ORIGINAL Article

Fluvastatin exerts an anti-tumor effect in vemurafenib-resistant melanoma cells

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Abstract

Although vemurafenib has been demonstrated to improve the overall survival of patients with metastatic melanoma harboring the BRAF V600E mutation, its efficacy is often hampered by drug resistance acquired within a relatively short period through several distinct mechanisms. In the present study, we investigated the effect of fluvastatin as a possible strategy to overcome such acquired resistance using a cultured cell line model.

We established vemurafenib-resistant (VR) cells from three BRAF (V600E)-mutated melanoma lines (C32, HMY-1 and SK-MEL-28) and evaluated the mechanism of acquired resistance of VR cells by water-soluble tetrazolium salts assay, western blot, real-time quantitative PCR and immunofluorescent microscopy. The efficacy of the combination on growth inhibitory effect of vemurafenib and fluvastatin on respective parental and VR cells were assessed by calculating combination index and western blot.

IC_{50} values of three VR cells were approximately 5- to 100-fold higher than those for the respective parental cells. The VR cells derived from HMY-1 and SK-MEL-28 showed constitutive activation of AKT kinase, and the specific AKT inhibitor MK-2208 or the PI3K inhibitor wortmannin increased the cellular sensitivity to vemurafenib. Intriguingly, application of a statin-related drug, fluvastatin, also resulted in a synergistic increase of sensitivity to vemurafenib in the VR cells (combination index 0.73-0.86) probably by alleviating constitutive AKT activation, while the same treatment did not notably alter the vemurafenib sensitivity of the parental cells.

Our results suggest the possible usefulness of statin-related drugs for overcoming vemurafenib resistance acquired through constitutive activation of the PI3K-AKT axis.

Introduction

Cutaneous malignant melanoma is a leading cause of death among the various skin cancers, exhibiting aggressive tumor behavior [1]. Whereas conventional chemotherapeutics and other strategies including classical immunotherapies have had a limited impact on the course of this disease [2,3], the emergence of two new modalities - immune checkpoint blockade and signal transduction inhibition - has opened a new era in the treatment.

Immune checkpoint blockade with monoclonal antibodies has achieved a durable long-term response after treatment despite a relatively low response rate in the short term; such treatment includes the antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4), ipilimumab [4], or those against programmed cell death ligand 1/receptor 1 (PD-L1/PD-1), nivolumab [5] and pembrolizumab [6]. National Comprehensive Cancer Network (NCCN) guidelines now recommend these antibodies for first-line treatment of metastatic melanomas [7]. In contrast, signal transduction inhibitors (vemurafenib/dabrafenib for BRAF and trametinib for MEK) have achieved rapid and high short-term response rates, whereas the durability of the responses is limited [8,9].

To achieve greater clinical benefit for patients with advanced melanoma, overcoming resistance to therapy in both the early and late phases is inevitable for both types of therapeutic approach. While 35-60% of melanoma patients exhibit minimal or no response to PD-1 and CTLA-4 blockade at the outset (primary resistance) [5,10], half of all patients with metastatic melanoma who initially respond to immune-based treatments develop resistance within 3 years (acquired resistance). The mechanisms underlying both primary and acquired resistance to immune checkpoint blockade have been unclear. No precise biomarker that can predict a patient's response has yet been developed, despite extensive investigation [11].

The mechanisms of primary/acquired resistance to signal transduction inhibitors have been relatively well clarified [12-16]. Although several molecular causes of vemurafenib resistance (VR)

have been proposed, either MAPK reactivation due to genetic alteration or activation of the PI3K/AKT/mTOR pathway appear to be most common. Mutations in NRAS [17] and MEK1 [18] that result in reactivation of the MAPK/ERK pathway are frequently detected in tumors with acquired VR. For these cases, combination therapy with dabrafenib and trametinib has been attempted in order to delay VR. For VR due to activation of the PI3K/AKT/mTOR pathway, the possible efficacy of AKT/mTOR inhibitors including approved drugs such as metformin, rapamycin, and resveratrol has been suggested [19-21]. Beside these cases, Lin et al. have demonstrated that activation of YAP/TAZ protein, targets of the Hippo pathway, promotes resistance to BRAF and MEK inhibitors. This observation suggests that combined repression of YAP and BRAF/MEK could be a promising therapy for some melanoma patients [22,23].

We have recently demonstrated that treatment with fluvastatin (a small-molecule inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A; HMG-CoA, i.e. a statin) compromised nuclear translocation of YAP/TAZ proteins in malignant mesothelioma cells thus downregulating transcriptional targets of YAP/TAZ including RHAMM [24]. Wang *et al.* have reported that simvastatin, another statin-related drug, also strongly perturbs the PI3K/AKT/mTOR pathway by enhancing the expression of PTEN [25]. These results may suggest that statins might function as an efficient modulator for melanoma with acquired VR either through YAP/TAZ or through PI3K/AKT/mTOR pathways.

In the present study, we established and characterized three VR melanoma cells initially focusing on the relationship between YAP/TAZ activation and VR in these cell lines. We found that fluvastatin increased the vemurafenib sensitivities of the VR cells not through YAP/TAZ but PI3K/AKT/mTOR.

Materials and Methods

Compounds

Vemurafenib (BRAF inhibitor, S1267), wortmannin (low-specificity, covalent inhibitor of phosphoinositide 3-kinases, S2758) and MK-2206 2HCl (high-specificity inhibitor of Akt1/2/3, S1078) were purchased from Selleckchem (Houston, TX, USA), and fluvastatin (PHR1620) was purchased from Sigma-Aldrich (St Louis, MO, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) at 10 mM.

Cell culture and media

All cells were cultured at 37 °C in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS in a humidified atmosphere with 5% CO_2. The C32 and SK-MEL-28 human melanoma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The HMY-1 human melanoma cell line was purchased from the Japanese Collection of Research Bioresources (JCRB, Tsukuba, Japan). To obtain vemurafenib-resistant (VR) cells, all cells were seeded and exposed to increasing concentrations of vemurafenib (1-4 μM) for 4 weeks. These cell lines were named C32/VR, HMY-1/VR, and SK-MEL-28/VR, respectively. This series of VR cells was maintained in medium containing 4 μM vemurafenib.

Cell viability assay

Cell viability was determined by water-soluble tetrazolium salts (WST) assay using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded into 96-well plates (3000 cells/ well) and cultured for 24 h before treatment with each reagent. After 72 h of treatment, the medium in each well was replaced with 100 μl of drug-free fresh medium and 10 μl of Cell

Counting Kit-8 solution, incubated for an additional 2-3 h, and the absorbance at 450 nm was measured using a Multiskan Spectrum spectrophotometer (Thermo Fisher Scientific).

Protein preparation and immunoblotting

Cells at 80-90% confluence were washed twice with ice-cold PBS, treated with PBS containing 10% trichloroacetic acid (TCA) for 30 min on ice, and scraped off into a tube. The cell pellet was washed twice with deionized water, and then lysed in 9 M urea, 2% Triton X-100 and 1% dithiothreitol (DTT). Protein concentration was measured with a BCA protein assay kit (Merck Millipore, Billerica, MA, USA) before addition of DTT. Equal amounts of protein (10 μg) per lane were electrophoresed on 10% SDS-PAGE gel for 60 min at 200 V and then transferred to polyvinylidene fluoride transfer membranes (Pall Corporation, Port Washington, NY, USA). The membranes were blocked with 5% non-fat dried milk (Cell Signaling Technology, Danvers, MA, USA) or 5% BSA (Sigma-Aldrich) in 1 × TBS-T for 1 h at room temperature. Then the membranes were incubated with an appropriate primary antibody overnight at 4°C and with an HRP-conjugated secondary antibody (GE Healthcare Life Sciences, Buckinghamshire, UK) for 1 h at room temperature. Signals were identified with ECL prime detection reagents (GE Healthcare Life Sciences) and ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA, USA). For immunodetection, primary antibodies were diluted as follows: rabbit anti-BRAF (20899-1-AP, Proteintech, HQ, USA) 1:1000, rabbit anti-MEK 1/2 (#8727, Cell Signaling Technology) 1:1000, rabbit anti-phospho-MEK 1/2 (#9154, Cell Signaling Technology) 1:1000, rabbit anti-ERK 1/2 (#4695, Cell Signaling Technology) 1:1000, rabbit anti-phospho-ERK 1/2 (#4370, Cell Signaling Technology) 1:2000, rabbit anti-AKT (#4691, Cell Signaling Technology) 1:1000, rabbit anti-phospho-AKT (Ser473) (#4060, Cell Signaling Technology) 1:1000, rabbit anti-phospho-AKT (Thr308) (#13038, Cell Signaling Technology) 1:1000, rabbit anti-PTEN (#9188, Cell Signaling Technology) 1:1000, rabbit anti-p-PTEN (#9549, Cell Signaling Technology) 1:1000, rabbit anti-COT

(sc-720, Santa Cruz) 1:500, rabbit anti-GAPDH (#5174, Cell Signaling Technology) 1:1000. Electrophoresis and immunoblotting were repeated at least three times with independent sample preparations and confirmed to be reproducible.

Immunofluorescence microscopy

Two thousand cells were grown on a poly-L-lysine-coated 18 mm-square coverslip at 37°C overnight, and then fixed with 4% paraformaldehyde at 37°C for 30 min. The cells were blocked with PBS containing 5% goat serum and 0.3% Triton X-100 at room temperature for 60 min, treated with rabbit anti-YAP antibody (#14074, Cell Signaling Technology) and mouse anti-TAZ antibody (#560235, BD Pharmingen, Franklin Lakes, NJ, USA) at 1:200 dilution in PBS containing 1% BSA and 0.3% Triton X-100 for 2 h, and subsequently with Alexa Fluor 488-conjugated anti-rabbit IgG goat antibody and Alexa Fluor 546-conjugated anti-mouse IgG goat antibody (Thermo Fisher Scientific) at 1:1000 dilution for 2 h. After washing, the coverslips were mounted with ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific) and observed using a confocal microscope (C1 and EZ-C1, Nikon, Tokyo, Japan).

siRNA transfection

Silencer Select predesigned siRNAs against YAP1 (ID s20367), WWTR1 (ID s24787), and control non-specific human siRNA, Lipofectamine RNAiMAX transfection reagent, and Opti-MEM were all obtained from Thermo Fisher Scientific. Cells were transfected with 10 nM siRNA using 7.5 μl of RNAiMAX diluted with Opti-MEM in a 6-well plate format in accordance with the manufacturer's instructions.

Real-time quantitative RT-PCR

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. RNA concentration was measured by NanoDrop (Thermo Fisher Scientific) and an equal amount of extracted RNA was reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific). cDNAs of YAP1, WWTR1, CTGF, ANKRD1 and GAPDH were quantified by real-time PCR (7500 Real Time PCR System, Thermo Fisher Scientific) using the TaqMan Gene Expression MasterMix and TaqMan Gene Expression Assays for YAP1 (Hs00902712_g1), WWTR1 (Hs00210007_m1), CTGF (Hs00170014_m1), ANKRD1 (Hs00173317_m1) and GAPDH (Hs02758991_g1).

Evaluation of drug combination effects

The combination indices (Comb.Is) and the drug reduction indices (DRIs) were calculated according to the method established by Chou and Talalay [26]. The mean values were calculated from three independent experiments.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) unless stated otherwise. Logistic function was fitted to the cell survival data points with the least squares estimates of the parameters. IC_{50} values were calculated from the fitted function. The statistical software R (version 3.4.2) was used [27]. Confidence intervals (Conf.Is) for IC_{50} were calculated with the "confint()" command.

Results

Establishment of vemurafenib-resistant (VR) cells

To obtain vemurafenib-resistant (VR) cells, we treated three malignant melanoma cell lines (C32, HMY-1, and SK-MEL-28) harboring the BRAF V600E mutation with step-wise increases in the concentration of vemurafenib (from 1 to 4 μM) for 4 weeks. This successfully gave rise to VR cells from all three cell lines, whose IC_{50}s were increased between 4.3-fold and 96.7-fold relative to the parental cells (Fig. 1 and Table 1).

We examined the expression level and phosphorylation status of MAPK-related proteins, as well as AKT, in the presence or absence of vemurafenib using immunoblotting (Fig. 2). Because of the BRAF V600E mutation, phosphorylation of both MEK and ERK was constitutively high in all of the three parental lines, and vemurafenib markedly reduced it. In contrast, vemurafenib barely affected the level of phosphorylation in the VR cells, suggesting that some bypass may operate at the level of or downstream of BRAF. Intriguingly, VR cells derived from two of the melanoma lines (HMY-1/VR and SK-MEL-28/VR) showed a dramatic increase of AKT phosphorylation (Thr308 and Ser473) regardless of vemurafenib treatment, in comparison with the respective parental cells (Fig. 2). It is noteworthy that these VR cells also showed a higher increase of IC_{50} than C32/VR line, the latter not showing any increase of AKT phosphorylation relative to the parental line. Although we examined the expression levels of COT and BRAF kinases as well as PTEN phosphatase as possible causes for the acquired vemurafenib-resistance, neither showed any notable change (Fig. 2 and Supplementary Figures 1, 2).

YAP/TAZ activity and drug resistance in VR cells

Several recent studies have shown that overexpression of YAP/TAZ transcription factors might

contribute to acquired vemurafenib resistance, possibly via expression of their targets involved in cell growth or survival. To assess the activity of the YAP/TAZ transcriptional axis in VR cells, we examined the expression levels of YAP/TAZ and their targets using quantitative PCR. Although the mRNA for ANKRD1, an established target of YAP/TAZ, showed a dramatic increase in all of the VR cells (3.2-142.6-fold; Supplementary Fig. 3), mRNAs for YAP and TAZ were only modestly increased in the VR cells. A degree of nuclear accumulation of YAP/TAZ protein was observed in some of the VR cells (YAP in HMY-1/VR and TAZ in C32/VR), but not in others. The level of accumulation did not seem to be closely associated with target gene expression (Supplementary Figures 3, 4). To directly assess the involvement of YAP/TAZ in the acquired resistance, we examined the effect of YAP/TAZ depletion on cell survival in the presence of vemurafenib. Transfection with siRNAs specific for YAP and TAZ resulted in an 80% and 20-40% decrease of ANKRD1 and CTGF mRNAs, respectively (Supplementary Fig. 5A), but barely changed the degree of vemurafenib resistance of the VR cells (decrease of IC_{50} values shown in Supplementary Fig. 5B). These results imply that activation of the YAP/TAZ transcriptional axis might not be a major cause of the acquired resistance in the VR cells.

Synergistic effect of a combination of vemurafenib and fluvastatin in VR melanoma cells

While examining the possible involvement of YAP/TAZ in vemurafenib resistance, we noticed a synergistic cell-killing effect of vemurafenib and the statin-related drug fluvastatin. A slight or moderate synergistic effect was observed in all of the VR cells (combination indices 0.73 - 0.86) but not in the corresponding parental cells (1.57 - 2.65, Table 1 and 2). The synergy may not be due to cell growth perturbation since the combination of the drugs at the concentration of IC_{50} started to induce apoptosis/necrosis in a certain population of the VR cells as early as 8 hours (Supplementary Fig. 6). We initially speculated that this effect occurred via the inhibitory action of statin-related drugs on the YAP/TAZ transcriptional axis, as reported recently. However, our observations did not support the

involvement of YAP/TAZ in the vemurafenib resistance, as described above.

Effect of fluvastatin on MEK/ERK/AKT phosphorylation

It has been suggested that inhibition of prenylation of small G proteins such as RAS or RHO by statins could result in down-regulation of the PI3K/AKT/mTOR pathway. We investigated how fluvastatin affects the phosphorylation status of MAPK-related proteins and AKT in both the parental and VR melanoma cells and its possible connection with acquired resistance. In the VR cells, fluvastatin did not markedly change the phosphorylation status of MEK and ERK proteins, whereas it reduced the phosphorylation of AKT (Thr308 and Ser473) in a dose-dependent manner (Fig. 3). The reduction did not coincide with an increase in the phosphorylation of PTEN protein, dismissing the involvement of PTEN in this context (Supplementary Fig. 2). These observations, together with the increase of basal AKT phosphorylation in two of the three VR lines, suggest a crucial role of AKT in acquired resistance. Consistent with the synergy of cell survival, the AKT activity was reduced further by a combination of vemurafenib with fluvastatin than by fluvastatin alone (Fig. 4). To demonstrate the involvement of AKT in vemurafenib resistance more directly, we investigated the effect of the AKT inhibitor MK-2206 or the PI3K inhibitor wortmannin on vemurafenib resistance in VR cells. In both cases, the combination indices were distinctly smaller than 1 (Supplementary Table 2), suggesting that activation of the PI3K-AKT pathway is crucial for the acquired resistance to vemurafenib, and also that the target of fluvastatin in this situation may be PI3K-AKT.

Figure legends

Fig. 1 Viability of the parental melanoma cell lines and vemurafenib-acclimated cells after treatment with vemurafenib for 3 days. The vemurafenib-acclimated cells showed an increase of IC_{50} ranging from 4.3- to 96.7-fold that of the parental cell lines (Table 1). PT, parental; VR, vemurafenib-resistant.

Fig. 2 Effects of vemurafenib treatment on the phosphorylation status of MEK/ERK/AKT proteins in the parental and VR melanoma cells. Cells were treated with 1 μM vemurafenib for 8 h and cell lysates were analyzed by immunoblotting. In the VR cells, phosphorylation of MEK and ERK was refractory to vemurafenib. Constitutive activation of AKT was observed in HMY-1 and SK-MEL-28. PT, parental; VR, vemurafenib-resistant.

Fig. 3 Effect of fluvastatin on MEK/ERK/AKT phosphorylation. Cells were treated with 1, 5 or 10 μM fluvastatin for 24 h and cell lysates were analyzed by immunoblotting. Fluvastatin did not affect the MAPK pathway in the VR cells, whereas it reduced phosphorylation of AKT in a dose-dependent manner.

Fig. 4 Synergistic effect of a combination of vemurafenib and fluvastatin. Cells were treated with 1 μM vemurafenib and/or 1 μM fluvastatin for 24 h. AKT phosphorylation in the VR cells was decreased further by the drug combination than by fluvastatin alone.

Discussion

Primary or acquired resistance to vemurafenib often involves reactivation of the MAPK pathway itself as well as constitutive activation of the PI3K/AKT pathway. Shi *et al.* reported that the MAPK and PI3K/PTEN/AKT pathways were altered in 70% and 22% of 44 patients with progressive tumors, respectively, and that in 20% of the cases, both pathways were activated simultaneously [16]. Consistent with these observations, in the present study we identified vemurafenib-refractory reactivation of the MAPK pathway in VR cells derived from all of the three melanoma lines and an increased level of constitutive phosphorylation of AKT kinase in cells from two of them.

In tumor cells with the BRAF V600E-mutated allele, the MAPK pathway becomes vemurafenib-refractory either through alterations that activate the target kinase(s) positioned downstream of BRAF, or acquisition of the BRAF bypass pathway through ARAF and/or CRAF, often coupled with upstream alterations [28,29]. The present study revealed no association between reactivation of the MAPK pathway and specific molecular changes. In our preliminary experiments, however, treatment with a pan-RAF kinase inhibitor (A/B/C RAF kinase inhibitor, LY3009120) or trametinib (MEK inhibitor) did not have a strong suppressive effect on ERK phosphorylation in VR cells. In addition, COT overexpression, a well-known bypass that activates ERK independent of BRAF [13], was not evident in any of the VR cells. These observations suggest that genetic alteration at the level of MEK or downstream may be involved in the reactivation. This possibility awaits clarification in a future study.

Constitutive activation of the PI3K/AKT pathway has been shown to occur through several distinct mechanisms such as *NRAS* mutation, overexpression of PDGFR or depletion of PTEN [30]. While we observed no notable reduction of PTEN phosphorylation, other possible causes were not explored in the present study.

Impairment of the Hippo signaling pathway or hyper-activation of its downstream effector YAP/TAZ has attracted attention as another mechanism for vemurafenib resistance in a subset of

malignant melanoma [23]. Lin *et al.* have shown that YAP functions as a parallel survival input to promote resistance to RAF-MEK inhibitor therapy [22]. On the basis of these findings, we treated VR cells with fluvastatin expecting that its inhibitory effect on YAP-dependent transcription would potentiate cell-killing by vemurafenib. A synergistic effect was evident, but it was found to occur through inhibition of PI3K/AKT. Although a connection between statin-related drugs and the PI3K/AKT pathway has been suggested previously [25,31-33], it has not been evaluated in the context of treatment for vemurafenib resistance. Pre-clinical studies have demonstrated synergy between inhibitors for PI3K/AKT and those for BRAF or MEK kinases [34-38]. Statin-related drugs may have an advantage over other PI3K/AKT inhibitors as their pharmacological safety has been well established. Our findings appear to provide evidence for the possible usefulness of statin-related drugs for overcoming resistance to vemurafenib.

It should be noted that apparent non-essentiality of YAP/TAZ in the present study may not be general features of the acquired vemurafenib resistance. Nevertheless, statin-related drugs might also be effective to overcome YAP-dependent form of vemurafenib resistance via the action on the mevalonate pathway, as we initially surmised. A recent study has demonstrated that the Hippo-YAP axis also contributes to suppression of anti-tumor immunity in addition to acquisition of VR [39]. In this study, inactivation of *LATS1/2* in mouse melanoma cells resulted in an increase of tumor immunogenicity and enhancement of tumor vaccine efficacy. Fluvastatin is attractive from this viewpoint also, with an ability to suppress PI3K/AKT and YAP, both acting to overcome vemurafenib resistance and the latter enhancing the degree of sensitivity to immune checkpoint therapy. Further research will be required to investigate these possibilities.

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Figure 1

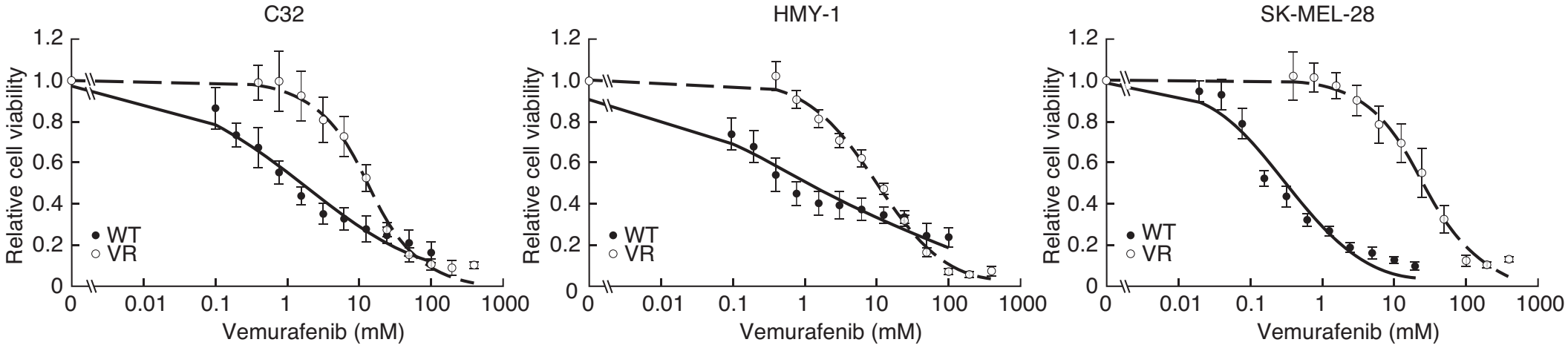

C32
HMY-1
SK-MEL-28
Relative cell viability
Vemurafenib (mM)
WT
VR
0
0.2
0.4
0.6
0.8
1.0
1.2
0
0.01
0.1
1
10
100
1000

Figure 2

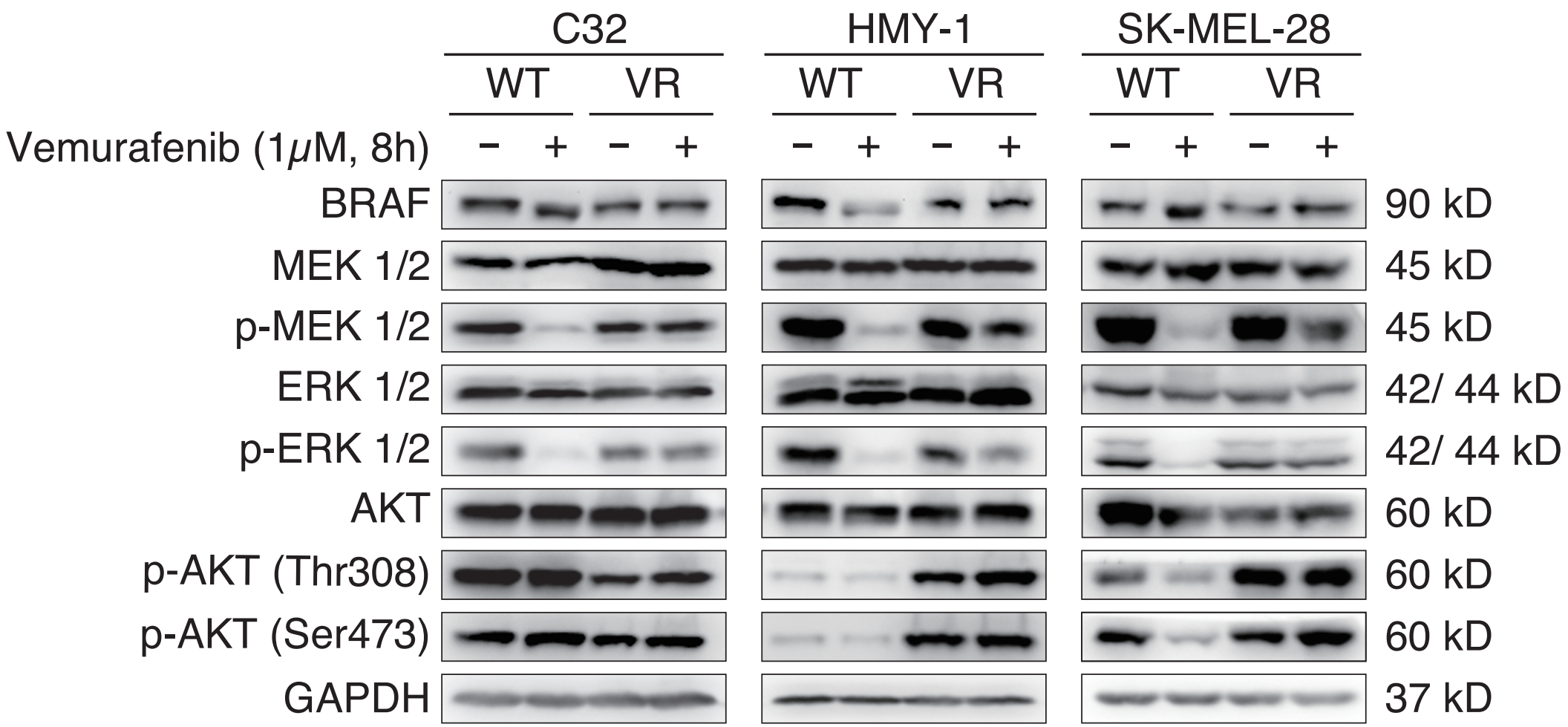

C32
HMY-1
SK-MEL-28
WT
VR
WT
VR
WT
VR
Vemurafenib (1μM, 8h)
− + − + − + − + − + − +
BRAF
MEK 1/2
p-MEK 1/2
ERK 1/2
p-ERK 1/2
AKT
p-AKT (Thr308)
p-AKT (Ser473)
GAPDH
90 kD
45 kD
45 kD
42/ 44 kD
42/ 44 kD
60 kD
60 kD
60 kD
37 kD

Figure 3

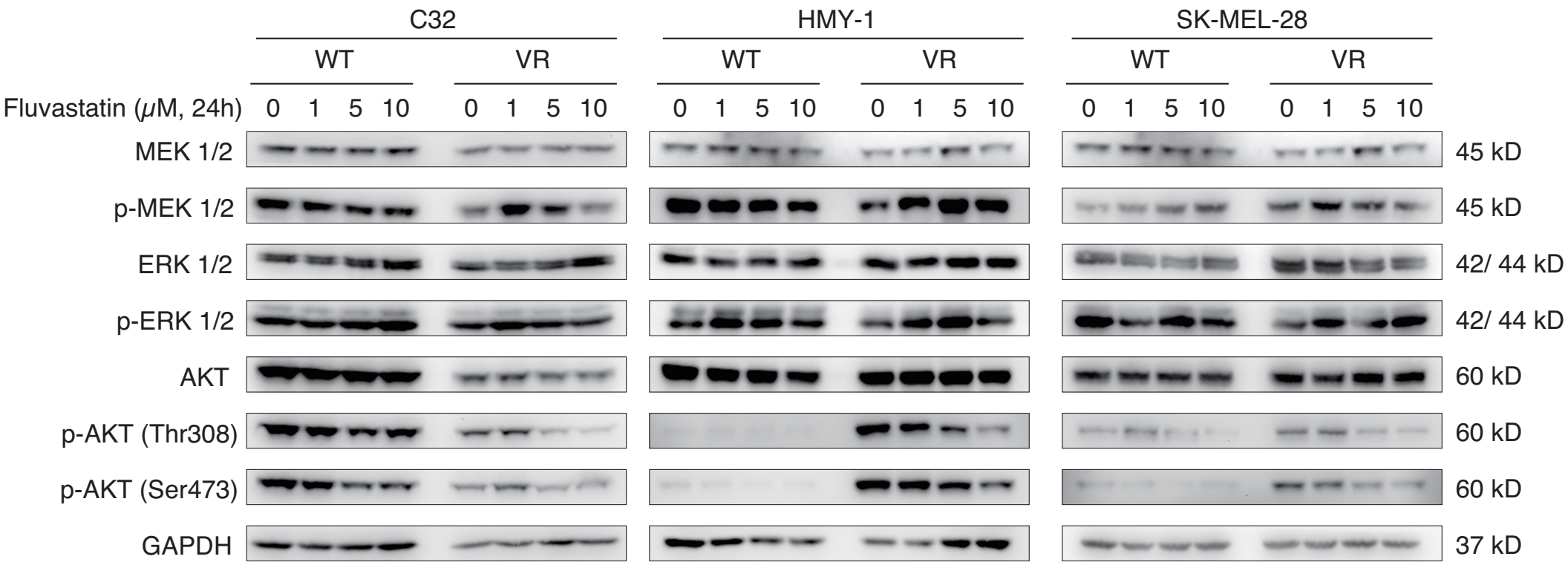

C32
HMY-1
SK-MEL-28
WT
VR
Fluvastatin (μM, 24h)
0 1 5 10
MEK 1/2
p-MEK 1/2
ERK 1/2
p-ERK 1/2
AKT
p-AKT (Thr308)
p-AKT (Ser473)
GAPDH
45 kD
45 kD
42/ 44 kD
42/ 44 kD
60 kD
60 kD
60 kD
37 kD

Figure 4

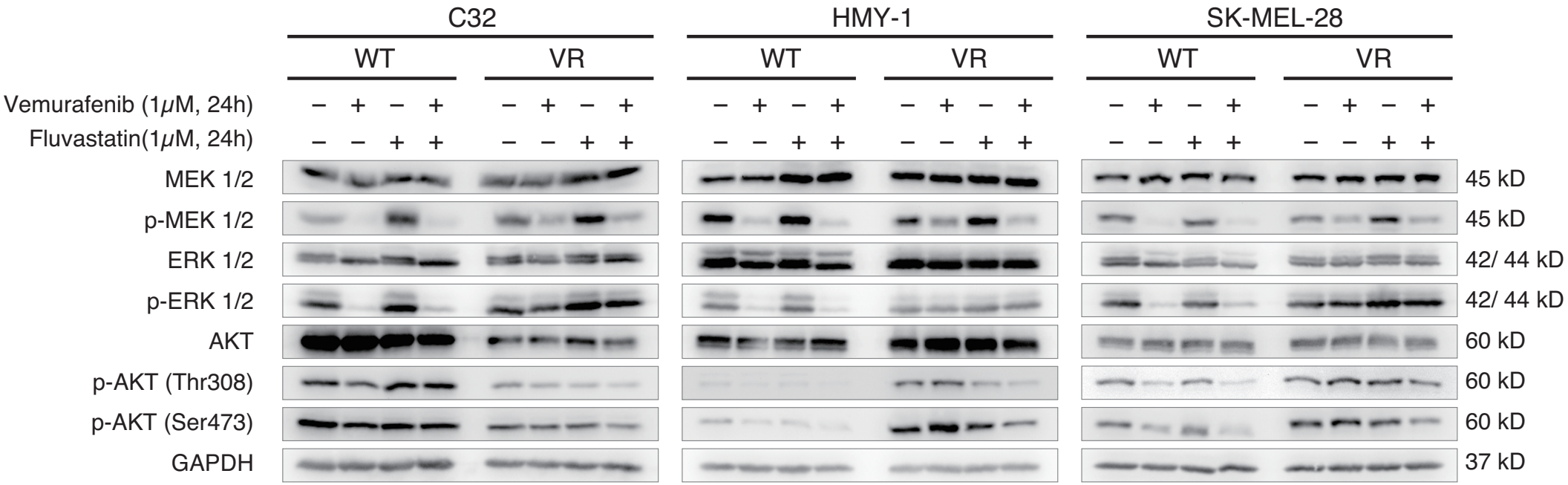

C32
HMY-1
SK-MEL-28
WT
VR
WT
VR
WT
VR
Vemurafenib (1μM, 24h)
Fluvastatin(1μM, 24h)
MEK 1/2
p-MEK 1/2
ERK 1/2
p-ERK 1/2
AKT
p-AKT (Thr308)
p-AKT (Ser473)
GAPDH
45 kD
45 kD
42/ 44 kD
42/ 44 kD
60 kD
60 kD
60 kD
37 kD

Table 1

Table 1 Drug-resistance profiles (IC_{50}) of parental and vemurafenib-resistant cells.

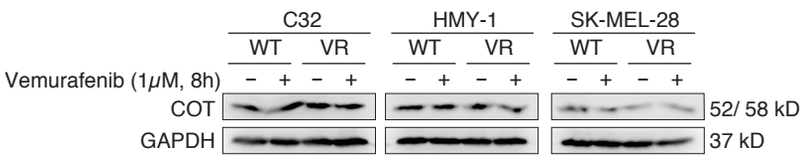
IC_{50} (μM)	Vemurafenib (95% Conf.I)	Fluvastatin (95% Conf.I)
C32/PT	3.34 (2.73 – 4.11)	26.16 (20.37 – 34.20)
C32/VR	14.52 (12.72 – 16.61)	12.45 (10.62 – 14.68)
HMY-1/ PT	1.97 (1.50 – 2.57)	20.84 (18.44 – 23.63)
HMY-1/VR	12.27 (11.10 – 13.14)	14.01 (12.52 – 15.70)
SK-MEL-28/ PT	0.25 (0.22 – 0.28)	10.32 (9.12 – 11.49)
SK-MEL-28/VR	24.18 (21.16 – 27.64)	11.18 (10.12 – 12.36)

IC_{50}, 50% inhibitory concentration; PT, parental; VR, vemurafenib-resistant; Conf.I, confidence interval.

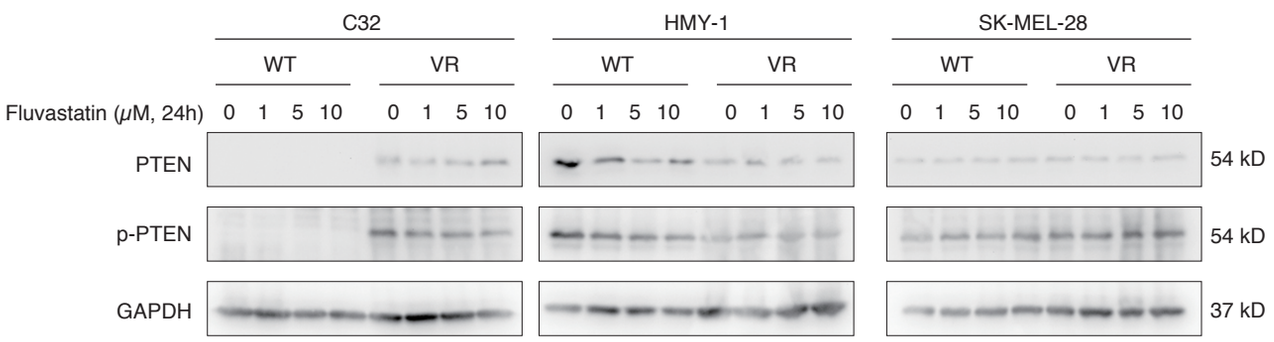
Table 2 Combination index analysis for the combination of vemurafenib with fluvastatin in the parental and the VR cells.

Cell line	Comb.I	DRI	
		Vemurafenib	Fluvastatin
C32/PT	1.59	1.11	1.45
C32/VR	0.78	2.33	2.86
HMY-1/PT	1.57	1.28	1.27
HMY-1/VR	0.86	2.38	2.30
SK-MEL-28/PT	2.65	0.62	0.96
SK-MEL-28/VR	0.73	2.51	3.01

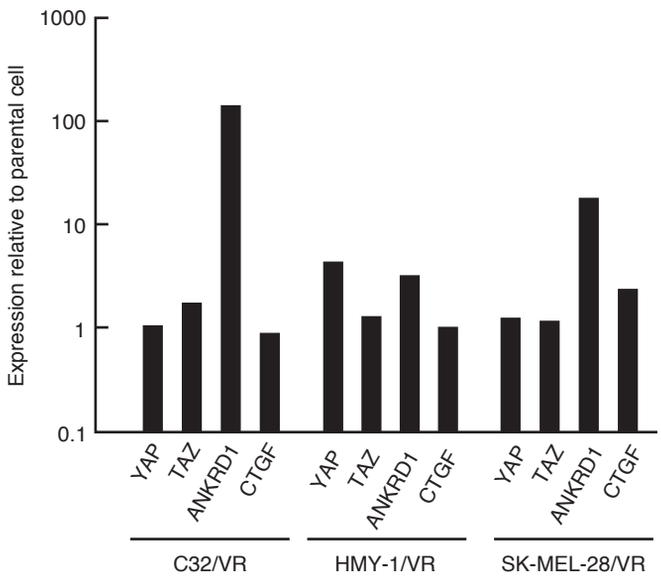
Comb.I, combination index; DRI, dose reduction index; PT, parental; VR, vemurafenib-resistant.



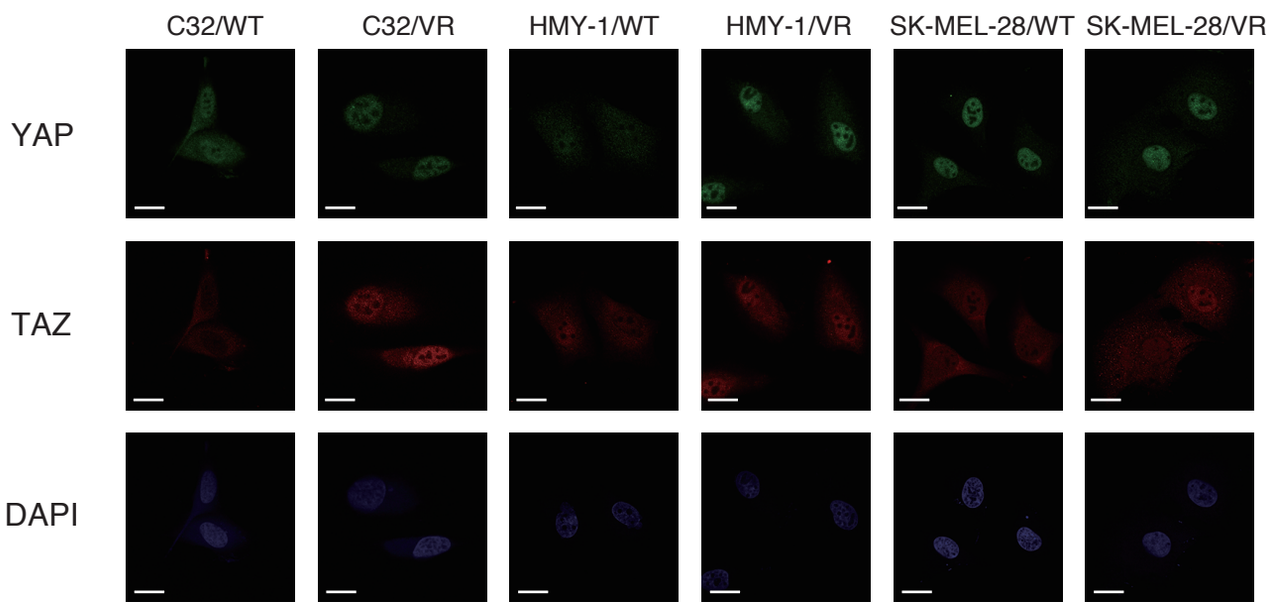
Supplementary Figure 1. Effects of vemurafenib-treatment on COT expression in melanoma cells. Cells treated with 1 μM vemurafenib for 8 h were lysed and analyzed with immunoblotting.



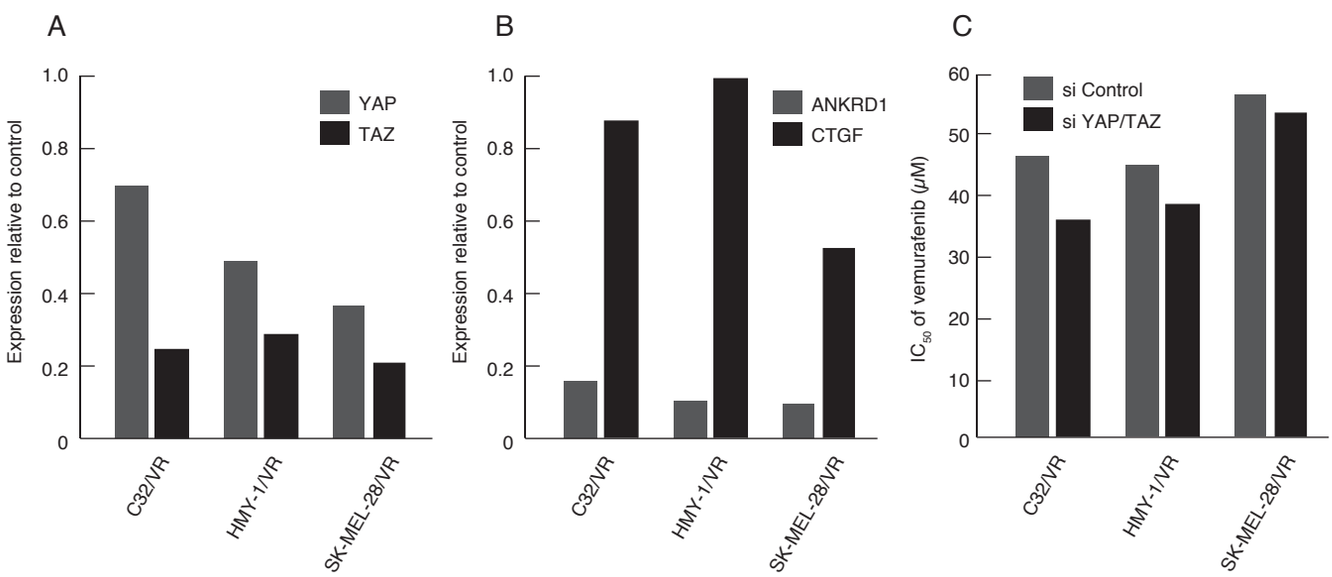
Supplementary Figure 2. Effects of fluvastatin-treatment on phosphorylation statuses of PTEN protein, in WT and VR melanoma cells. Cells treated with 1 μM vemurafenib for 8 h were lysed and analyzed with immunoblotting. WT, wild type; VR, vemurafenib-resistance.



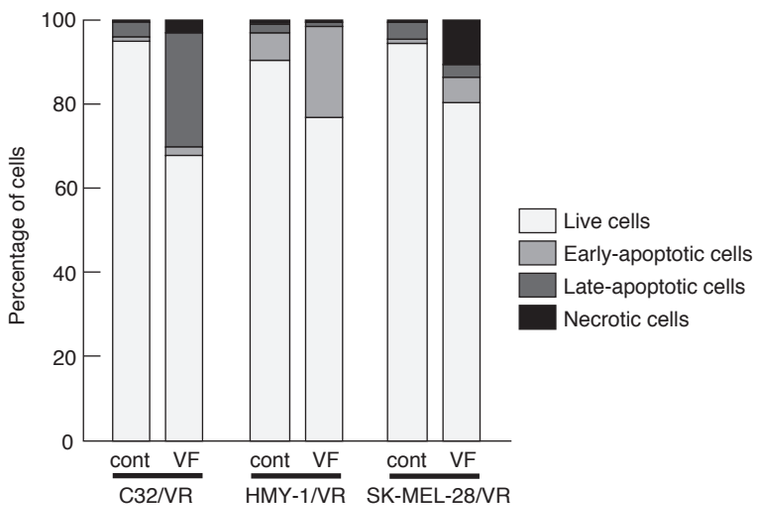
Supplementary Figure 3. Relative mRNA expression of YAP/TAZ and their transcription targets (ANKRD1/CTGF) in VR melanoma cells in comparison with WT cells.



Supplementary Figure 4. Immunofluorescence microscopy for YAP/TAZ protein in WT and VR melanoma cells. WT, wild type; VR, vemurafenib-resistance. Scale bar is 20 μm.



Supplementary Figure 5. Relative mRNA expression of YAP/TAZ (A) and their transcription targets (ANKRD1/CTGF) (B) and IC50 values of vemurafenib (C), when double knockdown of YAP/TAZ was performed with siRNA treatment. WT, wild type; VR, vemurafenib-resistance.



Supplementary Figure 6. Frequencies of apoptosis/necrosis induction after treatment with vemurafenib and fluvastatin. The cells were treated by the drugs at combinatorial IC50s, calculated as IC50s of respective drug alone divided by dose reduction indices (see Tables 1 and 2, 6.23 μM vemurafenib and 4.35 μM fluvastatin for C32/VR, 5.16 μM vemurafenib and 6.09 μM fluvastatin for HMY-1/VR, and 9.63 μM vemurafenib and 3.71 μM fluvastatin for SK-MEL-28/VR) for 8 hours. Apoptosis/necrosis was assessed with annexin/PI staining (Annexin V-FITC apoptosis detection kit, Nakarai Tesque, Inc., Kyoto, Japan) followed by fluorescence microscopy. At least 160 cells were counted for each treatment condition.