

## ORIGINAL ARTICLE

# Development of a potent small-molecule degrader against oncogenic BRAF<sup>V600E</sup> protein that evades paradoxical MAPK activation

Nobumichi Ohoka<sup>1</sup>  | Masanori Suzuki<sup>2</sup> | Takuya Uchida<sup>3</sup> | Yoshinori Tsukumo<sup>1</sup>  | Masayuki Yoshida<sup>2</sup> | Takao Inoue<sup>1</sup> | Hitoshi Ohki<sup>2</sup> | Mikihiro Naito<sup>1,4</sup> 

<sup>1</sup>Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences, Kawasaki, Kanagawa, Japan

<sup>2</sup>Modality Research Laboratories, Biologics Division, Daiichi Sankyo Co., Ltd., Tokyo, Japan

<sup>3</sup>Medicinal Chemistry Research Laboratories, R&D Division, Daiichi Sankyo Co., Ltd., Tokyo, Japan

<sup>4</sup>Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

## Correspondence

Nobumichi Ohoka, Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki, Kanagawa 210-9501, Japan.

Email: [n-ohoka@nihs.go.jp](mailto:n-ohoka@nihs.go.jp)

Mikihiro Naito, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

Email: [mnaito@mol.f.u-tokyo.ac.jp](mailto:mnaito@mol.f.u-tokyo.ac.jp)

## Funding Information

This work was supported in part by grants from the Japan Society for the Promotion of Science (KAKENHI grants JP18K06567 and JP21K06490 to N.O. and JP18H05502 and JP21H02777 to M.N.), the Japan Agency for Medical Research and Development (AMED grants JP19cm0106136, JP21ak0101073, and JP21mk0101197 to N.O. and JP20ak0101073 to M.N.), and the Princess Takamatsu Cancer Research Fund (to M.N.).

## Abstract

BRAF mutations are frequently observed in melanoma and hairy-cell leukemia. Currently approved rapidly accelerated fibrosarcoma (RAF) kinase inhibitors targeting oncogenic BRAF V600 mutations have shown remarkable efficacy in the clinic, but their therapeutic benefits are occasionally hampered by acquired resistance due to RAF dimerization-dependent reactivation of the downstream MAPK pathway, which is known as paradoxical activation. There is also a concern that paradoxical activation of the MAPK pathway may trigger secondary cancer progression. In this study, we developed chimeric compounds, proteolysis targeting chimeras (PROTACs), that target BRAF<sup>V600E</sup> protein for degradation. CRBN(BRAF)-24, the most effective chimera, potently degraded BRAF<sup>V600E</sup> in a ubiquitin-proteasome system (UPS)-dependent manner and inhibited the proliferation of BRAF<sup>V600E</sup>-driven cancer cells. In BRAF wild-type cells, CRBN(BRAF)-24 induced neither BRAF<sup>WT</sup> degradation nor paradoxical activation of the MAPK pathway. Biochemical analysis revealed that CRBN(BRAF)-24 showed more potent and sustained suppression of MAPK signaling than a BRAF<sup>V600E</sup> inhibitor, PLX-8394, in BRAF<sup>V600E</sup>-driven cancer cells. Targeted degradation of BRAF<sup>V600E</sup> by CRBN(BRAF)-24 could be a promising strategy to evade paradoxical activation of the RAF-MAPK pathway.

## KEYWORDS

BRAF, drug discovery, MAPK, PROTAC, ubiquitination

**Abbreviations:** CRBN, cereblon; IAP, inhibitor of apoptosis protein; MDM2, murine double minute 2; PROTAC, proteolysis targeting chimera; RAF, rapidly accelerated fibrosarcoma; RTK, receptor tyrosine kinase; SNIPER, specific and nongenetic inhibitor of apoptosis protein (IAP)-dependent protein eraser; UPS, ubiquitin-proteasome system; VHL, von Hippel-Lindau; WT, wild-type.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

## 1 | INTRODUCTION

RAF family kinases (ARAF, BRAF, and CRAF in humans) play a major role in cell proliferation, growth, differentiation, and survival.<sup>1-3</sup> When receptor tyrosine kinases (RTKs) are activated by binding of extracellular growth factors, the small GTPase RASs recruit RAFs to the plasma membrane, promoting their dimerization and activation.<sup>4</sup> Activated RAF phosphorylates and activates the downstream MEK and ERK kinases, thus transmitting growth signals through a kinase-activation cascade (known as the RAS-RAF-MEK-ERK pathway or MAPK pathway).

Among the genes encoding the three RAF proteins, *BRAF* is most prone to mutation in cancer cells. *BRAF* mutations are observed in melanomas, colorectal cancers, non-small cell lung cancers, and in almost all hairy-cell leukemia.<sup>5-8</sup> Most of these mutations are missense mutations found in the kinase domain and affect the biochemical characteristics of *BRAF* kinase.<sup>9-11</sup> Dysregulation of the MAPK signaling pathway by *BRAF* mutations is a potent driver of cancer development and progression.<sup>1-3</sup>

*BRAF* mutations are grouped into three classes. Class 1 *BRAF* mutations, sharing more than 90% of total *BRAF* alterations, include only *BRAF* V600 mutations (primarily *BRAF*<sup>V600E</sup> mutations).<sup>12,13</sup> These mutations produce constitutively active *BRAF* kinases capable of signaling as monomers, and their robust kinase activity results in high phosphorylation and activation levels of downstream ERK. Class 2 *BRAF* mutants, such as K601E, L597Q, and G469A, signal as constitutively active mutant dimers. The ability of class 1 and class 2 mutants to activate MAPK signaling is independent of upstream RAS activity, and highly activated ERK drives feedback inhibition of RAS activation.<sup>14-16</sup> In contrast, class 3 *BRAF* mutants, such as G466V and D594N, have little or no kinase activity, and increase ERK signaling through heterodimers formed with wild-type RAFs.<sup>17</sup> The class 3 *BRAF* mutants bind more tightly to RAS than wild-type *BRAF* and enhance the binding and activation of wild-type CRAF, the predominant partner for heterodimerization. Therefore, activation of ERK in tumors with the class 3 mutants requires RAS activation.

Selective RAF inhibitors, such as vemurafenib and dabrafenib, have shown remarkable clinical utility against highly active *BRAF*<sup>V600E</sup> mutant cancers.<sup>18</sup> However, as with many kinase inhibitors resistance develops, and patients become insensitive during long-term treatment.<sup>19</sup> In addition, these drugs paradoxically activate the MAPK pathway in *BRAF* wild-type cells, especially those with oncogenic RAS mutations or with elevated upstream receptor signaling.<sup>9-11</sup> The paradoxical activation of the MAPK pathway raises concerns that RAF inhibitors may promote cell proliferation and facilitate the progression of secondary RAS-driven cancers.<sup>13,20,21,22,23</sup> Therefore, there is a need for alternative therapeutic strategies targeting *BRAF* that can evade paradoxical activation of MAPK signaling.

Targeted protein degradation using bifunctional small molecules such as proteolysis-targeting chimeras (PROTACs) and specific and non-genetic inhibitor of apoptosis protein

(IAP)-dependent protein erasers (SNIPERs) is an emerging modality in drug discovery.<sup>24,25</sup> These molecules have a chimeric structure in which a ligand that binds to a target protein and another ligand that binds to an intracellular E3 ubiquitin ligase (e.g., CRBN, VHL, IAP, MDM2) are conjugated by a linker.<sup>26-31</sup> The chimeras cross-link target proteins of interest and E3 ligases and promote ubiquitination and degradation of the target proteins by the 26S proteasome. By substituting target ligands, numerous PROTACs and SNIPERs have been developed that degrade pathogenic proteins involved in various diseases.<sup>26-33</sup> In this study, we developed new chimeric molecules that degrade *BRAF* mutant proteins. The resulting molecule CRBN(*BRAF*)-24 shows potent activity to degrade *BRAF*<sup>V600E</sup> and does not induce paradoxical activation of the MAPK pathway in *BRAF*<sup>WT</sup> cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Design and synthesis of PROTAC compounds

We designed PROTAC compounds in which a *BRAF* inhibitor is connected to a ligand for an E3 ligase via a linker. The chemical synthesis, schemes, and physicochemical data for compounds are provided in the [Doc. S1](#) and [Scheme S1-S23](#).

### 2.2 | Reagents

Tissue culture plastics were purchased from Greiner Bio-One. RPMI 1640 medium, DMEM, Eagle's minimum essential medium, and kanamycin were from Merck. McCoy's 5a medium and Leibovitz's L-15 medium were from Thermo Fisher Scientific. Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (MG132) was from Peptide Institute. MLN7243 and MLN4924 were from Active Biochem.

### 2.3 | Cell culture

Human melanoma A375 cells, N-RAS mutant-A375 isogenic cells, and K-RAS mutant-A375 isogenic cells were maintained in DMEM containing 10% FBS and 100  $\mu\text{gml}^{-1}$  kanamycin. Human colon carcinoma COLO205, RKO, and NCI-H508 cells; human lung carcinoma NCI-H23, NCI-H1395, NCI-H2087, NCI-H1755, and NCI-H1666 cells; human melanoma SK-MEL-30 cells; human ovarian carcinoma OVCAR8 cells; and human B cell leukemia JVM-3 cells were maintained in RPMI 1640 medium containing 10% FBS (5% FBS for NCI-H1666) and 100  $\mu\text{gml}^{-1}$  kanamycin. Human melanoma SK-MEL-28 cells were maintained in Eagle's minimum essential medium containing 10% FBS and 100  $\mu\text{gml}^{-1}$  kanamycin. Human colon carcinoma HCT116 cells were maintained in McCoy's 5a medium containing 10% FBS and 100  $\mu\text{gml}^{-1}$  kanamycin. Human breast carcinoma MDA-MB-231 cells were maintained

in Leibovitz's L-15 medium containing 10% FBS and 100  $\mu\text{g ml}^{-1}$  kanamycin. SK-MEL-30 and JVM-3 cell lines were purchased from DSMZ. OVCAR8 cell line was described elsewhere.<sup>34</sup> The other cell lines were purchased from ATCC. These cells were treated with various concentrations of compounds for the indicated periods of time.

## 2.4 | Western blotting

Cells were lysed with SDS lysis buffer (0.1 M Tris-HCl at pH 8.0, 10% glycerol, 1% SDS) and immediately boiled for 10 minutes to obtain clear lysates. Protein concentrations were measured using the BCA method (Pierce). Lysates containing equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (Merck) for Western blot analysis using the appropriate antibodies. Immunoreactive proteins were visualized using the Immobilon Western chemiluminescent HRP substrate (Merck) or Clarity Western ECL substrate (Bio-Rad); light emission intensity was quantified using an LAS-3000 lumino-image analyzer equipped with Image Gauge v2.3 software. The antibodies used in this study were: anti-BRAF antibody (14814; Cell Signaling Technology), anti-CRAF antibody (53745; Cell Signaling Technology), anti-MEK1/2 antibody (8727; Cell Signaling Technology), anti-phospho-MEK1/2 (Ser217/221) antibody (9121; Cell Signaling Technology), anti-p44/42 MAPK (ERK1/2) antibody (4695; Cell Signaling Technology), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (4377; Cell Signaling Technology), anti-RAS antibody (8955; Cell Signaling Technology), anti-CRBN antibody (71810; Cell Signaling Technology), and anti- $\beta$ -actin antibody (A5316; Merck).

## 2.5 | siRNA transfection

A375 cells were transiently transfected with an equal mixture of three CRBN-specific stealth short interfering RNAs (stealth siRNAs) (HSS121807, HSS121808, HSS121809; Life Technologies) or a negative control stealth siRNA (12935300; Life Technologies) using Lipofectamine RNAi MAX reagent (Life Technologies) according to the protocols provided by the manufacturer.

## 2.6 | Cell viability assay

Cell viability was determined using water-soluble tetrazolium WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) reagent in a spectrophotometric assay according to the manufacturer's instructions (Dojindo). Cells treated with compounds were incubated with WST-8 reagent for 0.5 hour at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The absorbance of the medium at 450 nm or 490 nm was measured using an EnVision Multilabel Plate Reader (PerkinElmer).

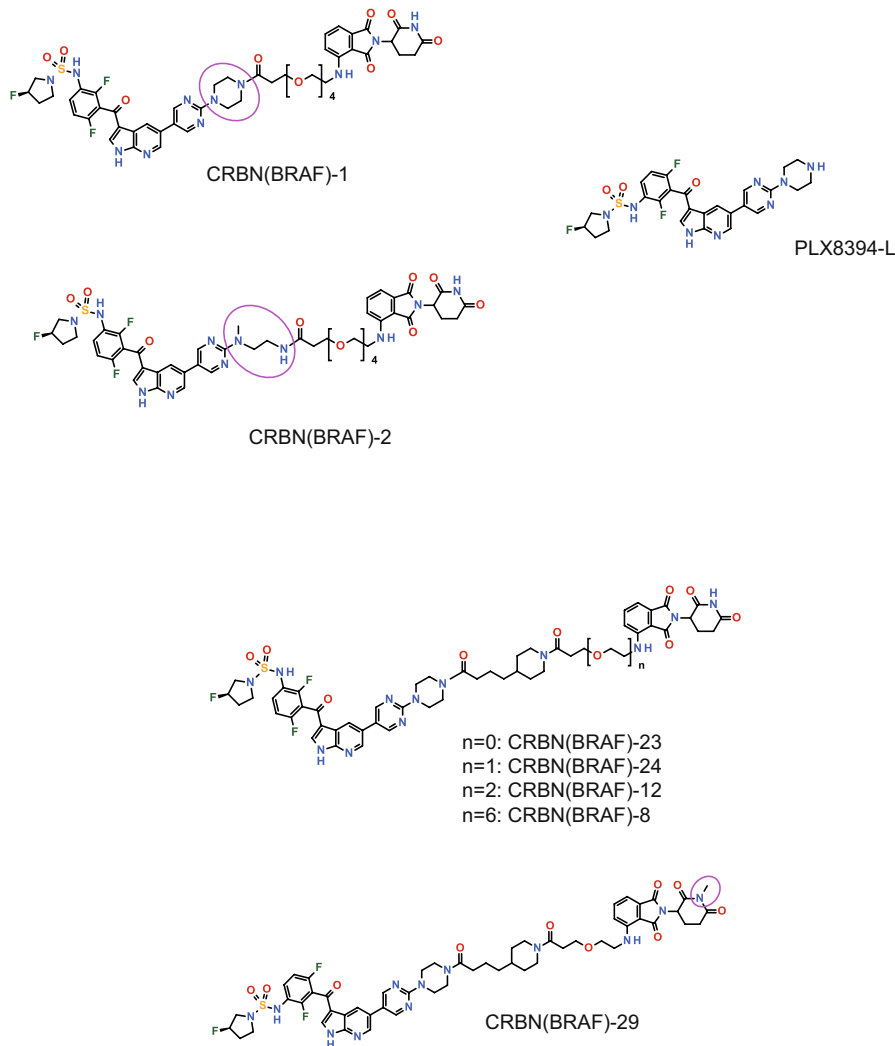
## 3 | Results

### 3.1 | CRBN(BRAF)-24 induces efficient and potent degradation of BRAF<sup>V600E</sup>

To target BRAF mutant proteins for degradation, we designed and synthesized several chimeric molecules that combine various BRAF inhibitors with four E3 ligase ligands (LCL161 for IAP,<sup>28</sup> VH032 for VHL,<sup>27</sup> pomalidomide for CRBN,<sup>35</sup> and RG7388 for MDM2<sup>30</sup>). After evaluating the ability of the chimeric compounds to degrade BRAF<sup>V600E</sup> mutant proteins in A375 cells (a cell line with homozygous BRAF<sup>V600E</sup>), we identified an active compound CRBN(BRAF)-1 composed of a clinical BRAF inhibitor PLX8394<sup>23,36,37</sup> and pomalidomide (Figure 1). As shown in Figure 2A, CRBN(BRAF)-1 effectively reduced BRAF protein levels after 24 hours of treatment. CRBN(BRAF)-2, in which the same ligands are conjugated with a different linker, showed attenuated activity to reduce BRAF<sup>V600E</sup> (Figure 1 and Figure 2A). Chimeric compounds recruiting other E3 ligases (IAP[BRAF]-7, VHL[BRAF]-2, and MDM2[BRAF]-2) that replaced the pomalidomide of CRBN(BRAF)-1 with LCL161, VH032, and RG7388, respectively, did not reduce BRAF levels (Figure S1).

We further developed a series of chimeric compounds (CRBN[BRAF]-23, CRBN[BRAF]-24, CRBN[BRAF]-12, and CRBN[BRAF]-8) with improved degradation activity by modifying CRBN(BRAF)-1 with linkers of different lengths and structures (Figure 1). CRBN(BRAF)-24, one of the most potent compounds, was selected as a representative on the basis of its ability to reduce BRAF protein levels in A375 cells (Figure 2B,C). CRBN(BRAF)-24 showed a DC<sub>50</sub> (the concentration at which 50% of maximum degradation was observed) value of 6.8 nM and D<sub>MAX</sub> (the percentage of maximum degradation observed) of approximately 80% in A375 cells at 24 hours after treatment (Figure 2B,C). The reduction in BRAF protein was maintained for at least 72 hours (Figure 2D). In SK-MEL-28 cells (another cell line with homozygous BRAF<sup>V600E</sup>), CRBN(BRAF)-24 also showed effective BRAF-reducing activity and inhibited the downstream pathway, MEK, as shown by suppressed ERK phosphorylation (Figure 2E). We further synthesized an inactive degrader, CRBN(BRAF)-29, by methylating the nitrogen of the glutarimide ring in the pomalidomide moiety of CRBN(BRAF)-24, which abolished the recruitment of CRBN<sup>35</sup> (Figure 1). CRBN(BRAF)-29 did not decrease BRAF protein levels, suggesting that the binding to CRBN with a pomalidomide moiety is necessary for the BRAF<sup>V600E</sup> reduction by CRBN(BRAF)-24 (Figure 2E). In addition, the activity of CRBN(BRAF)-29 to inhibit the downstream kinase signaling was approximately 10-fold weaker than that of CRBN(BRAF)-24, suggesting that the BRAF degradation activity of CRBN(BRAF)-24 contributes to better inhibition of downstream kinase signaling. A moderate hook effect was occasionally observed in the CRBN(BRAF)-24-induced BRAF reduction at higher concentrations (Figure 2B and D), probably because excess amounts of bivalent compounds inhibited the formation of the ternary complex composed of target (BRAF), degrader (CRBN[BRAF]-24), and E3 ligase (CRBN).<sup>24,27</sup>

**FIGURE 1** Chemical structures of chimeric degraders targeting BRAF mutant proteins. Chemical structures of proteolysis targeting chimera (PROTAC) compounds that showed degradation activity against BRAF mutant proteins, BRAF ligand, and negative control compounds. Circles show a characteristic structure



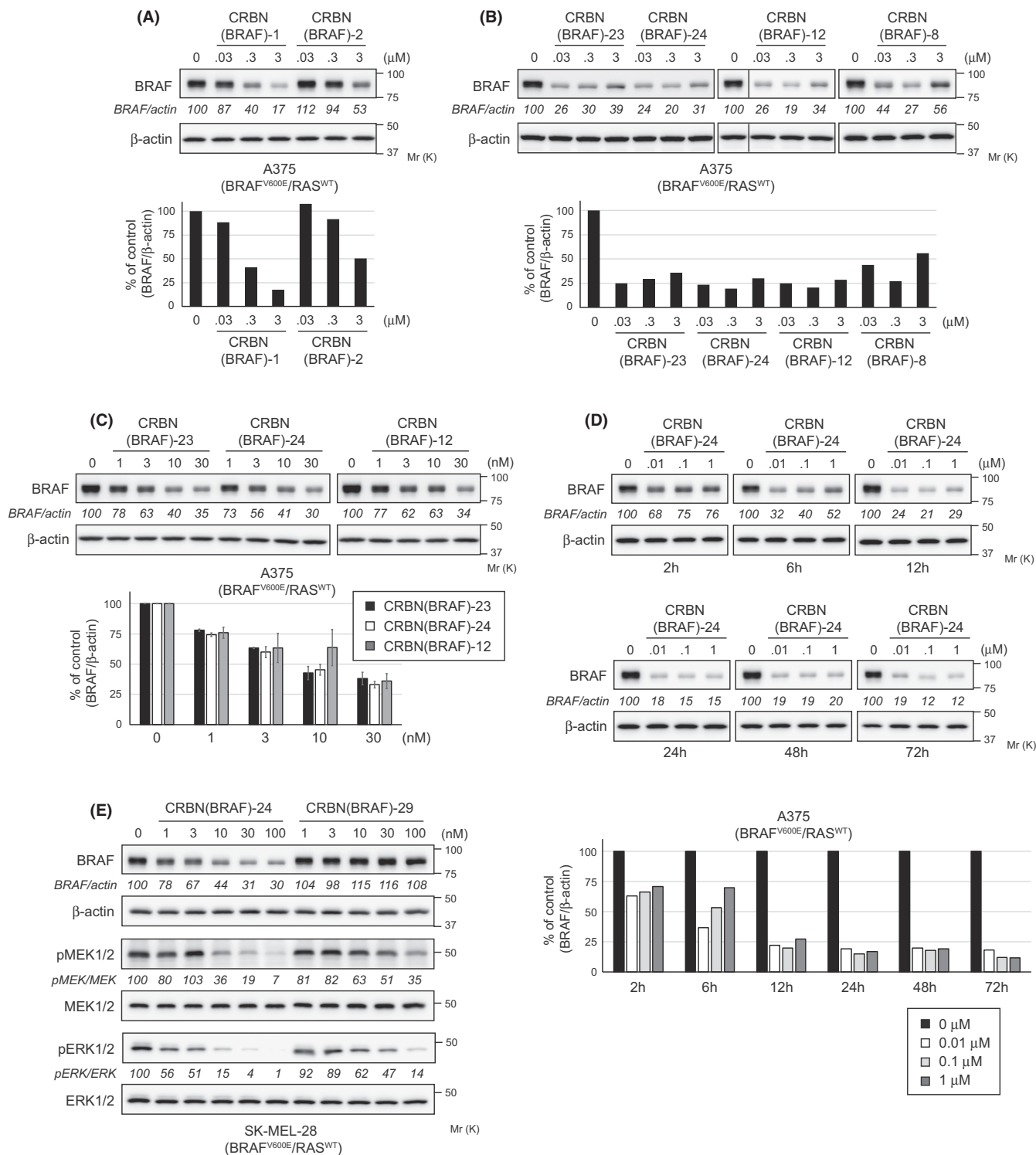
### 3.2 | CRBN(BRAF)-24 degrades BRAF<sup>V600E</sup> via UPS

To determine whether CRBN(BRAF)-24 reduces the protein level of BRAF<sup>V600E</sup> by the expected molecular mechanism, we first investigated the effect of UPS inhibitors. When A375 cells were treated with a proteasome inhibitor, MG132, or a ubiquitin-activating enzyme inhibitor, MLN7243, together with CRBN(BRAF)-24, the CRBN(BRAF)-24-induced reduction in BRAF levels was completely suppressed (Figure 3A). Similarly, the effect of CRBN(BRAF)-24 was also inhibited by MLN4924, a NEDD8-activating enzyme inhibitor that inhibits the activity of the Cullin-RING E3 ligases (CRLs), including CRL4<sup>CRBN</sup>. These results suggest that CRBN(BRAF)-24 degrades BRAF<sup>V600E</sup> via UPS using the CRL-type E3 ligases. It was also confirmed that treatment with a mixture of the BRAF ligand (PLX8394-L) and the CRBN ligand (pomalidomide) did not induce BRAF degradation (Figure 1; Figure 3B). In addition, cotreatment with an excess amount of PLX8394-L (target ligand competition) or pomalidomide (E3 ligand competition) inhibited the BRAF degradation by CRBN(BRAF)-24 (Figure 3B). Silencing the expression of *CRBN* by RNA interference (RNAi) inhibited the degradation of

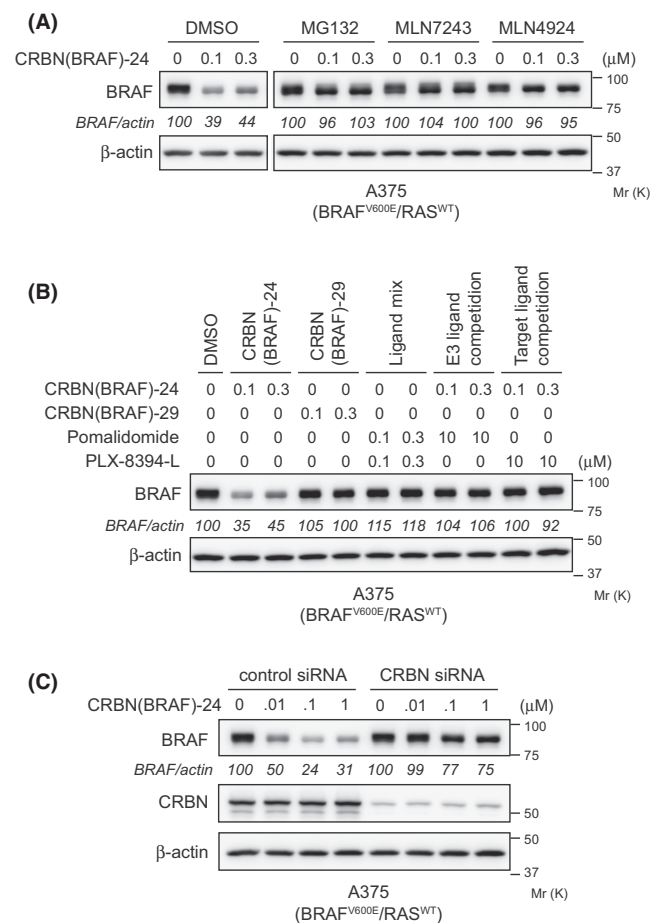
BRAF by CRBN(BRAF)-24 (Figure 3C). These results collectively indicate that CRL4<sup>CRBN</sup> is required for BRAF<sup>V600E</sup> degradation by CRBN(BRAF)-24.

### 3.3 | Neither BRAF degradation nor paradoxical activation of MAPK signaling is induced by CRBN(BRAF)-24 in BRAF<sup>WT</sup> cells

PLX8394, the BRAF ligand used for CRBN(BRAF)-24, has been shown to bind to BRAF<sup>V600E</sup>, BRAF<sup>WT</sup>, and CRAF, although the binding is approximately three- and sixfold more selective for BRAF<sup>V600E</sup> than for BRAF<sup>WT</sup> and CRAF, respectively.<sup>23</sup> Therefore, we next examined if CRBN(BRAF)-24 can degrade BRAF<sup>WT</sup> and CRAF proteins. In cell lines that do not bear the BRAF mutant alleles (HCT116, NCI-H23, SK-MEL-30, and OVCAR8), CRBN(BRAF)-24 had little effect on BRAF<sup>WT</sup> and CRAF protein levels (Figure 4B), strongly suggesting that CRBN(BRAF)-24 did not induce the degradation of BRAF<sup>WT</sup> and CRAF proteins. In cell lines bearing BRAF<sup>V600E</sup> alleles, CRBN(BRAF)-24 showed



**FIGURE 2** PLX8394-based proteolysis targeting chimeras (PROTACs) induced degradation of BRAF<sup>V600E</sup>. A-C, Protein knockdown activities of CRBN(BRAF)s. A375 cells were treated with the indicated concentrations of CRBN(BRAF)s for 24 h. D, CRBN(BRAF)-24 induced sustained downregulation of BRAF protein levels. A375 cells were treated with the indicated concentrations of CRBN(BRAF)s for the indicated periods. E, CRBN(BRAF)-24, but not CRBN(BRAF)-29, led to efficient suppression of BRAF protein levels and pathway signaling. SK-MEL-28 cells were treated with the indicated concentrations of CRBN(BRAF)-24 or CRBN(BRAF)-29 for 24 h. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. Numbers below the BRAF, pMEK1/2, and pERK1/2 panels represent the BRAF/actin, pMEK/MEK, and pERK/ERK ratios, respectively, normalized by designating the level of the vehicle control condition as 100%. Data in the bar graph are the mean ( $\pm$  SD) of two or three independent experiments



**FIGURE 3** Degradation of BRAF<sup>V600E</sup> was mediated by the ubiquitin-proteasome system (UPS) using a cereblon (CRBN) E3 ligase. **A**, Effect of UPS inhibitors on CRBN(BRAF)-24-induced BRAF degradation. A375 cells were treated with the indicated concentrations of CRBN(BRAF)-24 in the presence or absence of 10 μM MG132, MLN7243, or MLN4924 for 8 h. **B**, The combination of E3 ligand and target ligand did not induce BRAF degradation, and BRAF degradation induced by CRBN(BRAF)-24 was inhibited by excess E3 or target ligand. A375 cells were treated with the indicated compounds for 8 h. **C**, Depletion of CRBN suppressed CRBN(BRAF)-24-induced BRAF degradation. A375 cells were transfected with the indicated siRNA for 24 h and treated with CRBN(BRAF)-24 for 24 h. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. A mixture of three different siRNAs against CRBN was used to suppress expression

marginal (A375, COLO-205, and RKO) or moderate (SK-MEL-28) activity to reduce CRAF protein (Figure 4A). However, the CRAF reduction mechanism in these cells seems to be different from the BRAF<sup>V600E</sup> degradation because depletion of CRBN did not affect the CRAF reduction (Figure S2A), and CRAF protein levels were also reduced by PLX8394 (Figure S2B). Importantly, CRBN(BRAF)-24 did not activate MAPK signaling in any BRAF<sup>WT</sup> cells regardless of the presence of oncogenic RAS (Figure 4B), indicating that CRBN(BRAF)-24 does not induce paradoxical activation even in cells in which BRAF is constantly activated by the oncogenic RAS mutants.

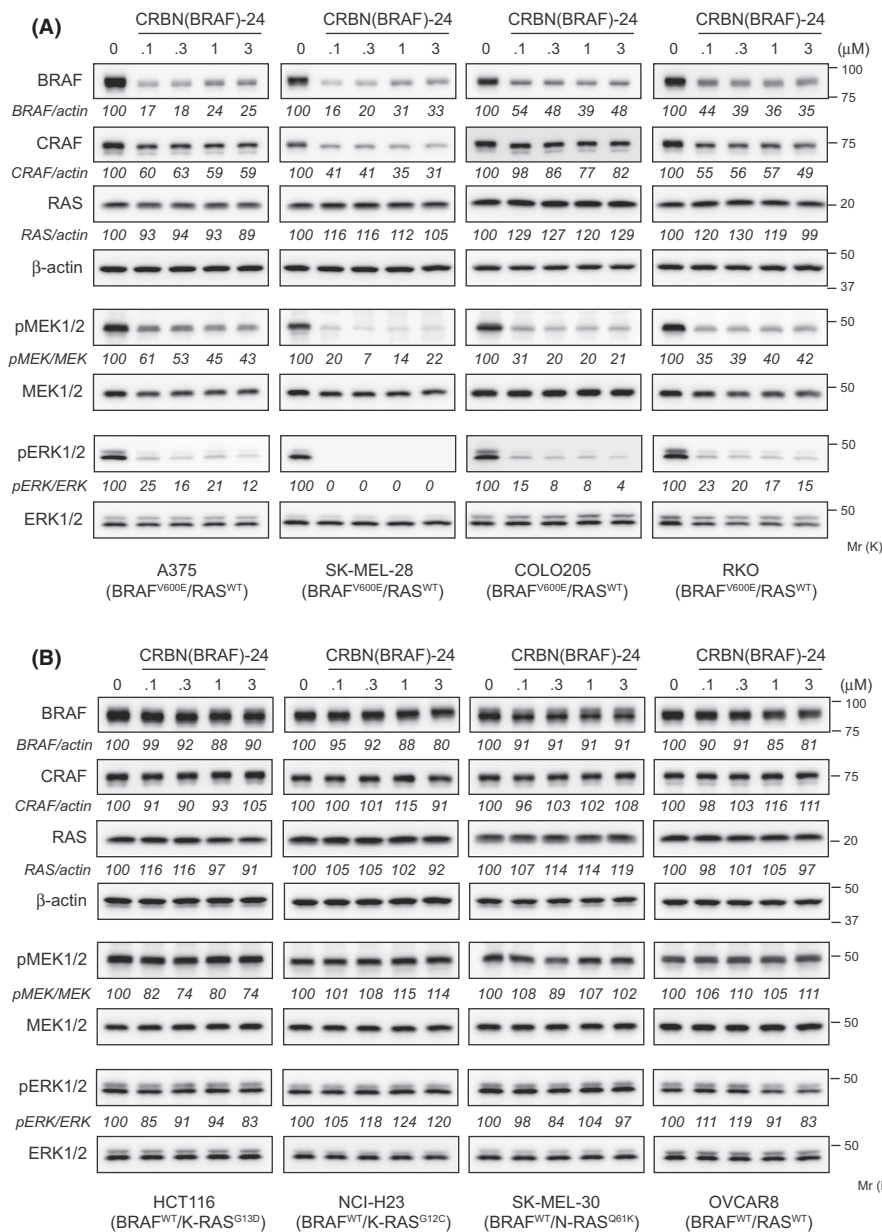
### 3.4 | CRBN(BRAF)-24 selectively induces growth inhibition of cell lines expressing BRAF<sup>V600E</sup>

Next, we investigated the effect of CRBN(BRAF)-24 on cancer cell proliferation and compared its efficacy with that of CRBN(BRAF)-29 and PLX8394. Consistent with the effective inhibition of the downstream MAPK pathway (Figure 4A), CRBN(BRAF)-24 strongly inhibited proliferation in three of the four BRAF<sup>V600E</sup> cell lines (A375, SK-MEL-28, and COLO205) at 10 nM and higher (Figure 5A). In these cell lines, CRBN(BRAF)-24 demonstrated better antiproliferative activity than CRBN(BRAF)-29 and PLX8394. In RKO cells, CRBN(BRAF)-24 showed a weak activity to inhibit proliferation. In contrast, none of the drugs showed antiproliferative activity against BRAF<sup>WT</sup> cell lines (Figure 5B). These results indicate that CRBN(BRAF)-24 selectively inhibits proliferation of cancer cells harboring BRAF<sup>V600E</sup> mutation, as does PLX8394.

### 3.5 | CRBN(BRAF)-24 exerts a potent and sustained inhibitory effect by degrading BRAF<sup>V600E</sup>

Because CRBN(BRAF)-24 uses a BRAF inhibitor as a warhead, it inhibits and degrades BRAF<sup>V600E</sup> protein. Hence, we investigated the advantage of CRBN(BRAF)-24 as a degrader/inhibitor over a mere inhibitor. First, to determine whether CRBN(BRAF)-24 potentially inhibits downstream signaling by degrading BRAF<sup>V600E</sup>, we compared ERK phosphorylation in BRAF<sup>V600E</sup>-positive cells treated with CRBN(BRAF)-24 and CRBN(BRAF)-29 in the presence or absence of a proteasome inhibitor, MG132. The active degrader CRBN(BRAF)-24 inhibited ERK phosphorylation more potently than the inactive degrader CRBN(BRAF)-29, but the better inhibitory effect of CRBN(BRAF)-24 was diminished by MG132 (Figure 6A). These results strongly suggest that CRBN(BRAF)-24 potently suppresses downstream ERK signaling by both inhibiting and degrading BRAF<sup>V600E</sup>.

As for the advantages of PROTACs, catalytic degradation of target proteins has been reported to result in sustained drug action.<sup>27,38,39</sup> To examine the sustained suppression of ERK phosphorylation, cells were treated with CRBN(BRAF)-24, PLX8394, and CRBN(BRAF)-29 for 24 hours, and then further incubated in drug-free medium (Figure 6B,C). After 24 hours of drug treatment (wash-out 0 hour), BRAF protein was reduced only in the cells treated with CRBN(BRAF)-24. At this timepoint, CRBN(BRAF)-24 and PLX8394 at 100 nM almost completely inhibited the level of phosphorylated ERK in SK-MEL-28 cells, while CRBN(BRAF)-29 required a concentration that was 10 times higher to achieve equivalent inhibition (Figure 6B). The suppressed ERK phosphorylation was maintained for 24 hours in CRBN(BRAF)-24-treated cells even after drug removal but was gradually restored in cells treated with PLX8394 and CRBN(BRAF)-29, especially at low concentrations. Surprisingly, the level of BRAF<sup>V600E</sup> protein was further reduced in the CRBN(BRAF)-24-treated cells during 24 hours incubation in drug-free medium. In line with this, treatment of SK-MEL-28 cells with CRBN(BRAF)-24



**FIGURE 4** CRBN(BRAF)-24 induced neither BRAF degradation nor paradoxical activation in BRAF<sup>WT</sup> cells. A, B, Effect of CRBN(BRAF)-24 on BRAF degradation and MAPK signaling in homozygous (A375 and SK-MEL-28) and heterozygous (COLO205 and RKO) BRAF<sup>V600E</sup> cells (A) or BRAF<sup>WT</sup> cells (B). Cells were treated with the indicated concentrations of CRBN(BRAF)-24 for 24 h. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies

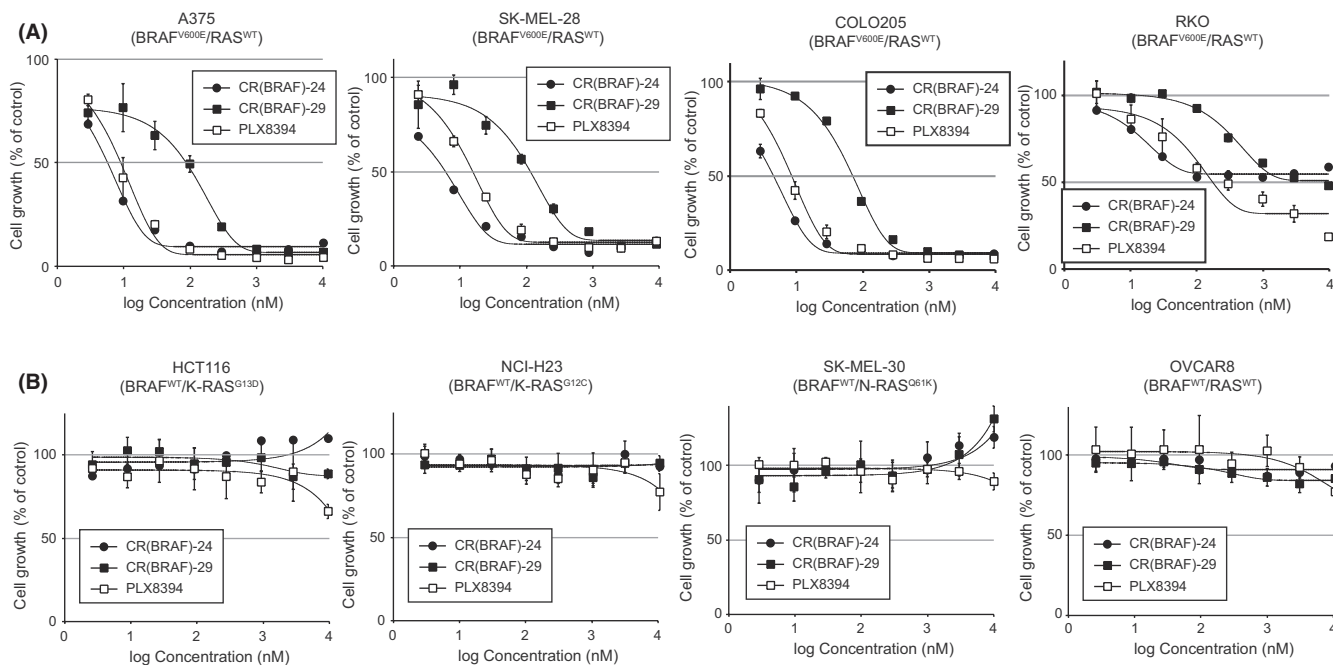
for 24 hours effectively inhibited cell proliferation in drug-free medium for the subsequent 72 hours (Figure 6C). In a parallel experiment, PLX8394 and CRBN(BRAF)-29 had little effect. These results suggest that CRBN(BRAF)-24 shows potent and sustained suppression of downstream ERK signaling by degrading BRAF<sup>V600E</sup>, thereby inhibiting the proliferation of BRAF<sup>V600E</sup>-driven cancer cells more potently than kinase inhibitor.

## 4 | DISCUSSION

The three currently FDA-approved RAF inhibitors (vemurafenib, dabrafenib, and encorafenib) provide significant benefits to patients with metastatic melanoma by potently inhibiting the MAPK pathway in tumors with BRAF<sup>V600</sup> mutations.<sup>19,40,41</sup> However, the emergence of acquired resistance seriously limits their clinical benefits.<sup>42,43</sup>

Combination with MEK inhibitors neutralizes the paradoxical activation in BRAF<sup>WT</sup> cells caused by these RAF inhibitors and supports an improved therapeutic profile, but resistance and tumor recurrence remain inevitable.<sup>13</sup> Therefore, there is the need for another approach to suppress oncogenic BRAF<sup>V600</sup> mutants more potently and selectively. In this study, we successfully developed PLX8394-based PROTACs that potently induce the degradation of BRAF<sup>V600E</sup> protein in a CRL4<sup>CRBN</sup> E3 ligase-dependent manner. CRBN(BRAF)-24 degrades BRAF<sup>V600E</sup> at nanomolar concentrations, and the effect was sustained for more than 72 hours. Biochemical analysis revealed that BRAF<sup>V600E</sup> degradation by CRBN(BRAF)-24 induces a more potent and sustained inhibition of the downstream MAPK signaling than PLX8394, especially after drug removal. Thus, CRBN(BRAF)-24 is a potent BRAF degrader that outcores a BRAF inhibitor, PLX8394.

PLX8394 is a potent inhibitor of BRAF<sup>V600</sup> mutant kinase (class 1) and can also disrupt both class 2 BRAF mutant homodimers and



**FIGURE 5** CRBN(BRAF)-24 suppressed cell proliferation of BRAF<sup>V600E</sup> cells but not BRAF<sup>WT</sup> cells. A, B, Effect of CRBN(BRAF)-24 on cell proliferation of homozygous (A375 and SK-MEL-28) and heterozygous (COLO205 and RKO) BRAF<sup>V600E</sup> cells (A) or BRAF<sup>WT</sup> cells (B). Cells were treated with the indicated concentrations of CRBN(BRAF)-24, CRBN(BRAF)-29, or PLX8394 for 72 h. Cell proliferation was evaluated by cell viability assay

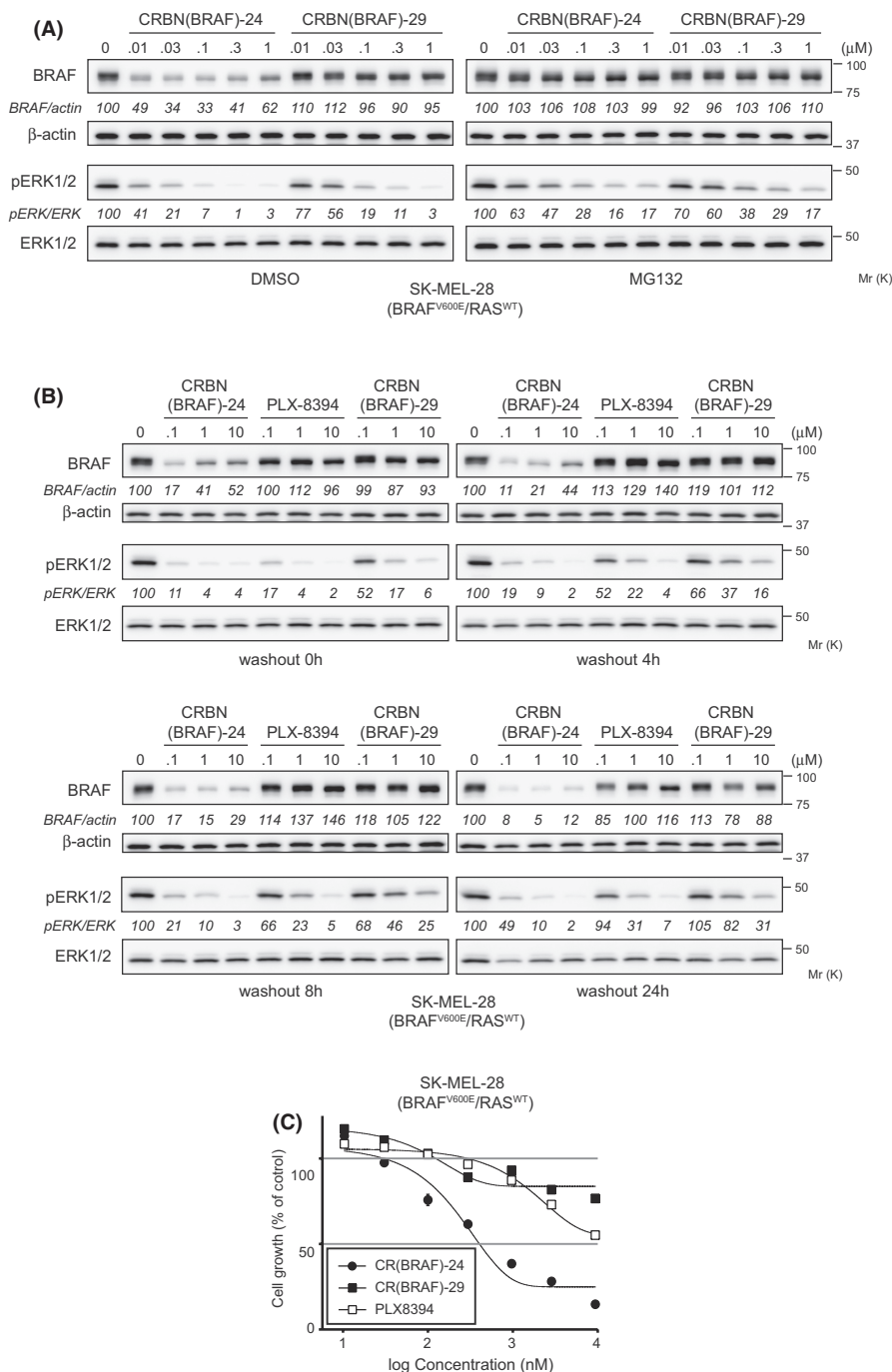
class 3 BRAF mutant heterodimers.<sup>36</sup> Therefore, we further investigated the effects of CRBN(BRAF)-24 on the levels of BRAF mutant proteins, downstream kinase signaling and cell proliferation in cells endogenously expressing class 2 and class 3 BRAF mutants (Figures S3 and S4). CRBN(BRAF)-24 showed weak or minimal activity to reduce the class 2 and 3 BRAF mutants and did not inhibit kinase signaling in these cells except in NCI-H508 cells (Figure S3). Consistent with this, CRBN(BRAF)-24 did not effectively inhibit the proliferation of these cell lines (Figure S4). In NCI-H508 cells (heterozygous BRAF<sup>G596R</sup>, class 3), CRBN(BRAF)-24 showed significant BRAF-degradation activity (Figure S3) but did not inhibit cell proliferation (Figure S4), suggesting that the proliferation of this cell line may not be solely dependent on the RAF-ERK pathway.

The currently approved RAF inhibitors selectively inhibit monomeric BRAF<sup>V600</sup> mutant proteins.<sup>13</sup> However, these inhibitors (collectively known as group 1 RAF inhibitors) are known to enhance the formation of BRAF homodimers or heterodimers between BRAF and other RAF proteins,<sup>44</sup> and binding of these inhibitors to one protomer of the RAF dimers results in a cooperative effect that activates the other protomer.<sup>13</sup> This mechanism leads to reactivation of the MAPK pathway and promotes acquired resistance to BRAF<sup>V600</sup> inhibition in cancer cells.<sup>36,45,46</sup> Furthermore, the paradoxical activation of this pathway in normal cells underlies the development of proliferative skin lesions such as keratoacanthomas<sup>20,21</sup> and may therefore trigger the progression of secondary RAS-driven skin tumors.<sup>22,47,48,49,50</sup> Group 2 RAF inhibitors (e.g., BGB659, TAK632, LY3009120), which have recently been reported as effective inhibitors of RAF monomers and dimers, induce only weakly paradoxical activation, but

their therapeutic window may be narrow because they inhibit ERK signaling and cell proliferation in normal cells as well as in cancer cells at concentrations effective for therapy.<sup>36</sup> PLX8394 is one of the next-generation BRAF inhibitors, which are often referred to as “paradoxical breakers,” disrupting BRAF dimerization and tending to prevent both inhibition and paradoxical activation of the MAPK pathway in BRAF<sup>WT</sup> cells.<sup>13,23,36</sup> In line with this, CRBN(BRAF)-24, which uses PLX8394 as a ligand, also induced neither inhibition nor paradoxical activation of ERK signaling in BRAF<sup>WT</sup> cells, even in the presence of oncogenic RAS mutations (Figure 4B) or upstream activation by EGF (Figure S5). Recently, other groups have reported CRBN-based PROTACs (P4B, compound 12, and 23) or a VHL-based PROTAC (SJF-0628), which incorporated vemurafenib or BI882370, that induce degradation of BRAF mutant proteins (mainly BRAF<sup>V600E</sup>) but not BRAF<sup>WT</sup>.<sup>51-53</sup> However, these PROTACs have been shown to inhibit or paradoxically activate the MAPK pathway in BRAF<sup>WT</sup> cells, especially in cells with oncogenic RAS mutations or elevated upstream receptor signaling.<sup>51,53</sup> Therefore, CRBN(BRAF)-24 is the first selective BRAF<sup>V600E</sup> degrader that showed little effects on BRAF<sup>WT</sup> cells, a feature not observed in previous BRAF degraders.

The development of clinical resistance to RAF inhibitors in BRAF<sup>V600E</sup>-driven cancers is associated with the upregulated function of RAS, which acts by driving the drug-responsive monomeric form of BRAF<sup>V600E</sup> into the drug-unresponsive dimeric form with CRAF.<sup>54,55</sup> Posternak et al. reported that the transduction of oncogenic RAS mutant isoforms into A375 cells causes marked resistance to BRAF<sup>V600E</sup> degradation by a BI882370-based PROTAC, P4B.<sup>51</sup> Similarly, we observed that A375 cells became resistant





**FIGURE 6** Potent and sustainable inhibitory activity of CRBN(BRAF)-24 via BRAF degradation. A, CRBN(BRAF)-24 potentially inhibited downstream signaling by degrading BRAF<sup>V600E</sup>. SK-MEL-28 cells were treated with the indicated concentrations of CRBN(BRAF)-24 or CRBN(BRAF)-29 in the presence or absence of 10  $\mu$ M MG132 for 8 h. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. B and C, Washout analysis of cells treated with CRBN(BRAF)s or PLX8394. SK-MEL-28 cells were treated with the indicated concentrations of CRBN(BRAF)-24, CRBN(BRAF)-29, or PLX8394 for 24 h followed by three washes with medium to remove compounds. B, Cells were further incubated in fresh medium for the indicated time periods. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. C, Cells were further incubated in fresh medium for 72 h. Cell proliferation was evaluated by cell viability assay

to CRBN(BRAF)-24 by the introduction of active RAS mutations (Figure S6B). However, BRAF<sup>V600E</sup> degradation by CRBN(BRAF)-24 in these cells was not affected (Figure S6A), suggesting that the BRAF<sup>V600E</sup>-degrading function of CRBN(BRAF)-24 is not suppressed by the activation of upstream RAS.

In summary, we have developed a new PROTAC against BRAF<sup>V600E</sup> protein by incorporating PLX8394 as a target ligand. CRBN(BRAF)-24 induced potent and selective degradation of BRAF<sup>V600E</sup> and exhibited antiproliferative activity against BRAF<sup>V600E</sup>-driven cancers without inducing paradoxical activation in BRAF<sup>WT</sup> cells. Although further optimization is required for its clinical development, CRBN(BRAF)-24 could be a novel lead as a BRAF<sup>V600E</sup>-targeted drug.

## ACKNOWLEDGMENTS

We thank H. Nikki March, PhD, from Edanz (<https://jp.edanz.com/ac>), for editing a draft of this manuscript. We would also like to acknowledge Daiichi Sankyo RD NOVARE Co., Ltd. for the high-resolution mass spectrometry and NMR analysis and the preparation of the compounds.

## DISCLOSURE

M. Suzuki, T. Uchida, M. Yoshida, and H. Ohki are employees of Daiichi Sankyo Co., Ltd. M. Naito is a member of the social cooperation program supported by Eisai Co., Ltd. and serves as a scientific advisor to UBiENCE Inc. M. Naito is an associate editor of *Cancer*

Science. The other authors declare no conflict of interest. All authors had full access to all the data in the study and had final responsibility for the decision to submit this manuscript for publication.

## ORCID

Nobumichi Ohoka  <https://orcid.org/0000-0002-0533-0610>

Yoshinori Tsukumo  <https://orcid.org/0000-0002-4470-2392>

Mikihiko Naito  <https://orcid.org/0000-0003-0451-1337>

## REFERENCES

- Lavoie H, Therrien M. Regulation of RAF protein kinases in ERK signalling. *Nat Rev Mol Cell Biol.* 2015;16:281-298.
- Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in human disease. *Cell.* 2017;170:17-33.
- Terrell EM, Morrison DK. Ras-mediated activation of the raf family kinases. *Cold Spring Harb Perspect Med.* 2019;9:a033746.
- Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene.* 2007;26:3291-3310.
- Barras D. BRAF Mutation in Colorectal Cancer: An Update. *Biomark Cancer.* 2015;7:9-12.
- Flemming A. Cancer: Targeting mutant BRAF in metastatic melanoma. *Nat Rev Drug Discov.* 2010;9:841.
- Nguyen-Ngoc T, Bouchaab H, Adjei AA, Peters S. BRAF alterations as therapeutic targets in non-small-cell lung cancer. *J Thorac Oncol.* 2015;10:1396-1403.
- Tiacci E, Trifonov V, Schiavoni G, et al. BRAF mutations in hairy-cell leukemia. *N Engl J Med.* 2011;364:2305-2315.
- Dankner M, Rose AAN, Rajkumar S, Siegel PM, Watson IR. Classifying BRAF alterations in cancer: new rational therapeutic strategies for actionable mutations. *Oncogene.* 2018;37:3183-3199.
- Forbes SA, Bindal N, Bamford S, et al. COSMIC: mining complete cancer genomes in the catalogue of somatic mutations in cancer. *Nucleic Acids Res.* 2011;39:D945-D950.
- Holderfield M, Deuker MM, McCormick F, McMahon M. Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond. *Nat Rev Cancer.* 2014;14:455-467.
- Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature.* 2002;417:949-954.
- Yaeger R, Corcoran RB. Targeting alterations in the RAF-MEK pathway. *Cancer Discov.* 2019;9:329-341.
- Lito P, Pratilas CA, Joseph EW, et al. Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. *Cancer Cell.* 2012;22:668-682.
- Pratilas CA, Taylor BS, Ye Q, et al. (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. *Proc Natl Acad Sci U S A.* 2009;106:4519-4524.
- Yao Z, Torres NM, Tao A, et al. BRAF mutants evade ERK-dependent feedback by different mechanisms that determine their sensitivity to pharmacologic inhibition. *Cancer Cell.* 2015;28:370-383.
- Yao Z, Yaeger R, Rodrik-Outmezguine VS, et al. Tumours with class 3 BRAF mutants are sensitive to the inhibition of activated RAS. *Nature.* 2017;548:234-238.
- Shelledy L, Roman D. Vemurafenib: First-in-class BRAF-mutated inhibitor for the treatment of unresectable or metastatic melanoma. *J Adv Pract Oncol.* 2015;6:361-365.
- Sosman JA, Kim KB, Schuchter L, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med.* 2012;366:707-714.
- Joseph EW, Pratilas CA, Poulikakos PI, et al. The RAF inhibitor PLX4032 inhibits ERK signaling and tumor cell proliferation in a V600E BRAF-selective manner. *Proc Natl Acad Sci U S A.* 2010;107:14903-14908.
- Lacouture ME, O'Reilly K, Rosen N, Solit DB. Induction of cutaneous squamous cell carcinomas by RAF inhibitors: cause for concern? *J Clin Oncol.* 2012;30:329-330.
- Su F, Viros A, Milagre C, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N Engl J Med.* 2012;366:207-215.
- Zhang C, Spevak W, Zhang Y, et al. RAF inhibitors that evade paradoxical MAPK pathway activation. *Nature.* 2015;526:583-586.
- Petterson M, Crews CM. PROTeolysis TARgeting Chimeras (PROTACs) - Past, present and future. *Drug Discov Today Technol.* 2019;31:15-27.
- Naito M, Ohoka N, Shibata N. SNIPERs-Hijacking IAP activity to induce protein degradation. *Drug Discov Today Technol.* 2019;31:35-42.
- Winter GE, Buckley DL, Paulk J, et al. DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation. *Science.* 2015;348:1376-1381.
- Bondeson DP, Mares A, Smith IE, et al. Catalytic in vivo protein knockdown by small-molecule PROTACs. *Nat Chem Biol.* 2015;11:611-617.
- Ohoka N, Okuhira K, Ito M, et al. In Vivo knockdown of pathogenic proteins via specific and nongenetic inhibitor of apoptosis protein (IAP)-dependent protein erasers (SNIPERs). *J Biol Chem.* 2017;292:4556-4570.
- Ohoka N, Morita Y, Nagai K, et al. Derivatization of inhibitor of apoptosis protein (IAP) ligands yields improved inducers of estrogen receptor alpha degradation. *J Biol Chem.* 2018;293:6776-6790.
- Hines J, Lartigue S, Dong H, Qian Y, Crews CM. MDM2-recruiting PROTAC offers superior, synergistic antiproliferative activity via simultaneous degradation of brd4 and stabilization of p53. *Cancer Res.* 2019;79:251-262.
- Ohoka N, Tsuji G, Shoda T, et al. Development of small molecule chimeras that recruit AhR E3 Ligase to Target Proteins. *ACS Chem Biol.* 2019;14:2822-2832.
- Ishida T, Ciulli A. E3 Ligase Ligands for PROTACs: How they were found and how to discover new ones. *SLAS Discov.* 2021;26:484-502.
- An S, Fu L. Small-molecule PROTACs: An emerging and promising approach for the development of targeted therapy drugs. *EBioMedicine.* 2018;36:553-562.
- Yamori T, Matsunaga A, Sato S, et al. Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an in vitro and in vivo human cancer cell line panel. *Cancer Res.* 1999;59:4042-4049.
- Lu J, Qian Y, Altieri M, et al. Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. *Chem Biol.* 2015;22:755-763.
- Yao Z, Gao Y, Su W, et al. RAF inhibitor PLX8394 selectively disrupts BRAF dimers and RAS-independent BRAF-mutant-driven signaling. *Nat Med.* 2019;25:284-291.
- Pickles OJ, Drozd A, Tee L, Beggs AD, Middleton GW. Paradox breaker BRAF inhibitors have comparable potency and MAPK pathway reactivation to encorafenib in BRAF mutant colorectal cancer. *Oncotarget.* 2020;11:3188-3197.
- Mares A, Miah AH, Smith IED, et al. Extended pharmacodynamic responses observed upon PROTAC-mediated degradation of RIPK2. *Commun Biol.* 2020;3:140.
- Watt GF, Scott-Stevens P, Gaohua L. Targeted protein degradation in vivo with proteolysis targeting chimeras: Current status and future considerations. *Drug Discov Today Technol.* 2019;31:69-80.
- Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med.* 2011;364:2507-2516.
- Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med.* 2010;363:809-819.

42. Poulidakos PI, Persaud Y, Janakiraman M, et al. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature*. 2011;480:387-390.
43. Fedorenko IV, Paraiso KH, Smalley KS. Acquired and intrinsic BRAF inhibitor resistance in BRAF V600E mutant melanoma. *Biochem Pharmacol*. 2011;82:201-209.
44. Durrant DE, Morrison DK. Targeting the Raf kinases in human cancer: the Raf dimer dilemma. *Br J Cancer*. 2018;118:3-8.
45. Hatzivassiliou G, Song K, Yen I, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature*. 2010;464:431-435.
46. Poulidakos PI, Zhang C, Bollag G, Shokat KM, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature*. 2010;464:427-430.
47. Zimmer L, Hillen U, Livingstone E, et al. Atypical melanocytic proliferations and new primary melanomas in patients with advanced melanoma undergoing selective BRAF inhibition. *J Clin Oncol*. 2012;30:2375-2383.
48. Callahan MK, Rampal R, Harding JJ, et al. Progression of RAS-mutant leukemia during RAF inhibitor treatment. *N Engl J Med*. 2012;367:2316-2321.
49. Andrews MC, Behren A, Chionh F, et al. BRAF inhibitor-driven tumor proliferation in a KRAS-mutated colon carcinoma is not overcome by MEK1/2 inhibition. *J Clin Oncol*. 2013;31:e448-e451.
50. Oberholzer PA, Kee D, Dziunycz P, et al. RAS mutations are associated with the development of cutaneous squamous cell tumors in patients treated with RAF inhibitors. *J Clin Oncol*. 2012;30:316-321.
51. Posternak G, Tang X, Maisonneuve P, et al. Functional characterization of a PROTAC directed against BRAF mutant V600E. *Nat Chem Biol*. 2020;16:1170-1178.
52. Han XR, Chen L, Wei Y, et al. Discovery of selective small molecule degraders of BRAF-V600E. *J Med Chem*. 2020;63:4069-4080.
53. Alabi S, Jaime-Figueroa S, Yao Z, et al. Mutant-selective degradation by BRAF-targeting PROTACs. *Nat Commun*. 2021;12:920.
54. Corcoran RB, Ebi H, Turke AB, et al. EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov*. 2012;2:227-235.
55. Prahallad A, Sun C, Huang S, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*. 2012;483:100-103.

#### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**How to cite this article:** Ohoka N, Suzuki M, Uchida T, et al. Development of a potent small-molecule degrader against oncogenic BRAF<sup>V600E</sup> protein that evades paradoxical MAPK activation. *Cancer Sci*. 2022;113:2828-2838. doi: [10.1111/cas.15401](https://doi.org/10.1111/cas.15401)