAF kinase activities in immunoprecipitates and MEK and ERK phosphorylation in cell lysates were well correlated in TAK-632–treated cells but not in BRAF inhibitor–treated cells, suggesting differences in the profile between pan-RAF and BRAF inhibitors.

Since the kinase activity of RAF dimers is suppressed only when both protomers are occupied by inhibitors, we suspected that a difference in inhibitor off-rate from RAF may contribute to the abovementioned gap. In fact, TAK-632 displayed a very long residence time to purified wild-type RAF (28) and PLX4720 demonstrated fast dissociation kinetics and a short residence time (13, 28). In addition to the data in cell-free conditions, washout assay revealed that the inhibition of MEK phosphorylation by TAK-632 was partially rescued but not as seen in control cells (Fig. 3C, bottom). Notably, TAK-632 suppressed MEK and ERK phosphorylation derived from dimerization-prone mutants (Fig. 3C, bottom). These data revealed that in contrast to existing BRAF inhibitors (13), TAK-632 suppresses activated BRAF–CRAF heterodimers (Fig. 3C). The interaction between dimerization-prone mutants was hardly detected in dimethyl sulfoxide (DMSO)-treated cells but was induced in RAF inhibitor–treated cells (Fig. 3C, top), suggesting that the endogenous BRAF(V600E)/MEK/ERK pathway stimulates a negative feedback mechanism to inhibit RAF dimerization in BRAF-mutant A375 cells (17).

The formation of CRAF–CRAF homodimers by PLX4720 reportedly mediated paradoxical RAF activation (13). To examine whether CRAF homodimer formation is also induced by TAK-632, we performed immunoprecipitation assays using A375 cells expressing CRAF with a distinct size tag (HA-FLAG-CRAF and GFP-CRAF). HA-FLAG-CRAF was coprecipitated with GFP-CRAF in a concentration-dependent manner by TAK-632 and vemurafenib (Fig. 3D, top). Furthermore, the RAF kinase assay revealed that TAK-632 slightly activated GFP-CRAF at low concentrations and suppressed it at higher concentrations in cells coexpressing HA-FLAG-BRAF/GFP-CRAF and HA-FLAG-CRAF/GFP-CRAF, respectively. Meanwhile, vemurafenib increased these activities in a concentration-dependent manner (Fig. 3D, bottom bars). Similar results were obtained by using SK-MEL-2 cells expressing dimerization-prone mutants (Supplementary Fig. S5). Thus, our findings suggest that TAK-632 blocks the activity of both RAF heterodimers and homodimers by prolonged binding to RAF.

Antiproliferative effect of TAK-632

To investigate the antiproliferative activity of TAK-632, we performed proliferation assays in various cell lines harboring mutated BRAF, NRAS, or KRAS. Although TAK-632 exhibited antiproliferative activity in various types of cancer cells, BRAF- and NRAS-mutated melanoma cells appeared to be more sensitive to TAK-632 (Table 1). These results are consistent with the observation that the MAPK pathway is easily suppressed by TAK-632 in BRAF- or NRAS-mutated melanoma cells (Fig. 1A and Supplementary Fig. S1A). Meanwhile, TAK-733 showed stronger antiproliferative effects than TAK-632 both in A375 and SK-MEL-2 cells (G50 of TAK-632 = 40–190 nmol/L, TAK-733 = 6 nmol/L in A375 cells and G50 of TAK-632 = 190–250 nmol/L, TAK-733 = 11 nmol/L in SK-MEL-2 cells). The data are also consistent with the inhibitory activities to ERK phosphorylation (Fig. 1A and Supplementary Fig. S1A and
Figure 3. TAK-632 suppresses the kinase activity of RAF dimer. A, SK-MEL-2 cells were treated with TAK-632 or vemurafenib (vem) at indicated concentrations for 2 hours. CRAF kinase activity was determined using the IP–kinase assay. B, SK-MEL-2 cells were treated with TAK-632 or vemurafenib at 10 μmol/L for 2 hours. Following this, immunoprecipitated CRAF was treated with TAK-632 or vemurafenib at the indicated concentrations for 30 minutes (in vitro–treated). CRAF kinase activity was determined using the IP–kinase assay in the presence of added inhibitors. C, A375 cells were transfected with plasmids as indicated. Twenty-four hours after transfection, cells were treated with TAK-632 or vemurafenib at the indicated concentrations for 2 hours. Immunoprecipitated proteins (IP) and cell lysates were analyzed by Western blot analysis. Individual panels with dividing lines are combined from a single electrophoresis gel. D, A375 cells were transfected with plasmids as indicated. Twenty-four hours after transfection, cells were treated with TAK-632 (632) or vemurafenib at the indicated concentrations for 2 hours. Immunoprecipitated proteins (IP) and cell lysates were analyzed by Western blot analysis. CRAF kinase activity was determined using the IP–kinase assay (bars). Individual panels with dividing lines are combined from a single electrophoresis gel.
Next, we examined the in vivo efficacy of TAK-632 in NRAS-mutant melanoma using a SK-MEL-2 xenograft model. TAK-632 exhibited potent antitumor efficacy when orally administered at 60 mg/kg once daily (T/C = 37%, P < 0.001) or at 120 mg/kg once daily (T/C = 29%, P < 0.001) for 21 days without severe toxicity (Fig. 4A). Western blot analysis in parallel studies of mice treated for 3 days revealed significant reductions of ERK phosphorylation in SK-MEL-2 tumors from animals treated with 60 or 120 mg/kg TAK-632 compared with that in vehicle-treated mice (Fig. 4B and C). The data indicate that antitumor activity and MAPK pathway inhibition are well correlated in the model.

TAK-632 suppresses the MAPK pathway in vemurafenib-resistant melanoma cells

One of the major clinical challenges is to overcome the acquired resistance to BRAF inhibitors for the treatment of patients with melanoma (11, 12). To preclinically examine the effects of TAK-632 on several known mechanisms of vemurafenib resistance (20, 21, 26), we expressed wild-type NRAS, NRAS with activating mutation (G12D, G13D, Q61K, or Q61R), KRAS with activating mutation (G12V), and COT in A375 cells. Although the MAPK pathway in cells expressing wild-type NRAS, mutated NRASs, and KRASG12V is resistant to vemurafenib as reported previously (15, 21), TAK-632 suppressed MEK and ERK phosphorylation in these cells as seen in control cells (Fig. 5A–C). On the other hand, COT conferred resistance to both TAK-632 and vemurafenib (Fig. 5D), consistent with a previous report that COT activates MEK and ERK in a RAF-independent manner (20). Notably, dimer formation was observed when NRASQ61K was expressed in A375 cells (Fig. 5E). The observed BRAF–CRAF dimer may be induced by high levels of GTP-bound NRAS, conferring insensitivity to vemurafenib in the cells (Fig. 5E) as reported previously (13–15, 17). In addition, aberrantly spliced BRAF has emerged as a mechanism of resistance to vemurafenib (16). The hyperactive spliced variants lack exons 2–10, including the RAS-binding domain, but retain the kinase domain and constitutively dimerize in a RAF-independent manner. Therefore, we expressed N-terminally truncated BRAF (ΔN-BRAF) that lacks exons 1–10 and examined the effect of TAK-632. Consistent with previous findings (16), MEK and ERK phosphorylation was increased by ΔN-BRAF and vemurafenib had a little effect on them (Fig. 5F). In contrast, TAK-632 suppressed MEK and ERK phosphorylation in cells expressing ΔN-BRAF (Fig. 5F). Interestingly, TAK-632 and vemurafenib strongly induced RAF heterodimer formation in cells expressing ΔN-BRAF compared with that in cells expressing wild-type BRAF (Fig. 5F), indicating that ΔN-BRAF easily forms RAF heterodimers both in a steady state and in the presence of RAF inhibitors. These observations indicate that TAK-632 can suppress the MAPK pathway in vemurafenib-resistant melanoma cells bearing RAS mutation or BRAF truncation.

Subsequently, we examined the effects of RAF inhibitors and TAK-733 on cell proliferation of vemurafenib-resistant melanoma cells. TAK-632, TAK-733, and vemurafenib displayed potent antiproliferative activities in parent or mock-transfected A375 cells (Table 2). We showed that cells expressing

Table 1. In vitro GI50 of TAK-632 in various cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Mutation status (from COSMIC database)</th>
<th>Mean GI50 (nmol/L)</th>
</tr>
</thead>
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<tr>
<td>A375</td>
<td>Melanoma</td>
<td>V600E WT WT</td>
<td>160</td>
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<tr>
<td>HT-144</td>
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<td>V600E WT WT</td>
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<td>Melanoma</td>
<td>WT Q61K WT</td>
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<td>G469V Q61K WT</td>
<td>60</td>
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<td>WT Q61R WT</td>
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<td>Lung cancer</td>
<td>WT WT Q61K</td>
<td>770</td>
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<td>WT WT WT</td>
<td>1,210</td>
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NOTE: Various cell lines were treated with TAK-632. The mean GI50 value was derived from the cell viability assay 72 hours after drug treatment.
NRAS<sup>Q61K</sup> and AN-BRAF demonstrated resistance to vemurafenib, as reported previously (16, 21), but retained sensitivity to TAK-632 and TAK-733, albeit at higher doses than parent or mock-transfected cells (Table 2). Consistent with these results, Western blot analysis revealed that both TAK-632 and TAK-733 significantly suppressed ERK phosphorylation not only in mock-transfected cells but also in NRAS<sup>Q61K</sup> and AN-BRAF-expressing cells (Supplementary Fig. S6A). Given that some dabrafenib-resistant BRAF-mutated melanoma cells reportedly had NRAS mutation or BRAF truncation (34, 35), TAK-632 may also have the potential to show efficacy in such dabrafenib-resistant melanoma cells. Further investigation will clarify the point in the future.

**Combination of TAK-632 with a MEK inhibitor enhances antiproliferative activities in BRAF- and NRAS-mutated melanoma cells**

Given that near complete inhibition of the MAPK pathway may be required for significant antitumor effects in melanomas (7), vertical combinations of agents targeting multiple steps along the MAPK pathway can be a reasonable therapeutic strategy. Therefore, we examined combinatorial effects of TAK-632 and TAK-733 on MAPK pathway activation and cell proliferation in melanomas. Compared with single treatment, the combination robustly suppressed ERK phosphorylation in A375, HMV-II, and SK-MEL-2 (Fig. 6A–C). As shown in a previous study (36), TAK-733 induced the accumulation of phosphorylated MEK in NRAS-mutant HMV-II and SK-MEL-2 cells but not in BRAF-mutant A375 cells (Fig. 6A–C), because treatment with a MEK inhibitor induces the accumulation of phosphorylated MEK by relieving the negative feedback loop from ERK in BRAF wild-type cells (37, 38). In contrast, in BRAF-mutant cells, the negative feedback mechanism is abrogated; therefore, the accumulation of phosphorylated MEK does not occur (37, 38). Notably, when HMV-II and SK-MEL-2 cells were cotreated with TAK-632 and TAK-733, the increase in MEK phosphorylation by a MEK inhibitor was prevented by TAK-632 (Fig. 6B and C). To further examine the combination effects of TAK-632 and TAK-733 in NRAS-mutated melanoma cells, we investigated the expression level of several cell-cycle/translation/apoptosis markers in SK-MEL-2 cells. Western blot analysis on the downstream markers shows the benefit of the combination over the single agents in cell cycle as measured by cyclin D1, translation as measured by S6 phosphorylation, and apoptosis as measured by cleaved PARP and lamin (Fig. 6C).

Consistent with the potent inhibition of ERK phosphorylation and downstream markers, TAK-632 showed a stronger inhibitory effect on cell viability when combined with TAK-733 in HMV-II and SK-MEL-2 cells (Fig. 6D and E). The calculated CI value at EC<sub>50</sub> was 0.64 in HMV-II cells and 0.46 in SK-MEL-2 cells, indicating synergy between the drugs in blocking the proliferation of NRAS-mutant melanoma cells. The combination displayed similar synergistic effects in other NRAS-mutant cells, HMCB and GAK cells (data not shown). On the other hand, additive antiproliferative effects were observed in A375 cells, indicated by a CI value of 1.1 at the EC<sub>50</sub> concentration (Fig. 6F). To determine whether the combined effects preferentially occurred in these melanomas, we tested 293T and HeLa cells, both of which bear wild-type RAF and RAS. We found that the combination did not induce potent inhibition of their proliferation (Supplementary Fig. S7A and S7B). Collectively, our data indicate that the combination of TAK-632 and a MEK inhibitor effectively inhibits the proliferation of melanoma cells with BRAF or NRAS mutation.

Figure 4. Effect of TAK-632 on xenograft proliferation. A, mice bearing SK-MEL-2 xenografts were treated once daily for 21 consecutive days with vehicle or TAK-632 SD at the indicated concentrations (10 mice per each treatment group). B, tumor volumes were calculated using the formula: volume = length × width<sup>2</sup> ÷ 2. Points, mean tumor volumes; bars, SE. C, mean tumor volumes were calculated using the formula: volume = length × width<sup>2</sup> ÷ 2. Points, mean tumor volumes; bars, SE. **P < 0.01. ***P < 0.001.

Figure 5. Effect of TAK-632 on xenograft proliferation. A, mice bearing SK-MEL-2 xenografts were treated once daily for 21 consecutive days with vehicle or TAK-632 SD at the indicated concentrations (10 mice per each treatment group). Day 0 indicates the beginning of treatment. Tumors were measured twice a week. Points, mean tumor volumes; bars, SE. **P < 0.01. ***P < 0.001.
TAK-632 shows synergy with a MEK inhibitor in vemurafenib-resistant melanoma models

Since levels of MEK and ERK phosphorylation in A375 cells expressing NRASQ61K or ΔN-BRAF were increased compared with the levels in parent or mock-transfected cells (Supplementary Fig. S6B), reactivation of the MAPK pathway is expected to warrant combined treatment with TAK-632 and a MEK inhibitor to further inhibit the MAPK pathway and consequently suppress cell proliferation. The combination of TAK-632 and TAK-733 demonstrated highly
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Table 2. In vitro GI$_{50}$ (nmol/L) of RAF or MEK inhibitor in A375 cells expressing ΔN-BRAF or NRAS$^{Q61K}$

<table>
<thead>
<tr>
<th></th>
<th>TAK-632</th>
<th>Vemurafenib</th>
<th>TAK-733</th>
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</thead>
<tbody>
<tr>
<td>Parent</td>
<td>40</td>
<td>180</td>
<td>6</td>
</tr>
<tr>
<td>Mock</td>
<td>60</td>
<td>250</td>
<td>6</td>
</tr>
<tr>
<td>ΔN-BRAF</td>
<td>500</td>
<td>&gt;10,000</td>
<td>240</td>
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<tr>
<td>NRAS-Q61K</td>
<td>1,710</td>
<td>&gt;10,000</td>
<td>460</td>
</tr>
</tbody>
</table>

NOTE: A375 cells expressing mock, NRAS$^{Q61K}$, and ΔN-BRAF were treated with TAK-632, vemurafenib, or TAK-733. The mean GI$_{50}$ value was derived from the cell viability assay 72 hours after drug treatment.

synergistic antiproliferative effects in NRAS$^{Q61K}$ and ΔN-BRAF cells (Fig. 6G and H; CI = 0.30 and 0.36, respectively). Consistent with these results, the combined treatment robustly suppressed ERK phosphorylation in NRAS$^{Q61K}$ and ΔN-BRAF cells (Supplementary Fig. S8A; 2 hours of treatment and Fig. 6I and S8B; 24 hours of treatment). The 24-hour treatment assay also revealed that the cotreatment led to more drastic inhibition of cell-cycle progression, as indicated by decreased cyclin D1 expression in these cells (Fig. 6I and Supplementary Fig. S8B). Given the strong inhibitory effects of the combination of TAK-632 and TAK-733 on the MAPK pathway and cell proliferation, reactivation of the MAPK pathway appears to play a major role in the resistance to vemurafenib in the models.

Discussion

NRAS is mutated in approximately 18% of melanomas and 10% of hematologic cancers (4). Our data using siRNA or the pan-RAF inhibitor TAK-632 indicate that NRAS-mutated melanoma highly depends on the RAF/MEK/ERK pathway (Fig. 1). Given that melanoma possesses outstanding mutation rates in components of the MAPK pathway, it is not surprising that this pathway is particularly important for the initiation and/or maintenance of melanoma. Although we have not examined the dependence of hematologic cancers on RAF, further investigations will provide us with an insight into therapeutic interventions for the cancers.

The inhibitor-induced conformational change in the activation loop of a kinase is observed in cocrystral structures of a DFG-out (inactive conformation) type inhibitor and offers a slower dissociation rate than a DFG-in (active conformation) type inhibitor (39). We have shown that TAK-632 binds to BRAF in the DFG-out state (28), whereas several BRAF inhibitors, including vemurafenib, are known to associate with BRAF in the DFG-in state (7, 13). In fact, TAK-632 had a much slower off-rate from RAF than BRAF inhibitors (13, 28). Consistent with these observations, it has been reported that an investigational RAF inhibitor AZ-628 binds to BRAF in the DFG-out state, has slow off-rate, and does not significantly induce RAF paradoxical activation (13, 40). Notably, our data show that vemurafenib elevates RAF kinase activity in immune-noprecipitates even at high concentrations that suppress MEK and ERK phosphorylation in cell lysates (Figs. 2A and 3A). This discrepancy provides supporting evidence that vemurafenib has a rapid off-rate from RAF and thereby easily dissociates from RAF during immunoprecipitation and subsequent kinase assays. The difference in dissociation rates between the pan-RAF inhibitor TAK-632 and BRAF inhibitors illustrates the following model: TAK-632 (pan-RAF inhibitors) continuously binds to both protomers of RAF dimers and thereby inhibits kinase activity of the RAF dimer so that minimal RAF paradoxical activation occurs only at low concentrations. Meanwhile, BRAF inhibitors induce RAF dimerization but do not persistently occupy both protomers, thereby robustly stimulating paradoxical RAF activation.

Notably, the RAF inhibitory activity of TAK-632 appears to be several times higher than that of vemurafenib, as shown in our data and existing findings from enzymatic assays and cellular assays (Supplementary Fig. S1A; refs. 7, 15, 28). However, such moderate differences in the potencies of these inhibitors may not be sufficient to explain the remarkable difference in the RAF dimer inhibitory activities (Fig. 3). We show that in vitro-treated vemurafenib suppresses paradoxically activated RAF (Fig. 3B and S3B), suggesting that the varying RAF dimer inhibitory activity may be derived from the difference in the dissociation rate from RAF.

The model complies with a recently reported model that RAF inhibitors mediate RAF paradoxical activation by relieving inhibitory autophosphorylation (33). Loss of glycine-rich phosphate-binding (P)-loop phosphorylation is considered to destabilize the inactive DFG-out conformation and allow a shift to the active DFG-in conformation (41, 42). Consistent with the report (33), we noted that vemurafenib did not induce significant paradoxical RAF activation in HMV-II cells that bear not only NRAS mutation but also BRAF mutation at G469, which may disrupt P-loop autoinhibition (data not shown). As the formation of RAF dimers and inhibition of P-loop autophosphorylation are not mutually exclusive, these mechanisms are expected to cooperatively induce RAF paradoxical activation.

Paradoxical RAF activation by BRAF inhibitors plays a significant role in the development of squamous cell carcinoma and keratoacanthoma in the clinic (8–11, 43). Several BRAF inhibitors have paradoxically stimulated normal cell proliferation in mouse epithelial cells in vivo (13, 44). When we performed pharmacodynamic analysis using melanoma xenograft rat models, we found that TAK-632 suppressed ERK phosphorylation in xenograft tumors but did not induce RAF paradoxical activation in normal skin tissues (data not shown). Our toxicologic studies also showed no proliferative effects on skin epithelial cells in animals (data not shown), suggesting that TAK-632 may avoid the risk of secondary skin tumors in the clinic. However, whereas vemurafenib did not have skin proliferative effects in preclinical animal models (7), skin tumor incidence was observed in patients (8, 9). Moreover, sorafenib, which is a multi-kinase inhibitor with pan-RAF inhibitory effects (14, 45), is associated with the emergence of skin tumors during treatment (46). Collectively, we should cautiously consider whether TAK-632 has an effect on cutaneous cell proliferation.
Figure 6. TAK-632 shows synergy with a MEK inhibitor. A–C, A375, HMV-II, or SK-MEL-2 cells were cotreated with TAK-632 and TAK-733 at the indicated concentrations for 24 hours. Cell lysates were analyzed by Western blot analysis. Individual panels with dividing lines are combined from a single electrophoresis gel. D–F, HMV-II, SK-MEL-2, or A375 cells were cotreated with TAK-632 and TAK-733 at the indicated concentrations for 72 hours. Cell viability was measured. The CI value at EC50 was calculated. G and H, A375 cells stably expressing NRASQ61K or ΔN-BRAF were cotreated with TAK-632 and TAK-733 at the indicated concentrations for 72 hours. Cell viability was measured. The CI value at EC50 was calculated. I, A375 cells stably expressing mock, NRASQ61K, or ΔN-BRAF were cotreated with TAK-632 and TAK-733 or vemurafenib and TAK-733 at the indicated concentrations for 24 h. Cell lysates were analyzed by Western blot analysis. Individual panels with dividing lines are combined from a single electrophoresis gel.
Since resistance to BRAF inhibitor treatment is associated with reactivation of the MAPK pathway, the combination of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib in patients with BRAF-mutant melanomas has been clinically tested. Phase I and II trials showed significant improvements in progression-free survival of the combination group compared with that of the monotherapy group (47). Notably, we showed that TAK-632 exhibits highly synergistic effects with TAK-733 in BRAF inhibitor-resistant melanoma cells (Fig. 6), suggesting the potential clinical benefit of the combination of pan-RAF inhibitors and MEK inhibitors. In the abovementioned clinical trials, the rate of skin proliferative lesions was reduced by the combination therapy (47). Although the potential risk of TAK-632 inducing skin tumors in patients remains unknown, combined therapy with MEK inhibitors would be a reasonable strategy not only to enhance the antitumor activity but also to possibly reduce the risk of skin tumor incidence. Given that amplification of the upstream oncogenic driver of the MAPK pathway has been identified as a mechanism for MEK inhibitor resistance in colorectal cancer cell lines, the combination of pan-RAF inhibitors and MEK inhibitors may also contribute to overcoming the drug resistance in tumors besides melanoma (48–50).

In this preclinical study, we demonstrated that TAK-632 has favorable characteristics in terms of killing NRAS-mutant melanoma and BRAF inhibitor-resistant BRAF-mutant melanoma cells. TAK-632 can prevent RAF paradoxical activation by suppressing the kinase activity of the RAF dimer (Supplementary Fig. 59). Furthermore, our findings suggest that TAK-632 has the potential to delay the emergence of drug resistance caused by NRAS mutation and BRAF truncation. In addition, TAK-632 potentially targets other tumors whose growth and survival are dependent on the MAPK pathway. Further investigations will be required to identify such sensitive patient populations.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Tsushuya, J. Donelan
Writing, review, and/or revision of the manuscript: A. Nakamura, T. Arita, J. Donelan, E. Carideo, K. Galvin, M. Okaniwa, T. Ishikawa, S. Yoshida
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Carideo, M. Okaniwa
Study supervision: T. Arita, J. Donelan, S. Yoshida

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References


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