PLOS ONE

RESEARCH ARTICLE

# Combination of a Selective HSP90α/β Inhibitor and a RAS-RAF-MEK-ERK Signaling Pathway Inhibitor Triggers Synergistic Cytotoxicity in Multiple Myeloma Cells

**Rikio Suzuki[1,2☯], Shohei Kikuchi[1☯], Takeshi Harada[1☯], Naoya Mimura[1], Jiro Minami[1], Hiroto Ohguchi[1], Yasuhiro Yoshida[1], Morihiko Sagawa[1], Gullu Gorgun[1], Diana Cirstea[1], Francesca Cottini[1], Jana Jakubikova[1], Yu-Tzu Tai[1], Dharminder Chauhan[1], Paul G. Richardson[1], Nikhil Munshi[4], Kiyoshi Ando[2], Teruhiro Utsugi[3], Teru Hideshima[1], Kenneth C. Anderson[1]***

**1** Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, United States of America, **2** Department of Hematology/Oncology, Tokai University School of Medicine, Isehara, Kanagawa, Japan, **3** Tsukuba Research Center, Taiho Pharmaceutical Co., Ltd., Tsukuba, Japan, **4** VA Boston Healthcare System, Jerome Lipper Multiple Myeloma Center, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, United States of America

☯ These authors contributed equally to this work.
* kenneth_anderson@dfci.harvard.edu



OPEN ACCESS

**Citation:** Suzuki R, Kikuchi S, Harada T, Mimura N, Minami J, Ohguchi H, et al. (2015) Combination of a Selective HSP90α/β Inhibitor and a RAS-RAF-MEK-ERK Signaling Pathway Inhibitor Triggers Synergistic Cytotoxicity in Multiple Myeloma Cells. PLoS ONE 10 (12): e0143847. doi:10.1371/journal.pone.0143847

**Editor:** Nikolas K. Haass, University of Queensland Diamantina Institute, AUSTRALIA

**Received:** June 8, 2015

**Accepted:** November 9, 2015

**Published:** December 2, 2015



**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** This study was supported by the National Institutes of Health Grants (http://grants.nih.gov/grants/oer.htm) P50-100707, PO1-CA078378, and PO1-155258 (K.C.A., N.M.); RO1-CA050947 (K.C.A.) and RO1-178264 (T.H., K.C.A.); IO1-BX001584 (N. M.); and SENSHIN Medical Research Foundation (http://www.mt-pharma.co.jp/zaidan/) (R.S.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

Heat shock protein (HSP)90 inhibitors have shown significant anti-tumor activities in preclinical settings in both solid and hematological tumors. We previously reported that the novel, orally available HSP90α/β inhibitor TAS-116 shows significant anti-MM activities. In this study, we further examined the combination effect of TAS-116 with a RAS-RAF-MEK-ERK signaling pathway inhibitor in *RAS*- or *BRAF*-mutated MM cell lines. TAS-116 monotherapy significantly inhibited growth of *RAS*-mutated MM cell lines and was associated with decreased expression of downstream target proteins of the RAS-RAF-MEK-ERK signaling pathway. Moreover, TAS-116 showed synergistic growth inhibitory effects with the farnesyltransferase inhibitor tipifarnib, the BRAF inhibitor dabrafenib, and the MEK inhibitor selumetinib. Importantly, treatment with these inhibitors paradoxically enhanced p-C-Raf, p-MEK, and p-ERK activity, which was abrogated by TAS-116. TAS-116 also enhanced dabrafenib-induced MM cytotoxicity associated with mitochondrial damage-induced apoptosis, even in the *BRAF*-mutated U266 MM cell line. This enhanced apoptosis in *RAS*-mutated MM triggered by combination treatment was observed even in the presence of bone marrow stromal cells. Taken together, our results provide the rationale for novel combination treatment with HSP90α/β inhibitor and RAS-RAF-MEK-ERK signaling pathway inhibitors to improve outcomes in patients with in *RAS*- or *BRAF*-mutated MM.

**Competing Interests:** The authors of this manuscript have the following competing interests: T.U. is an employee at Taiho Pharmaceutical Co., Ltd. Y.-T.T. is a consultant for Onyx. D.C. is a consultant for Oncopeptides AB. P.G.R. is a member of the advisory boards for Celgene, Millennium, Johnson & Johnson, Novartis, and Bristol-Myers Squibb. N.M. is a member of the advisory boards for Millennium, Celgene, and Novartis. T.H. is a consultant for Acetylon Pharmaceuticals. K.C.A. is a member of the advisory boards for Onyx, Celgene, Gilead, and Sanofi-Aventis, and is a scientific founder of Acetylon and Oncopep. J.M. receives research funding from Bristol-Myers Squibb. The other authors declare no conflicts of interest. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

## Introduction

Multiple myeloma (MM) is characterized by proliferation of clonal plasma cells in the bone marrow (BM) microenvironment, monoclonal protein in the blood and/or urine, bone lesions, and immunodeficiency [1,2]. In recent years, the introduction of high-dose chemotherapy and stem cell transplantation, as well as novel therapies including bortezomib, thalidomide, and lenalidomide, have prolonged the survival of patients with MM [1,3]. However, relapses are common, and novel therapies are needed.

An inhibitor of heat shock protein (HSP)90 is a promising novel targeted therapy [4]. Since the prototype geldanamycin was first isolated in 1970 from *Streptomyces higroscopicus var geldanus*, HSP90 inhibitors have been developed as treatment options for specific molecularly-defined subgroups of cancer [5]. Most HSP90 inhibitors bind to the ATP-binding sites in the N-terminal domain of HSP90, thereby inhibiting the interaction between HSP90 and client proteins as well as the folding and maturation of client proteins, leading to the destruction of client proteins via the ubiquitin-proteasome system [5,6]. Compared with normal cells, malignant cells including MM cells are highly dependent on HSP90 systems to overcome cellular stress induced by abnormal fusion proteins and/or a hypoxic, acidotic, and nutrient-deprived microenvironment [6–9]. Moreover, in malignant tissues HSP90 forms specific multi-chaperone complexes, which have higher affinity for oncoproteins than HSP90 in normal tissues [5,10]. Thus, HSP90 is an attractive molecular target for cancer therapy.

Since first-generation HSP90 inhibitors have shown various toxicities, i.e. a geldanamycin analog causes liver toxicity with poor solubility [11,12], second-generation HSP90 inhibitors have been developed and are currently in clinical trials. However, the effectiveness of some HSP90 inhibitor monotherapies may be limited in the clinical setting [4,5,13]. Moreover, HSP90 inhibitor SNX-5422-induced ocular toxicity has been observed, resulting in discontinuation of its clinical evaluation [5,14]. Thus, ongoing efforts are developing less toxic, second-generation HSP90 inhibitors and identifying biomarkers algorithm to identify the most appropriate patient populations to benefit.

Our group reported that several HSP90 inhibitors (17-AAG, SNX-2112, and TAS-116) show promising anti-MM effects [7,15,16]. TAS-116 is an orally active, ATP competitive inhibitor of HSP90α/β [17,18]. In particular, TAS-116 shows favorable pharmacokinetics and a reduced ocular toxicity profile, possibly due to its lower distribution in retinal tissue than in plasma in rats [17]. Moreover, TAS-116 shows superior anti-tumor effects in several tumors including MM and lung carcinoma *in vitro* and *in vivo* [16,17]. Therefore, TAS-116 represents a promising therapeutic potential.

The rat sarcoma (RAS)-v-raf murine sarcoma viral oncogene homolog (RAF)-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling pathway is one of the most important oncogenic pathways which plays a central role in regulation of cell proliferation and survival [19]. Aberrant signaling through this pathway is common in a wide variety of malignancies, including MM, making it an attractive candidate for development of novel targeted therapies [20]. Many cytokines (i.e., interleukin (IL)-6, insulin-like growth factor-1, stromal cell derived factor-1α (SDF1α), and BAFF (B cell activating factor)) activate the RAS-RAF-MEK-ERK signaling cascade and mediate MM cell proliferation [21,22].

A recognized genetic difference between monoclonal gammopathy of undetermined significance (MGUS) and MM is *RAS* mutation, which is extremely rare in MGUS but present in 20–30% of newly diagnosed MM [23]. The RAS pathway plays a main role in switching of MGUS to MM, since activating *RAS* mutations (mainly *neuroblastoma ras viral oncogene homolog (NRAS)* or *v-ki-ras2 kirsten rat sarcoma viral oncogene homolog (KRAS)*) are found in 32–50%

of patients with MM [20]. Our group and others have previously reported that *RAS* mutation is an independent prognostic factor in MM [24], and that *NRAS* mutation significantly reduces MM sensitivity to single-agent bortezomib therapy [25]. Many RAS pathway inhibitors, including RAF inhibitors and MEK inhibitors, have been developed and show superior effects in the treatment of malignant melanoma, Her2-positive breast cancer, and anaplastic lymphoma kinase (ALK)-positive NSCLC [19]. However, RAF inhibitors and MEK inhibitors essentially produce a cytostatic effect and show limited efficacy as a monotherapy [20]. Therefore, a second type of therapy that synergizes with the anti-tumor effects of RAF or MEK inhibitors is needed. Recently, some groups have reported that the combination of RAF inhibitors and MEK inhibitors shows significant synergistic anti-tumor effects in melanoma with v-raf murine sarcoma viral oncogene homolog B1 (BRAF) V600E mutation [26,27]. However, dabrafenib shows paradoxical effects, in which proliferation of tumors harboring wild-type *RAF* and *RAS* mutation is promoted rather than inhibited [28]. Moreover, acquisition of resistance to dabrafenib has recently been described [29,30]. Therefore, an optimal partner that overcomes these resistance mechanisms is needed.

Another group reported that the combination of ganetespib with MEK inhibitors shows significant synergistic anti-tumor effects against NSCLCs with *RAS* mutations *in vitro* and *in vivo* [31]. In the present study, we demonstrate that TAS-116 in combination with an inhibitor of the RAS-RAF-MEK-ERK signaling pathway shows significant synergistic anti-myeloma effects in *RAS*- or *BRAF*-mutated MM cell lines *in vitro*, providing the framework for its clinical evaluation to improve MM patient outcome.

## Methods

### Reagents

The HSP90 inhibitor TAS-116 was synthesized at Taiho Pharmaceutical Co., Ltd. (Tsukuba, Japan). Bortezomib, doxorubicin, tipifarnib, dabrafenib, and AZD6244 were obtained from Selleck Chemicals (Houston, TX, USA). Recombinant human IL-6 was from R&D Systems (Minneapolis, MN, USA).

### Human cell lines

The dexamethasone (Dex)-sensitive MM.1S MM cell line was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL, USA). RPMI-8226, U266, and NCI-H929 human MM cell lines were obtained from ATCC (Manassas, VA, USA). The doxorubicin (Dox)-resistant RPMI-8226 DOX40 cell line was kindly provided by Dr. William Dalton (Lee Moffitt Cancer Center, Tampa, FL, USA). The IL-6-dependent INA6 human cell line was provided by Dr. Renate Burger (University of Kiel, Kiel, Germany). KMS-11 was kindly provided by Dr. Takemi Otsuki (Kawasaki Medical School, Okayama, Japan). All MM cell lines were cultured in RPMI 1640 containing 10% FBS (Sigma-Aldrich, St Louis, MO, USA), 2 μM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), plus 2.5 ng/mL IL-6 only for INA6 cells.

### Primary cells

Patient MM cells and bone marrow stromal cells (BMSCs) were obtained from BM samples after informed consent was obtained. All participants provided their written informed consent to participate in this study. This study was performed in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the Dana-Farber Cancer Institute. Mononuclear cells were separated using Ficoll-Hypaque density sedimentation, and

plasma cells were purified (>95% $CD138^+$) by positive selection with anti-CD138 magnetic-activated cell separation microbeads (Miltenyi Biotec, San Diego, CA, USA). Tumor cells were also purified from the BM of MM patients using the RosetteSep negative selection system (StemCell Technologies, Vancouver, BC, Canada). BMSCs were generated by culturing BM mononuclear cells for 4 to 6 weeks in DMEM supplemented with 15% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

## Growth inhibition assay

The growth inhibitory effect of TAS-116 or RAS-RAF-MEK-ERK inhibitors in MM cell lines was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT; Sigma-Aldrich) dye absorbance as described [32].

## Western blotting

MM cells were treated with or without novel or conventional agents, harvested, washed, and lysed as reported [32,33]. Cell lysates were subjected to SDS-PAGE, transferred to membranes, and immunoblotted with the following antibodies: anti-p27, cyclin D1, phospho-B-Raf (Ser445), B-Raf, phospho-v-raf-1 murine leukemia viral oncogene homolog 1 (C-Raf) (Ser338), C-Raf, phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-ERK (Thr202/Tyr204), ERK, phospho-v-akt murine thymoma viral oncogene homolog (Akt) (Ser473), Akt, caspase 3, poly (ADP-ribose) polymerase (PARP), β-actin, Bim, α-tubulin (all from Cell Signaling, Beverly, MA, USA), as well as NRAS and KRAS (both from Santa Cruz Biotechnology, Dallas, TX, USA).

## Transient transfection

INA6 cells were transiently transfected with non-targeting short interfering RNA (siRNA) or *NRAS* siRNA siGENOME SMARTpool siRNA (Dharmacon, Inc., Lafayette, CO, USA). RPMI-8226 and RPMI-8226 DOX40 cells were transiently transfected with non-targeting siRNA or *KRAS* siRNA siGENOME SMARTpool siRNA (Dharmacon) using Nucleofector Kit V (Amaxa Biosystems, Cologne, Germany). Cells were harvested 24–72 h after transfection and analyzed with immunoblotting and the cell viability assay.

## Detection of apoptosis with annexin V/propidium iodide (PI) staining

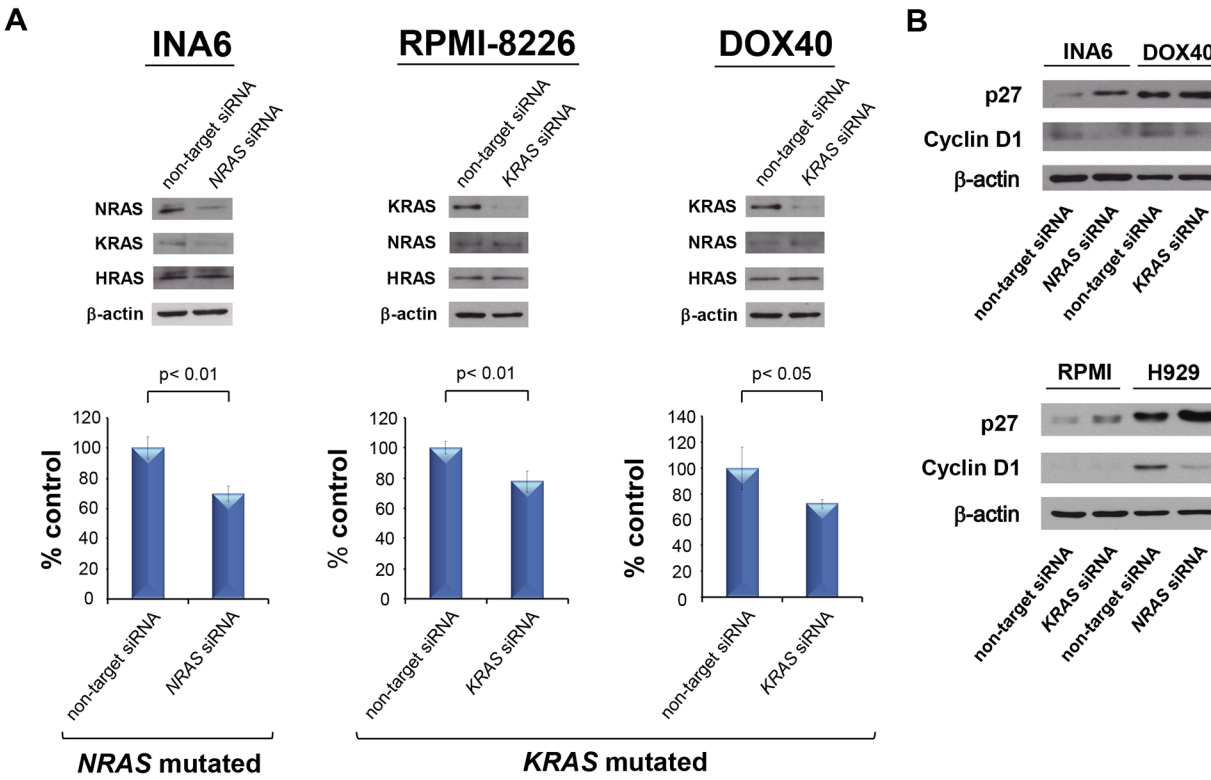
Detection of apoptotic cells was done with the annexin V/ PI detection kit (Immunotech/Beckman Coulter, Indianapolis, IN, USA) as described [34]. Apoptotic cells were analyzed on a BD FACSCanto II (BD Biosciences) using FACSDiva (BD Biosciences). Cells that were annexin V positive and PI negative were considered early apoptotic cells, whereas positivity for both annexin V and PI was associated with late apoptosis or necrosis.

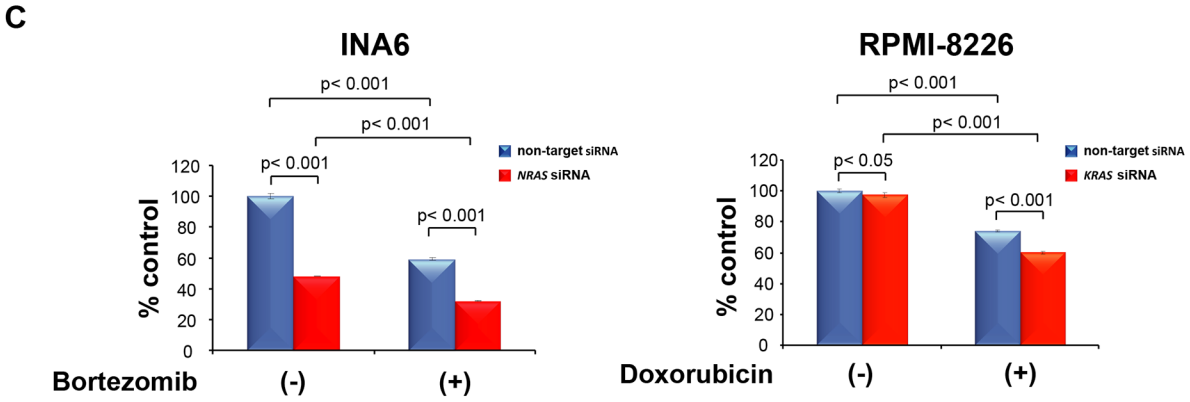
## Mitochondrial membrane potential

To evaluate the effect of TAS-116 on alterations of mitochondrial membrane potential, MM cells were treated with or without novel or conventional agents with addition of MitoCapture reagent (MitoCapture Apoptosis Detection kit®, Calbiochem) for the last 20 minutes, followed by flow cytometric analysis on a BD FACSCanto II (BD Biosciences) using FACSDiva® (BD Biosciences) [35].

## Statistical analysis

Statistical significance was determined with the Student's t-test. The minimal level of significance was $P < 0.05$. The combination index (CI) values were calculated by isobologram analysis using


A
INA6
RPMI-8226
DOX40
non-target siRNA
NRAS siRNA
KRAS siRNA
NRAS
KRAS
HRAS
β-actin
p< 0.01
p< 0.05
% control
NRAS mutated
KRAS mutated
B
INA6
DOX40
RPMI
H929
p27
Cyclin D1
β-actin


C
INA6
RPMI-8226
p< 0.001
p< 0.05
non-target siRNA
NRAS siRNA
KRAS siRNA
% control
Bortezomib
Doxorubicin
(-)
(+)

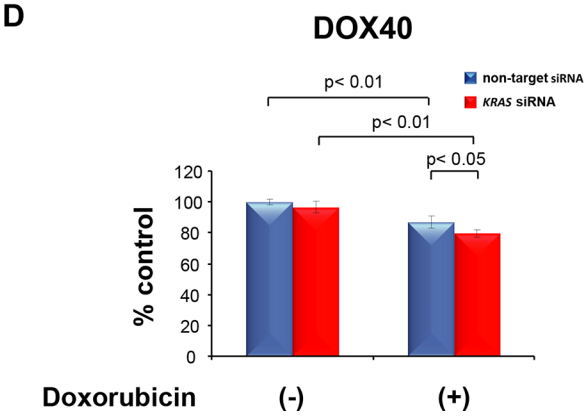

D
DOX40
p< 0.01
p< 0.05
non-target siRNA
KRAS siRNA
% control
Doxorubicin
(-)
(+)

**Fig 1. Downregulation of RAS inhibits growth and enhances cytotoxicity of doxorubicin and bortezomib in *RAS*-mutated MM cell lines.** (A) INA6 cells were transiently transfected with non-targeting or *NRAS* siRNA, and RPMI-8226 and RPMI-8226 DOX40 cells were transiently transfected with non-targeting or *KRAS* siRNA. The cell number and viability 48 h later were assessed with trypan blue exclusion. Whole-cell lysates were subjected to western blotting to confirm the downregulation of NRAS and KRAS expression using NRAS, KRAS, HRAS, and β-actin Abs. Data are the mean ± SD of triplicate wells. (B) INA6 and NCI-H929 cells were transiently transfected with non-targeting or *NRAS* siRNA, and RPMI-8226 and RPMI-8226 DOX40 cells were transiently transfected with non-targeting or *KRAS* siRNA. After 48 h, whole-cell lysates were subjected to western blotting using p27, cyclin D1, NRAS and β-actin Abs. (C) INA6 cells were transiently transfected with non-targeting or *NRAS* siRNA and then treated with or without bortezomib (5 nM) for 48 h. RPMI-8226 cells were transiently transfected with non-targeting or *KRAS* siRNA and then treated with or without doxorubicin (0.1 μM) for 48 h. In each case, cell viability was assessed with the MTT assay of triplicate cultures and expressed as the percentage of the untreated control. Data are the mean ± SD. (D) RPMI-8226 DOX40 cells were transiently transfected with non-targeting or *KRAS* siRNA and then treated with or without doxorubicin (1 μM) for 24 h. Cell viability was assessed with the MTT assay of triplicate cultures and expressed as the percentage of the untreated control. Data are the mean ± SD.

doi:10.1371/journal.pone.0143847.g001

the CompuSyn Version 1.0 software program (ComboSyn, Paramus, NJ, USA). CI $<$ 1.0 indicates synergism; CI = 1.0 indicates an additive effect; and CI $>$ 1.0 indicates antagonism.

## Results

### Downregulation of RAS inhibits growth and enhances cytotoxicity of doxorubicin and bortezomib in *RAS*-mutated MM cell lines

First, we assessed the functional significance of NRAS and KRAS in *RAS*-mutated MM cells using a siRNA strategy. The viability of *NRAS*-mutated INA6 cells was markedly inhibited by *NRAS* siRNA compared with non-targeting siRNA and was associated with significant downregulation of NRAS expression. Similarly, the viability of *KRAS*-mutated RPMI-8226 and RPMI-8226 DOX40 cells was inhibited by *KRAS* siRNA compared with non-targeting siRNA, associated with significant downregulation of KRAS expression (Fig 1A).

Others have shown that the RAS-RAF-MEK-ERK signaling pathway is necessary for G1 progression and that it also regulates cyclin D1 transcription and p27 expression [36–38]. We therefore hypothesized that *RAS* siRNA-induced growth inhibition may be associated with cell cycle arrest. Indeed, we observed downregulation of cyclin D1 and upregulation of p27 in both *NRAS* siRNA-transfected INA6 and NCI-H929 as well as *KRAS* siRNA-transfected RPMI-8226 and RPMI-8226 DOX40 cells, compared with non-targeting siRNA-transfected cells (Fig 1B). These results suggest that downregulation of RAS in *RAS*-mutated MM cells regulates MM cell survival, at least in part, via cell cycle modulation.

We and others have previously shown that *NRAS* or *KRAS* mutation contributes to resistance to conventional chemotherapy or bortezomib [25,39]. Therefore, we next examined whether RAS inhibition enhances conventional chemotherapy- or bortezomib-induced cytotoxicity in *RAS*-mutated MM cells. Bortezomib- or doxorubicin-triggered cytotoxicity was significantly enhanced in NRAS knockdown INA6 and NCI-H929 cells, but not in KMS11 cells (Fig 1C left and S2 Fig); moreover, cytotoxicity of doxorubicin was also enhanced in KRAS knockdown RPMI-8226 cells and doxorubicin-resistant RPMI-8226 DOX40 cells (Fig 1C right and Fig 1D). These results indicate that RAS inhibition enhances conventional chemotherapy- or bortezomib-induced cytotoxicity in *RAS*-mutated MM cells.

### TAS-116 induces cytotoxicity and targets degradation of the HSP90 client RAS-RAF-MEK-ERK signaling pathway proteins in *RAS*-mutated MM cells

We next examined the cytotoxicity of TAS-116 in a panel of MM cell lines selected for expression of *NRAS* or *KRAS* mutations. TAS-116 is a selective inhibitor of cytoplasmic HSP90α/β that does not inhibit HSP90 paralogs, such as endoplasmic reticulum GRP94 or mitochondrial TRAP1 (S1 Fig). TAS-116 significantly inhibited the viability in all *RAS*-mutated MM cells in a

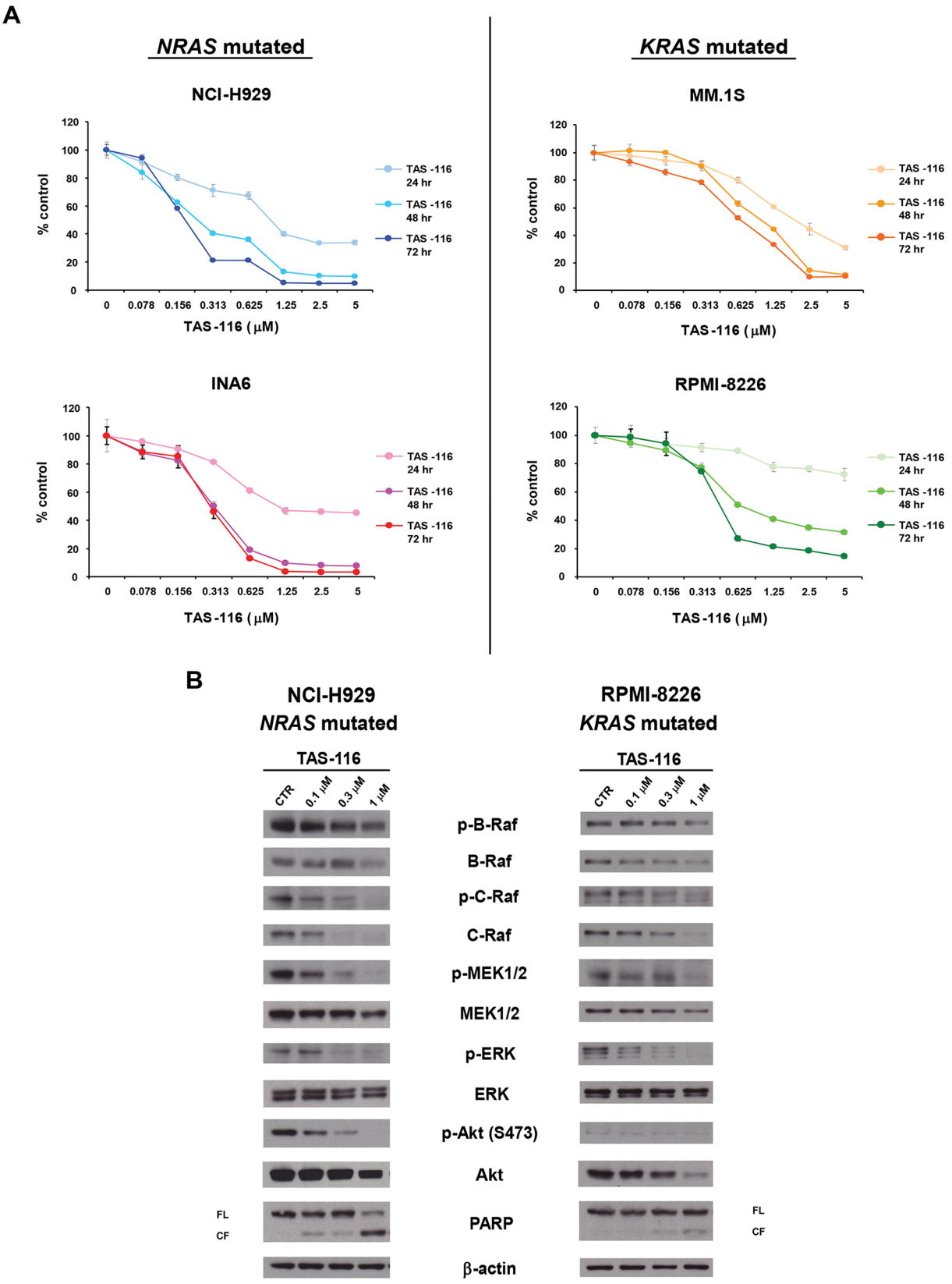

A
NRAS mutated
KRAS mutated
NCI-H929
MM.1S
INA6
RPMI-8226
% control
TAS-116 (μM)
0 0.078 0.156 0.313 0.625 1.25 2.5 5
TAS -116 24 hr
TAS -116 48 hr
TAS -116 72 hr
B
NCI-H929
NRAS mutated
RPMI-8226
KRAS mutated
TAS-116
CTR 0.1 μM 0.3 μM 1 μM
p-B-Raf
B-Raf
p-C-Raf
C-Raf
p-MEK1/2
MEK1/2
p-ERK
ERK
p-Akt (S473)
Akt
PARP
FL
CF
β-actin

**Fig 2. TAS-116 effects on cell viability and RAS-RAF-MEK-ERK signaling in *RAS*-mutated MM cell lines.** (A) NCI-H929, INA6, MM.1S, and RPMI-8226 MM cell lines were cultured with TAS-116 (0–5 μM) for 24, 48, or 72 h. In each case, cell viability was assessed with the MTT assay of triplicate cultures and expressed as the percentage of the untreated control. Data are the mean ± SD. (B) NCI-H929 and RPMI-8226 cells were treated with the indicated doses of TAS-116 for 24 h. Whole-cell lysates were subjected to western blotting using p-B-Raf, B-Raf, p-C-Raf, C-Raf, p-MEK1/2, MEK1/2, p-ERK, ERK, p-Akt (S473), Akt, PARP, and β-actin Abs. FL, full-length; CF, cleaved form.

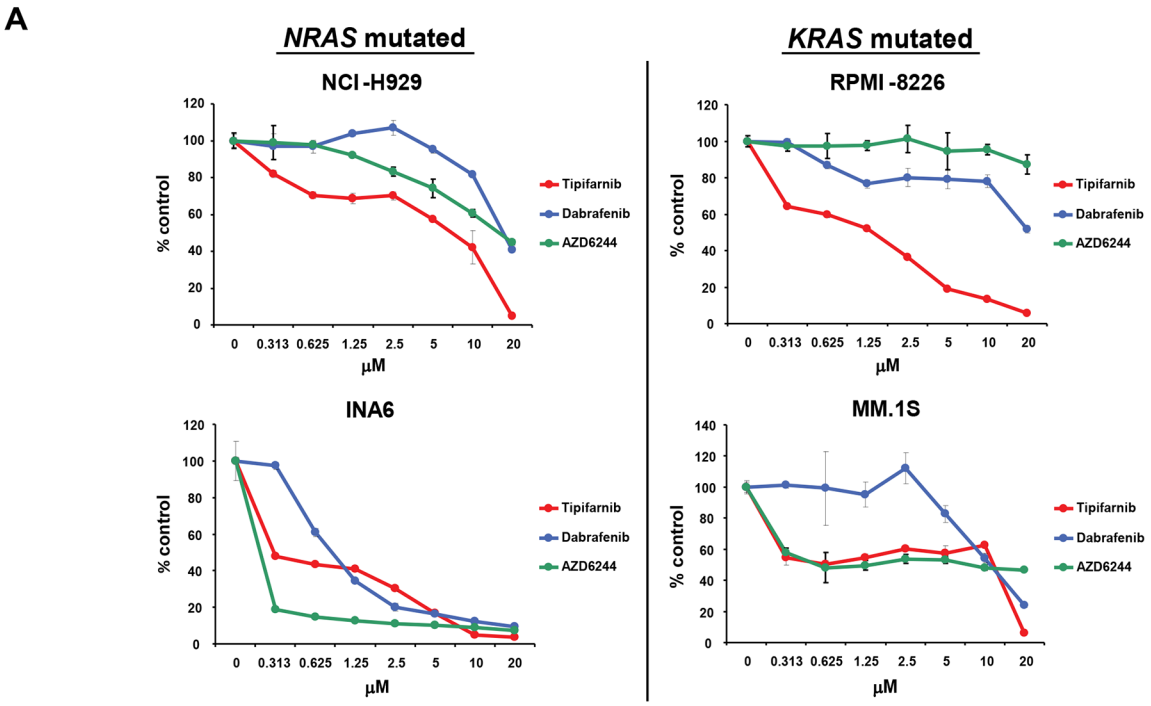
doi:10.1371/journal.pone.0143847.g002

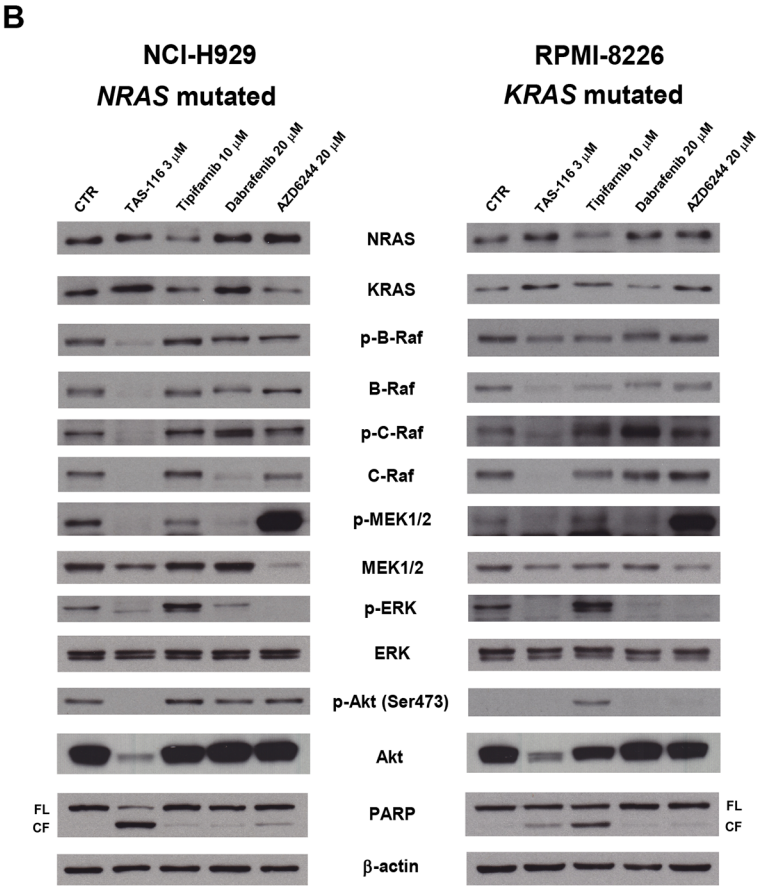
dose- and time-dependent manner (Fig 2A). We also measured expression levels of RAS-RAF-MEK-ERK pathway client proteins following TAS-116 treatment. TAS-116 triggered significant degradation of key RAS-RAF-MEK-ERK pathway regulators (p-C-Raf, p-MEK1/2, and p-ERK) in a dose-dependent manner in *RAS*-mutated MM cell lines (Fig 2B). In these cell lines, especially NCI-H929 cells, we observed that a modest reduction in p-B-Raf, marked induction of PARP cleavage, and inhibition of p-Akt were induced by TAS-116 in a dose-dependent manner (Fig 2B). Furthermore, we confirmed that TAS-116 triggered a decrease in mitochondria transmembrane potential in a time-dependent manner (S3 Fig). Taken together, these results indicate that TAS-116 potently targets HSP90 client RAS-RAF-MEK-ERK signaling pathway proteins, and induces cytotoxicity, associated with mitochondrial alterations and apoptosis in *RAS*-mutated MM cell lines.

## Farnesyltransferase inhibitor and selective RAF or MEK inhibitors trigger cytotoxicity and induce apoptosis in *RAS*-mutated MM cells

We next examined the cytotoxicity of the farnesyltransferase inhibitor tipifarnib, the selective RAF inhibitor dabrafenib, or the selective MEK inhibitor AZD6244, in *RAS*-mutated MM cell lines. These inhibitors induced modest to moderate cytotoxicity in each *RAS*-mutated MM cell line, with the IL-6 dependent INA6 cells being the most sensitive (Fig 3A left, lower panel). Interestingly, a broad range of tipifarnib and AZD6244 (0.313 μM to 10 μM) induced equivalent cytotoxicity, suggesting cytostatic effects in this cell line (Fig 3A right, lower panel). We next examined the effect of these inhibitors on degradation of RAS-RAF-MEK-ERK signaling pathway client proteins. Tipifarnib inhibited NRAS and KRAS in *NRAS*-mutated NCI-H929 cells, as well as NRAS in *KRAS*-mutated RPMI-8226 cells. However, tipifarnib markedly increased the RAS-RAF-MEK-ERK signaling pathway proteins p-B-Raf, p-C-Raf, and p-ERK in NCI-H929 cells; as well as p-C-Raf, p-MEK1/2, and p-ERK in RPMI-8226 cells. In addition, PARP cleavage and p-Akt (Ser473) were increased in both cell lines, associated with a feedback loop mechanism (Fig 3B). Interestingly, dabrafenib increased NRAS and KRAS in NCI-H929 cells, and NRAS in RPMI-8226 cells. Moreover, dabrafenib increased p-C-Raf and decreased p-MEK1/2 and p-ERK in both cell lines (Fig 3B). Although others have previously shown that RAF inhibitors paradoxically activate ERK signaling in wild-type *BRAF* and *RAS*-mutated tumors [28,40–42], we here confirmed that dabrafenib (1.25 to 2.5 μM) activated p-MEK1/2 and p-ERK in both cell lines, suggesting that paradoxical activation also occurs in MM (S4 Fig).

We next investigated the mechanism of cytotoxicity in *RAS*-mutated MM cells triggered by RAS-RAF-MEK-ERK signaling pathway inhibitors using annexin V/PI staining. We observed a dose-dependent increase in annexin V-positive cells in these *RAS*- mutated MM cell lines after treatment with each inhibitor ($P < 0.01$ in each tipifarnib treatment doses versus control groups in each cell lines; $P < 0.05$ in AZD6244 treatment doses versus control groups in each cell lines; $P < 0.05$ in 5, 10, and 20 μM dabrafenib treatment doses versus control groups in NCI-H929 and MM.1S cells) (Fig 3C). We also confirmed that these inhibitors induced mild or moderate PARP cleavage in NCI-H929 and RPMI-8226 cells (Fig 3B). Taken together, these results indicate that a farnesyltransferase inhibitor and selective RAF or MEK inhibitors trigger cytotoxicity and induce apoptosis in *RAS*-mutated MM cells.


A
NRAS mutated
KRAS mutated
NCI-H929
RPMI-8226
INA6
MM.1S
% control
μM
Tipifarnib
Dabrafenib
AZD6244


B
NCI-H929
NRAS mutated
RPMI-8226
KRAS mutated
CTR
TAS-116 3 μM
Tipifarnib 10 μM
Dabrafenib 20 μM
AZD6244 20 μM
NRAS
KRAS
p-B-Raf
B-Raf
p-C-Raf
C-Raf
p-MEK1/2
MEK1/2
p-ERK
ERK
p-Akt (Ser473)
Akt
PARP
FL
CF
β-actin

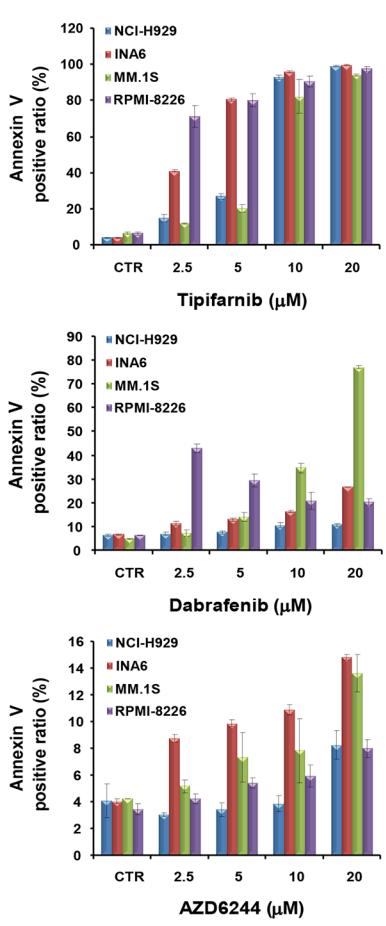

C
Annexin V positive ratio (%)
NCI-H929
INA6
MM.1S
RPMI-8226
Tipifarnib (μM)
Dabrafenib (μM)
AZD6244 (μM)
CTR
2.5
5
10
20

**Fig 3. RAS pathway inhibitors induce cytotoxicity and apoptosis in *RAS*-mutated MM cell lines.** (A) NCI-H929, INA6, MM.1S, and RPMI-8226 MM cell lines were cultured with tipifarnib (0–20 μM), dabrafenib (0–20 μM), or AZD6244 (0–20 μM) for 72 h. In each case, cell viability was assessed with the MTT assay of triplicate cultures and expressed as the percentage of the untreated control. Data are the mean ± SD. (B) NCI-H929 and RPMI-8226 cells were treated with TAS-116 (3 μM), tipifarnib (10 μM), dabrafenib (20 μM), or AZD6244 (20 μM) for 24 h. Whole-cell lysates were subjected to western blotting using NRAS, KRAS, p-B-Raf, B-Raf, p-C-Raf, C-Raf, p-MEK1/2, MEK1/2, p-ERK, ERK, p-Akt (S473), Akt, PARP, and β-actin Abs. FL, full-length; CF, cleaved form. (C) NCI-H929, INA6, MM.1S, and RPMI-8226 cells were treated with tipifarnib (0–20 μM), dabrafenib (0–20 μM), or AZD6244 (0–20 μM) for 48 h. Apoptotic cells were analyzed with flow cytometry using annexin V/PI staining. Each treatment was tested in triplicate wells, and apoptosis was assessed as the percentage of annexin V-positive cells.

doi:10.1371/journal.pone.0143847.g003

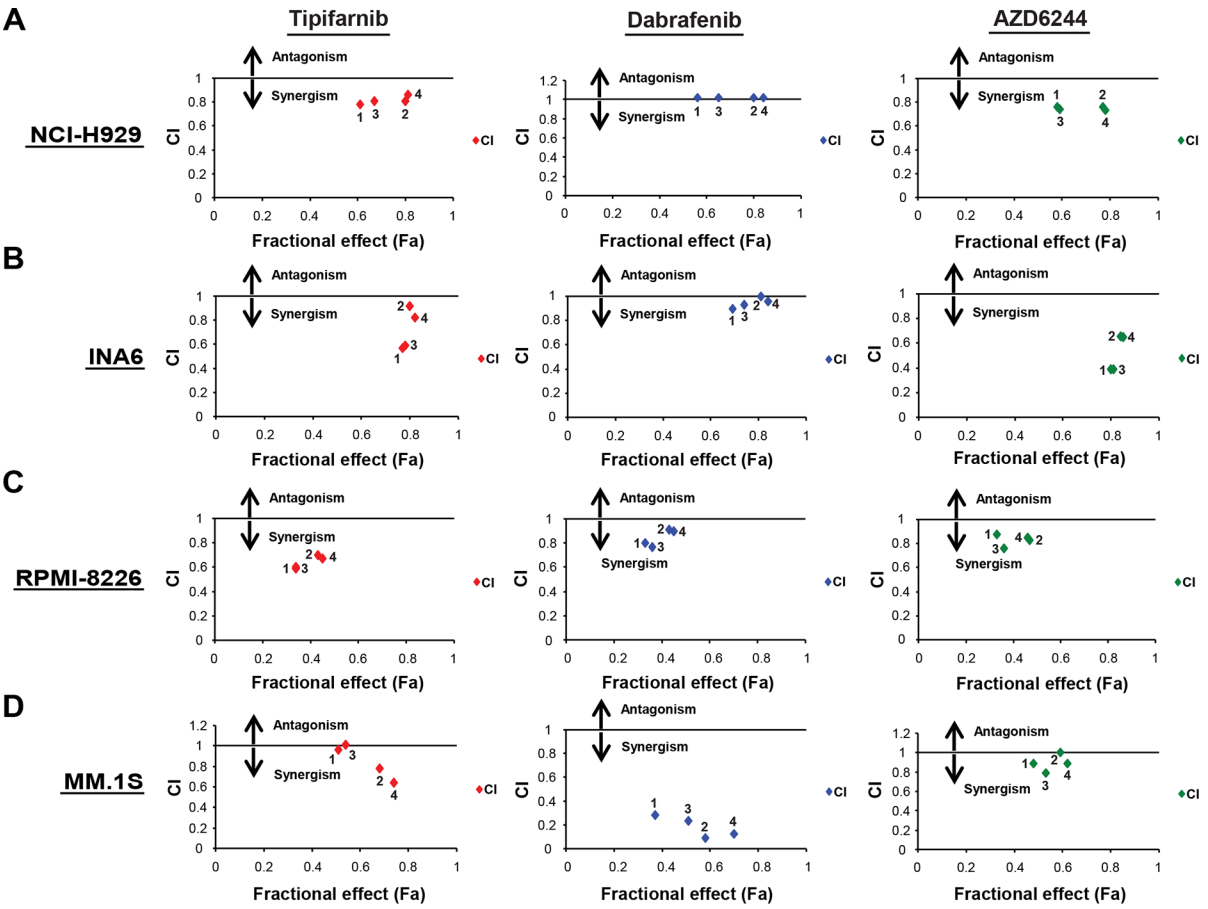
## The combination of TAS-116 and tipifarnib, dabrafenib, or AZD6244 triggers synergistic anti-MM activity

We next assessed the anti-MM effect of TAS-116 in combination with tipifarnib, dabrafenib, or AZD6244 using the MTT assay. The combination of TAS-116 plus one of these inhibitors induced additive or synergistic cytotoxicity in *RAS*-mutated MM cell lines (Fig 4A, 4B, 4C and 4D and S1–S4 Tables). In addition, annexin V/PI staining showed that TAS-116 significantly enhanced apoptosis induced by these inhibitors in *RAS*-mutated MM cell lines ($P < 0.01$ in each combination treatment versus either monotherapy or control in NCI-H929 and MM.1S cell lines; $P < 0.05$ in each combination treatment versus either monotherapy or control in RPMI-8226 cell line; $P < 0.01$ in dabrafenib and AZD6244 combination treatment versus either monotherapy or control, and $P < 0.01$ in tipifarnib combination treatment versus monotherapy or control in INA6 cell line) (Fig 5A).

We next examined the combination effect on the modulation of the RAS-RAF-MEK-ERK signaling pathway cascades in *RAS*-mutated cell lines using western blot analysis. Treatment with RAS-RAF-MEK-ERK inhibitors induced p-C-Raf activation, and AZD6244 treatment induced significant accumulation of p-MEK1/2, which was significantly inhibited by TAS-116. p-ERK induced by tipifarnib was also inhibited by TAS-116. In NCI-H929 cells, treatment with RAS-RAF-MEK-ERK signaling pathway inhibitors upregulated p-Akt, which was also significantly inhibited by TAS-116. These results suggest that TAS-116 markedly inhibits p-C-Raf, p-MEK1/2, and p-ERK, which were paradoxically activated by these inhibitors. Importantly, cleavage of PARP and caspase 3 was significantly enhanced by treatment with TAS-116 in combination with RAS-RAF-MEK-ERK signaling pathway inhibitors in both cell lines. On the other hand, TAS-116 in combination with dabrafenib paradoxically enhanced p-ERK level, possibly via a feedback mechanism. These results indicate with RAS-RAF-MEK-ERK signaling pathway inhibitors can paradoxically trigger RAS-RAF-MEK-ERK signaling pathway activation via a feedback mechanism, and that combination therapy with TAS-116 blocks this response and induces synergistic cytotoxicity and caspase-dependent apoptosis.

Previous studies have reported that RAS-RAF-MEK-ERK pathway inhibition upregulates Bim, which translocates to mitochondria, releases cytochrome-c, and induces caspase-dependent apoptosis [43]. We therefore hypothesized that TAS-116 could enhance mitochondrial damage-induced apoptosis induced by RAS-RAF-MEK-ERK inhibitors. Importantly, TAS-116 or RAS-RAF-MEK-ERK pathway inhibitors upregulate Bim expression, and the combination further enhances Bim expression (S5A Fig). Moreover, TAS-116 also enhances mitochondrial damage-induced apoptosis by RAS-RAF-MEK-ERK pathway inhibitors (S5B Fig). Taken together, these results suggest that TAS-116 enhances apoptosis induced by RAS-RAF-MEK-ERK pathway inhibitors including dabrafenib via mitochondrial damage in MM cells.

We previously showed that the MEK inhibitor AZD6244 targets both MM cells and the BM microenvironment milieu [44]. Thus, we next hypothesized that TAS-116 enhances the AZD6244-induced anti-MM effect even in the BM microenvironment. For this purpose, we employed BMSC culture supernatant to avoid the effect of these agents on BMSCs, which may


A
Tipifarnib
Dabrafenib
AZD6244
NCI-H929
B
INA6
C
RPMI-8226
D
MM.1S
Antagonism
Synergism
CI
Fractional effect (Fa)

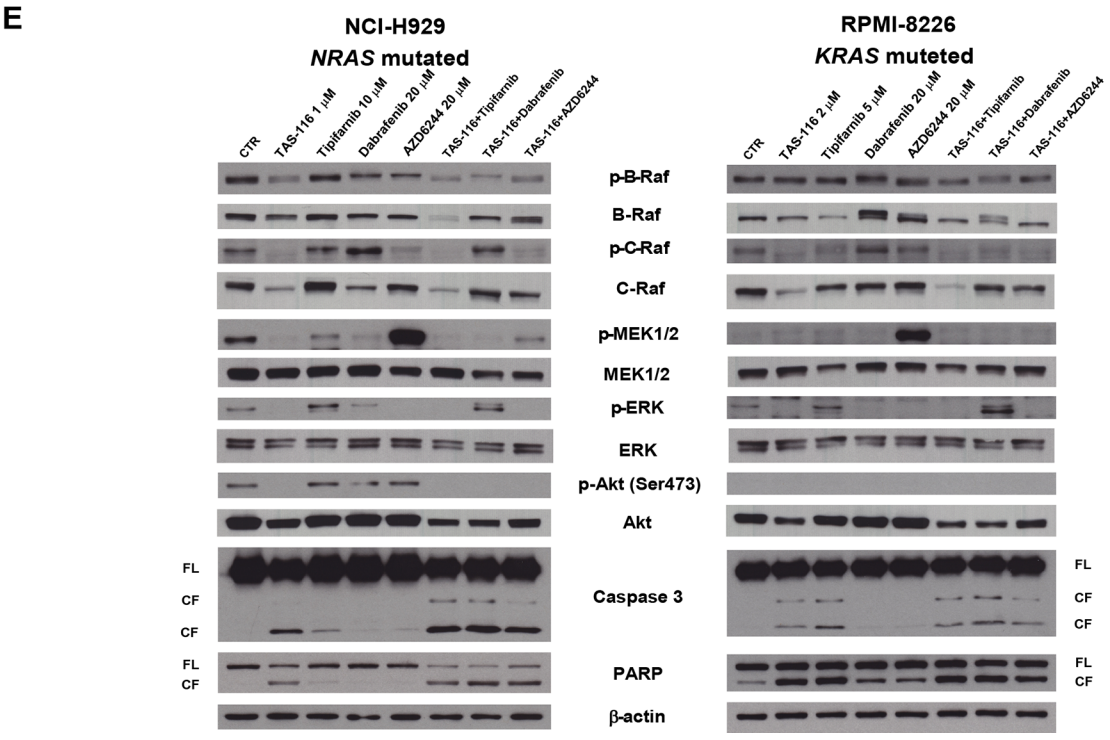

E
NCI-H929
NRAS mutated
RPMI-8226
KRAS muteted
CTR
TAS-116 1 μM
Tipifarnib 10 μM
Dabrafenib 20 μM
AZD6244 20 μM
TAS-116+Tipifarnib
TAS-116+Dabrafenib
TAS-116+AZD6244
TAS-116 2 μM
Tipifarnib 5 μM
p-B-Raf
B-Raf
p-C-Raf
C-Raf
p-MEK1/2
MEK1/2
p-ERK
ERK
p-Akt (Ser473)
Akt
FL
CF
Caspase 3
PARP
β-actin

**Fig 4. Combination of TAS-116 plus tipifarnib, dabrafenib, or AZD6244 triggers synergistic anti-MM activity.** (A) NCI-H929 cells, (B) INA6, (C) RPMI8226, and (D) MM1.S cells were treated with TAS-116 (0–1 μM) in combination with tipifarnib (0–2 μM), dabrafenib (0–5 μM), or AZD6244 (0–4 μM) for 48 h, and then the viability was analyzed with the MTT assay. Isobologram analysis shows the synergistic or additive cytotoxic effect of TAS-116 and each drug. The graphs are derived from the values given in S1 Table (A), S2 Table (B), S3 Table (C), and S4 Table (D). The numbers 1–4 in each graph correspond to the combinations shown in S1 Table. CI values < 1.0 indicate synergism; CI = 1.0 indicate an additive effect; and CI > 1.0 indicates antagonism. (E) NCI-H929 and RPMI-8226 cells were treated with the indicated concentrations of TAS-116 either alone or in combination with tipifarnib, dabrafenib, or AZD6244 for 24 h. Whole-cell lysates were subjected to western blotting using p-B-Raf, B-Raf, p-C-Raf, C-Raf, p-MEK1/2, MEK1/2, p-ERK, ERK, p-Akt (S473), Akt, caspase 3, PARP, and β-actin Abs. FL, full-length; CF, cleaved form.

doi:10.1371/journal.pone.0143847.g004

downregulate growth/anti-apoptotic soluble factors (ie, IL-6, IGF1). Importantly, western blotting showed that TAS-116 in combination with AZD6244 enhanced cleavage of PARP and caspase 3 in *KRAS*-mutated MM.1S cells, in the presence or absence of BMSC supernatant (Fig 5B). Furthermore, annexin V/PI analysis showed that TAS-116 in combination with AZD6244 enhanced apoptosis compared with TAS-116 or AZD6244 monotherapy ($P < 0.01$, respectively, in both NCI-H929 and MM.1S cell lines), even with BMSC supernatant ($P < 0.05$, respectively, in both cell lines) (Fig 5C). Taken together, these results indicate that the combination of TAS-116 and tipifarnib, dabrafenib, or AZD6244 triggers synergistic anti-MM activity, even in the BM microenvironment.

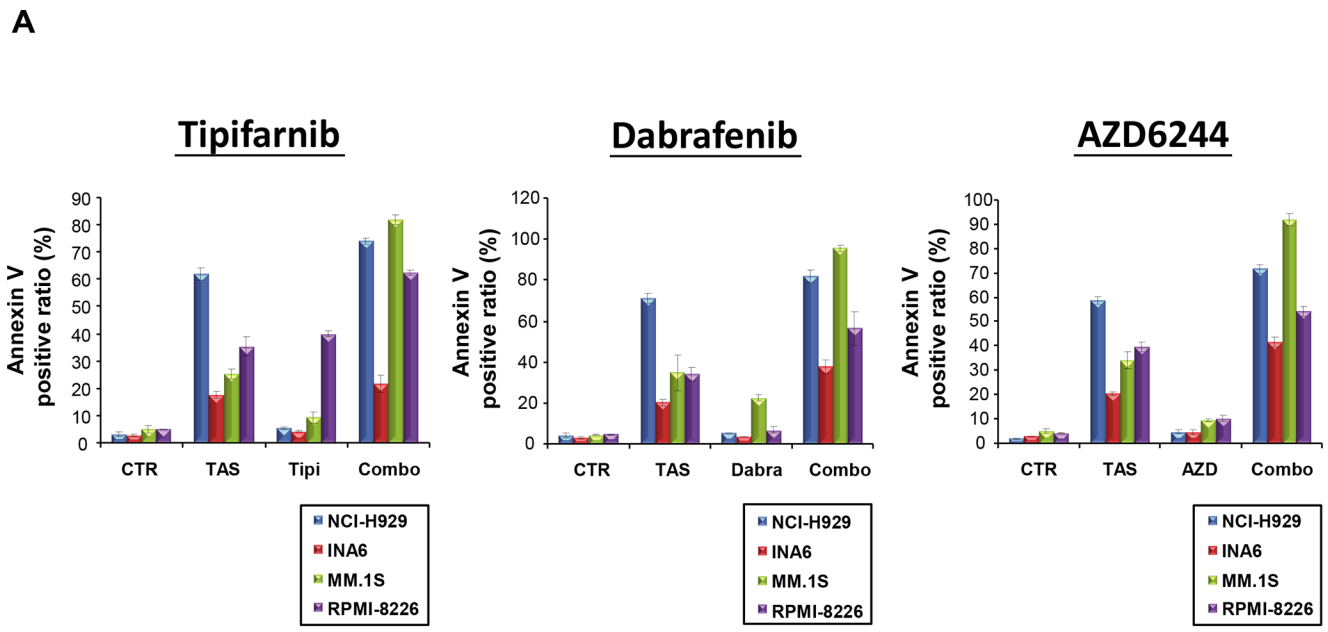
## TAS-116 induces synergistic cytotoxicity with dabrafenib in the *BRAF*-mutated U266 MM cell line

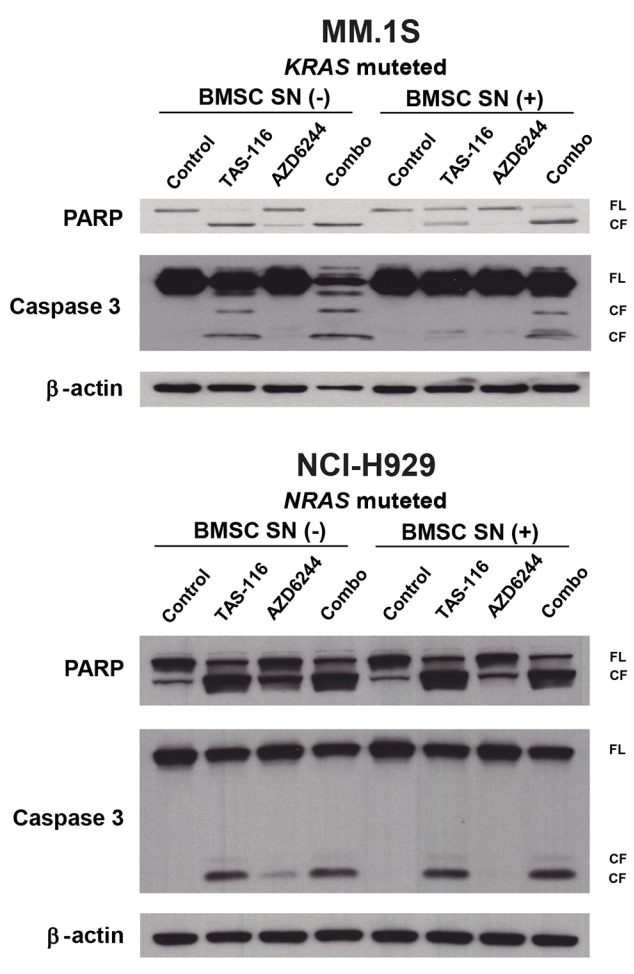
We next examined the cytotoxicity of TAS-116 or dabrafenib in the *BRAF*-mutated (K601N) U266 MM cell line using the MTT assay. TAS-116 or dabrafenib significantly inhibited the viability of U266 MM cells in a dose- and time-dependent manner (Fig 6A and 6B). TAS-116 in combination with dabrafenib induced synergistic cytotoxicity in the U266 MM cell line, evidenced by MTT assay (Fig 6C). In addition, annexin V/PI staining showed that TAS-116 significantly enhanced apoptosis induced by dabrafenib in the U266 MM cell line ($P < 0.001$ in the combination treatment versus TAS-116 monotherapy; $P < 0.01$ in the combination treatment versus dabrafenib monotherapy) (Fig 6D). Western blot analysis showed that TAS-116 markedly inhibited p-C-Raf, which was paradoxically activated by dabrafenib in U266 MM cells (Fig 6E). Importantly, TAS-116 and dabrafenib synergistically inhibited p-MEK1/2 and p-ERK (Fig 6E). Furthermore, cleavage of PARP and caspase 3 was significantly enhanced by TAS-116 in combination with dabrafenib (Fig 6E). Taken together, these results indicate that TAS-116 enhances dabrafenib-induced MM cytotoxicity and apoptosis, even in the *BRAF*-mutated U266 MM cells.

## Discussion

The RAS-RAF-MEK-ERK signaling pathway plays a crucial role in tumorigenesis, cell proliferation, inhibition of apoptosis, and drug resistance [45,46]. Furthermore, *RAS* mutations may play a key role in malignant transformation of clonal plasma cells and MM pathogenesis [23]. In particular, other groups have shown that *NRAS* or *KRAS* mutations confer resistance to conventional or molecularly-targeted therapies in various malignancies, including MM [25,47–49]. Therefore, a novel therapy that overcomes these mechanisms of resistance is needed. In the present study, we focused on the combination of the novel, oral, selective HSP90α/β inhibitor TAS-116 and RAS-RAF-MEK-ERK inhibitors in *RAS*-mutated or *BRAF*-mutated MM cells.

Our previous studies have shown that TAS-116 more potently and significantly targets HSP90 client proteins including C-Raf and MEK1/2 in MM cell lines than 17-AAG [16]. Importantly, we and others have previously reported that *NRAS* mutation significantly reduces


A
Tipifarnib
Dabrafenib
AZD6244
Annexin V positive ratio (%)
CTR
TAS
Tipi
Dabra
AZD
Combo
NCI-H929
INA6
MM.1S
RPMI-8226


B
MM.1S
KRAS muteted
BMSC SN (-)
BMSC SN (+)
Control
TAS-116
AZD6244
Combo
PARP
Caspase 3
β-actin
FL
CF
NCI-H929
NRAS mutated

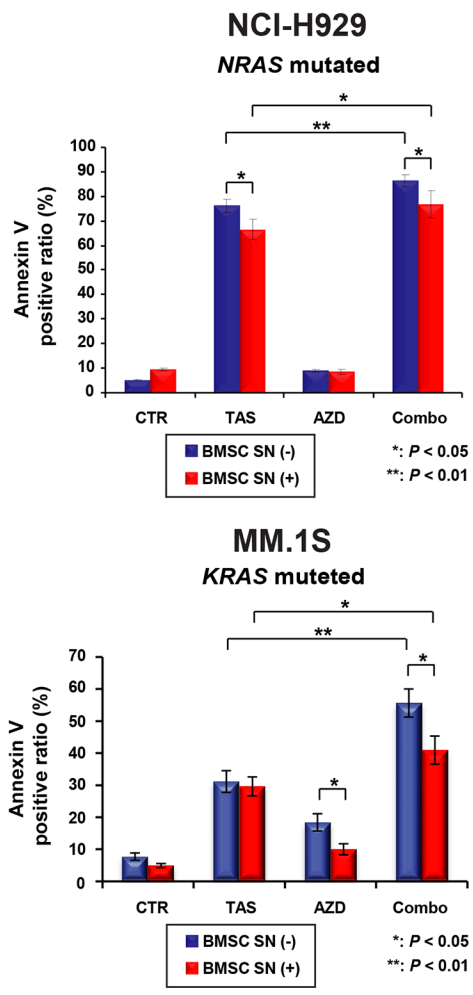

C
NCI-H929
NRAS mutated
Annexin V positive ratio (%)
CTR
TAS
AZD
Combo
BMSC SN (-)
BMSC SN (+)
*: P < 0.05
**: P < 0.01
MM.1S
KRAS muteted

**Fig 5. Combination of TAS-116 plus tipifarnib, dabrafenib, or AZD6244 blocks the growth stimulatory effect of the bone marrow microenvironment.** (A) NCI-H929, INA6, MM.1S, and RPMI-8226 cells were treated with TAS-116 (1 μM) either alone or in combination with tipifarnib (NCI-H929: 2 μM, INA6: 0.5 μM, MM.1S: 2 μM, RPMI-8226: 2 μM), dabrafenib (NCI-H929: 10 μM, INA6: 2 μM, MM.1S: 10 μM, RPMI-8226: 10 μM), or AZD6244 (NCI-H929: 10 μM, INA6: 2 μM, MM.1S: 20 μM, RPMI-8226: 20 μM) for 48 h. Apoptotic cells were analyzed with flow cytometry using annexin V/PI staining. Each treatment was tested in triplicate wells, and apoptosis was assessed as the percentage of annexin V-positive cells. TAS, TAS-116; Tipi, tipifarnib; Dabra, dabrafenib; AZD, AZD6244. (B) MM.1S and NCI-H929 cells were cultured with TAS-116 (2 μM), AZD6244 (20 μM), or TAS-116 plus AZD6244 for 24 h in the presence or absence of BMSC supernatant. Whole-cell lysates were subjected to western blotting using PARP, caspase 3, and β-actin Abs. FL, full-length; CF, cleaved form; SN, supernatant. (C) MM.1S cells were cultured with TAS-116 (1 μM), AZD6244 (20 μM), or TAS-116 plus AZD6244; and NCI-H929 cells were cultured with TAS-116 (1 μM), AZD6244 (10 μM), or TAS-116 plus AZD6244 for 48 h in the presence or absence of BMSC supernatant. Apoptotic cells were analyzed with flow cytometry using annexin V/PI staining. Each treatment was tested in triplicate wells, and apoptosis was assessed as the percentage of annexin V-positive cells. TAS, TAS-116; AZD, AZD6244; SN, supernatant (*: $P < 0.05$; **: $P < 0.01$).

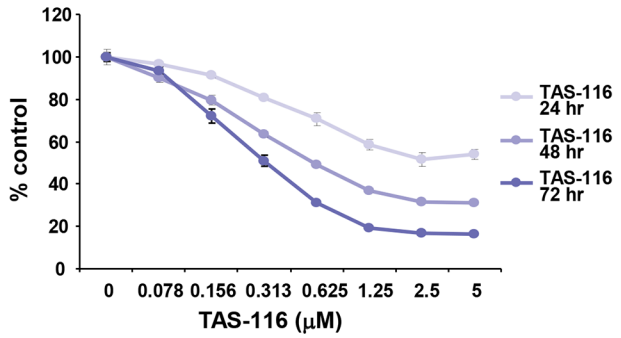
doi:10.1371/journal.pone.0143847.g005

MM sensitivity to single-agent bortezomib therapy [25]. Patients with *RAS* mutations also have significantly shorter progression-free and overall survival than patients without this mutation [24]. In this study, we demonstrated that TAS-116 potently induces cytotoxicity and significantly inhibits RAS-RAF-MEK-ERK pathway client proteins in *NRAS*- or *KRAS*-mutated MM cell lines. These results suggest that TAS-116 potently overcomes the resistance mechanism induced by *RAS* mutations.

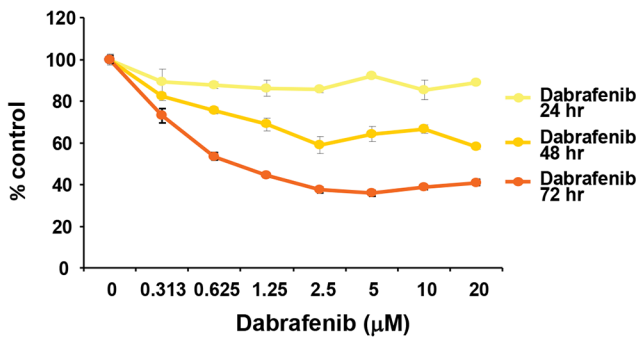
The RAS-RAF-MEK-ERK pathway is activated in a variety of tumors, including MM [20,50]; therefore, RAS-RAF-MEK-ERK pathway inhibitors are promising therapeutic options for cancer therapy. However, inhibition of the RAS-RAF-MEK-ERK pathway may be mainly cytostatic [20] and show significant efficacy primarily at an early stage of treatment, since resistance develops due to activation of other signaling pathways via feedback mechanisms or paradoxical activation mechanisms [51]. Therefore, there is a need to combine inhibitors of the RAS-RAF-MEK-ERK with a drug to target and overcome these resistance mechanisms. In the present study, we show that TAS-116 triggers significant synergistic anti-MM effects when used in combination with a RAS-RAF-MEK-ERK pathway inhibitor. Importantly, TAS-116 inhibited the paradoxical activation of p-C-Raf, p-MEK, or p-ERK triggered by these inhibitors, and markedly enhanced RAS-RAF-MEK-ERK pathway inhibitor-induced apoptosis. Moreover, this enhanced apoptosis in *RAS*-mutated MM triggered by combination treatment was observed even in the presence of BMSCs. Taken together, our results provide the rationale for a novel treatment strategy combining selective HSP90α/β inhibitor and RAS-RAF-MEK-ERK signaling pathway inhibitors to improve patient outcome in *RAS*-mutated MM.

Other groups have shown that dabrafenib shows significant *in vitro* and *in vivo* anti-tumor effects in malignant melanoma with the *BRAF V600E* mutation. However, the therapeutic effects are often temporary due to development of several resistance mechanisms [40]. One mechanism is ERK signaling resistant to treatment with RAF inhibitors due to increased RAF dimers in cells, and another involves bypassing the dependence of the tumor on mutant RAF. Moreover, tumors with wild-type BRAF, including those with *RAS* mutation, exhibit a paradoxical activation of ERK signaling when treated with these drugs [28,40,41]. Importantly, many proteins in the RAS-RAF-MEK-ERK pathway are client proteins of HSP90; therefore, a combination of TAS-116 and a RAS-RAF-MEK-ERK pathway inhibitor may abrogate the feedback mechanisms that promote resistance to dabrafenib. In the present study, we focused on whether TAS-116 induces a synergistic effect or overcomes these resistance mechanisms when combined with dabrafenib to treat MM cells with *RAS* and *BRAF* mutations. We show that TAS-116 induced synergistic anti-MM cytotoxicity and enhanced dabrafenib-induced apoptosis in *RAS*-mutated MM cell lines. However, TAS-116 paradoxically enhanced p-ERK when combined with dabrafenib,suggesting that MEK-ERK signaling may not contribute to MM cell growth inhibition triggered by this combination treatment. Because HSP90 inhibitors block multiple pathways crucial to MM survival [7,9,20], the combination of TAS-116 and

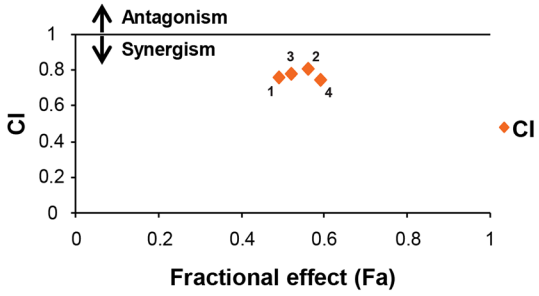
A




B

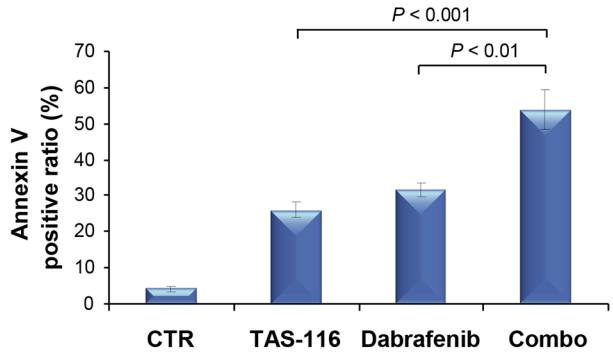



C




| Combination dosage number | Dabrafenib (μM) | TAS-116 (μM) | Fa (Combination) | CI |
|---|---|---|---|---|
| 1 | 1 | 0.5 | 0.49 | 0.76 |
| 2 | 1 | 1 | 0.56 | 0.81 |
| 3 | 2 | 0.5 | 0.52 | 0.78 |
| 4 | 2 | 1 | 0.59 | 0.75 |

D




E

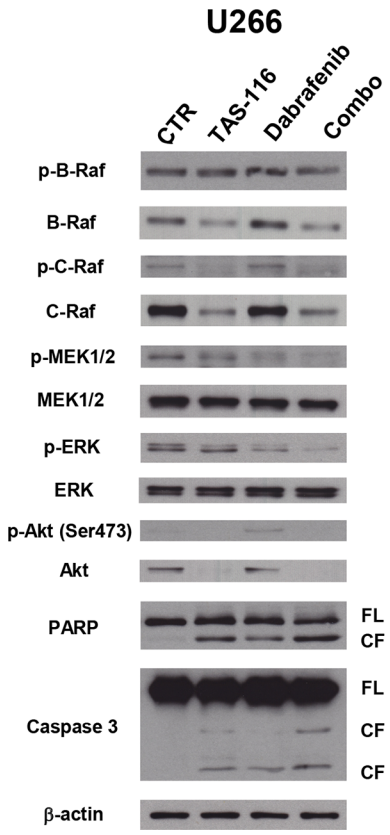


**Fig 6. TAS-116 induces synergistic cytotoxicity with dabrafenib in the BRAF-mutated U266 MM cell line.** U266 cells were cultured with TAS-116 (0–5 μM) (A) or dabrafenib (0–5 μM) (B) for 24, 48, or 72 h. Cell viability was assessed with the MTT assay of triplicate cultures and expressed as the percentage of the untreated control. Data are the mean ± SD. (C) U266 cells were treated with the indicated concentrations of TAS-116, dabrafenib, or TAS-116 plus dabrafenib for 48 h, and then the viability was analyzed with the MTT assay. Isobologram analysis shows the synergistic cytotoxic effect of TAS-116 and dabrafenib. The graph (left) is derived from the values given in the table (right). The numbers 1–4 in the graph correspond to the combinations shown in the table. CI values < 1.0 indicate synergism; CI = 1.0 indicates an additive effect; and CI > 1.0 indicates antagonism. (D) U266 cells were treated with TAS-116 (0.5 μM), dabrafenib (1 μM), or TAS-116 plus dabrafenib for 48 h. Apoptotic cells were analyzed with flow cytometry using annexin V/PI staining. Each treatment was tested in triplicate wells, and apoptosis was assessed as the percentage of annexin V-positive cells. (E) U266 cells were treated with TAS-116 (1 μM), dabrafenib (2 μM), or TAS-116 plus dabrafenib for 24 h. Whole-cell lysates were subjected to western blotting using p-B-Raf, B-Raf, p-C-Raf, C-Raf, p-MEK1/2, MEK1/2, p-ERK, ERK, p-Akt (S473), Akt, PARP, caspase 3, and β-actin Abs. FL, full-length; CF, cleaved form.

doi:10.1371/journal.pone.0143847.g006

dabrafenib enhances the anti-MM effect in *RAS*-mutated and wild-type *BRAF* MM cells via a mechanism other than ERK pathway inhibition.

Whole-genome sequencing data have recently revealed that a subset of patients carry an activating mutation (V600E) in the BRAF kinase [52]. In addition, other groups recently reported a significant response to the BRAF inhibitor vemurafenib in *BRAF V600E*-mutated MM patients in the clinical setting [52,53]. In the laboratory setting, BRAF inhibition also significantly downregulates MEK-ERK pathway activity in *BRAF K601N*-mutated U266 MM cells [54]. Therefore, these data indicate that *BRAF* mutation is a promising target for MM therapy. However, several mechanisms of resistance to RAF inhibitors have been proposed [40], and several combinatorial therapy approaches have already been pursued to improve the outcome of patients with BRAF V600E melanomas treated with BRAF inhibitors [40]. In the present study, we focused on the significance of a combination of TAS-116 and dabrafenib. TAS-116 triggered significant synergistic anti-MM effects and enhanced apoptosis induced by dabrafenib in the U266 MM cell line. Importantly, TAS-116 enhanced the inhibition of p-MEK1/2 or p-ERK induced by dabrafenib. Moreover, TAS-116 markedly inhibited p-C-Raf or p-Akt, which were paradoxically activated by dabrafenib in the U266 MM cell line. Taken together, these results suggest that the combination of TAS-116 and dabrafenib has a synergistic anti-MM effect, even in *BRAF*-mutated MM cells.

In conclusion, TAS-116 in combination with a RAS-RAF-MEK-ERK signaling pathway inhibitor shows significant synergistic anti-MM effects in *RAS*- or *BRAF*-mutated MM cell lines *in vitro*, providing the framework for its clinical evaluation to improve the outcome of this subset of patients with MM.

## Supporting Information

**S1 Fig. TAS-116 is a novel, oral, selective HSP90α/β inhibitor.** Chemical structure of TAS-116.
(EPS)

**S2 Fig. RAS knockdown induces cell cycle modulation and enhances cytotoxicity induced by bortezomib and doxorubicin in *RAS*-mutated MM cells.** NCI-H929 and KMS11 cells were transiently transfected with non-targeting or *NRAS* siRNA and then treated with or without bortezomib (0–10 nM) or doxorubicin (0–400 nM) for 48 h. In each case, cell viability was assessed with the MTT assay of triplicate cultures and expressed as the percentage of the untreated control. Data are the mean ± SD.
(EPS)

**S3 Fig. TAS-116 triggered a decrease in mitochondria transmembrane potential.** MM.1S cells were treated with or without 2 μM TAS-116 for 4, 8, or 24 h, with addition of MitoCapture reagent (MitoCapture Apoptosis Detection kit, Calbiochem) for the last 20 minutes, followed

by flow cytometric analysis.
(EPS)

**S4 Fig. The RAF inhibitor dabrafenib induces paradoxical activation of ERK signaling in *RAS*-mutated MM cells.** NCI-H929 and RPMI-8226 cells were treated with the indicated concentrations of dabrafenib for 24 h. Whole-cell lysates were subjected to western blotting using p-B-Raf, B-Raf, p-C-Raf, C-Raf, p-MEK1/2, MEK1/2, p-ERK, ERK, and β-actin Abs.
(EPS)

**S5 Fig. TAS-116 enhances mitochondrial apoptosis induced by RAS-RAF-MEK-ERK pathway inhibitors.** (A) NCI-H929 and RPMI-8226 cells were treated with the indicated concentrations of TAS-116 either alone or in combination with tipifarnib, dabrafenib, or AZD6244 for 24 h. Whole-cell lysates were subjected to western blotting using Bim and α-tubulin Abs. (B) NCI-H929 cells were treated with the indicated concentrations of TAS-116 either alone or in combination with tipifarnib, dabrafenib, or AZD6244 for 12 h, with addition of MitoCapture reagent (MitoCapture Apoptosis Detection kit, Calbiochem) for the last 20 minutes, followed by flow cytometric analysis. MFI, mean fluorescent intensity.
(EPS)

**S1 Table. TAS-116 combination indices (CI) with tipifarnib, dabrafenib, or AZD6244 in NCI-H929 cells.** The tables correspond to the Fa and CI values in Fig 4A. CI was calculated using CompuSyn software.
(EPS)

**S2 Table. TAS-116 combination indices (CI) with tipifarnib, dabrafenib, or AZD6244 in INA6 cells.** The tables correspond to the Fa and CI values in Fig 4B. CI was calculated using CompuSyn software.
(EPS)

**S3 Table. TAS-116 combination indices (CI) with tipifarnib, dabrafenib, or AZD6244 in RPMI-8226 cells.** The tables correspond to the Fa and CI values in Fig 4C. CI was calculated using CompuSyn software.
(EPS)

**S4 Table. TAS-116 combination indices (CI) with tipifarnib, dabrafenib, or AZD6244 in MM.1S cells.** The tables correspond to the Fa and CI values in Fig 4D. CI was calculated using CompuSyn software.
(EPS)

## Author Contributions

Conceived and designed the experiments: RS SK T. Harada D. Cirstea JJ YTT D. Chauhan KA TU T. Hideshima KCA. Performed the experiments: RS SK T. Harada N. Mimura JM HO YY MS GG FC T. Hideshima. Analyzed the data: RS SK T. Harada D. Chauhan PGR N. Munshi T. Hideshima KCA. Wrote the paper: RS SK T. Harada T. Hideshima KCA.

## References

1. Palumbo A, Anderson K (2011) Multiple myeloma. N Engl J Med 364: 1046–1060. doi: 10.1056/NEJMra1011442 PMID: 21410373
2. Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson KC (2009) Multiple myeloma. Lancet 374: 324–339. doi: 10.1016/S0140-6736(09)60221-X PMID: 19541364
3. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, et al. (2008) Improved survival in multiple myeloma and the impact of novel therapies. Blood 111: 2516–2520. PMID: 17975015

4. Neckers L, Workman P (2012) Hsp90 molecular chaperone inhibitors: are we there yet? Clin Cancer Res 18: 64–76. doi: 10.1158/1078-0432.CCR-11-1000 PMID: 22215907
5. Garcia-Carbonero R, Carnero A, Paz-Ares L (2013) Inhibition of HSP90 molecular chaperones: moving into the clinic. Lancet Oncol 14: e358–369. doi: 10.1016/S1470-2045(13)70169-4 PMID: 23896275
6. Whitesell L, Lindquist SL (2005) HSP90 and the chaperoning of cancer. Nat Rev Cancer 5: 761–772. PMID: 16175177
7. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Kung AL, Davies FE, et al. (2006) Antimyeloma activity of heat shock protein-90 inhibition. Blood 107: 1092–1100. PMID: 16234364
8. Zhang L, Fok JH, Davies FE (2014) Heat shock proteins in multiple myeloma. Oncotarget 5: 1132–1148. PMID: 24675290
9. Khong T, Spencer A (2011) Targeting HSP 90 induces apoptosis and inhibits critical survival and proliferation pathways in multiple myeloma. Mol Cancer Ther 10: 1909–1917. doi: 10.1158/1535-7163.MCT-11-0174 PMID: 21859842
10. Barrott JJ, Haystead TA (2013) Hsp90, an unlikely ally in the war on cancer. FEBS J 280: 1381–1396. doi: 10.1111/febs.12147 PMID: 23356585
11. Banerji U, O'Donnell A, Scurr M, Pacey S, Stapleton S, Asad Y, et al. (2005) Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J Clin Oncol 23: 4152–4161. PMID: 15961763
12. Grem JL, Morrison G, Guo XD, Agnew E, Takimoto CH, Thomas R, et al. (2005) Phase I and pharmacologic study of 17-(allylamino)-17-demethoxygeldanamycin in adult patients with solid tumors. J Clin Oncol 23: 1885–1893. PMID: 15774780
13. Scaltriti M, Dawood S, Cortes J (2012) Molecular pathways: targeting hsp90—who benefits and who does not. Clin Cancer Res 18: 4508–4513. doi: 10.1158/1078-0432.CCR-11-2138 PMID: 22718860
14. Rajan A, Kelly RJ, Trepel JB, Kim YS, Alarcon SV, Kummar S, et al. (2011) A phase I study of PF-04929113 (SNX-5422), an orally bioavailable heat shock protein 90 inhibitor, in patients with refractory solid tumor malignancies and lymphomas. Clin Cancer Res 17: 6831–6839. doi: 10.1158/1078-0432.CCR-11-0821 PMID: 21908572
15. Okawa Y, Hideshima T, Steed P, Vallet S, Hall S, Huang K, et al. (2009) SNX-2112, a selective Hsp90 inhibitor, potently inhibits tumor cell growth, angiogenesis, and osteoclastogenesis in multiple myeloma and other hematologic tumors by abrogating signaling via Akt and ERK. Blood 113: 846–855. doi: 10.1182/blood-2008-04-151928 PMID: 18948577
16. Suzuki R, Hideshima T, Mimura N, Minami J, Ohguchi H, Kikuchi S, et al. (2015) Anti-tumor activities of selective HSP90alpha/beta inhibitor, TAS-116, in combination with bortezomib in multiple myeloma. Leukemia 29: 510–514. doi: 10.1038/leu.2014.300 PMID: 25306900
17. Ohkubo S, Kodama Y, Muraoka H, Hitotsumachi H, Yoshimura C, Kitade M, et al. (2015) TAS-116, a Highly Selective Inhibitor of Heat Shock Protein 90alpha and beta, Demonstrates Potent Antitumor Activity and Minimal Ocular Toxicity in Preclinical Models. Mol Cancer Ther 14: 14–22. doi: 10.1158/1535-7163.MCT-14-0219 PMID: 25416789
18. Nakashima T, Ishii T, Tagaya H, Seike T, Nakagawa H, Kanda Y, et al. (2010) New molecular and biological mechanism of antitumor activities of KW-2478, a novel nonansamycin heat shock protein 90 inhibitor, in multiple myeloma cells. Clin Cancer Res 16: 2792–2802. doi: 10.1158/1078-0432.CCR-09-3112 PMID: 20406843
19. Montagut C, Settleman J (2009) Targeting the RAF-MEK-ERK pathway in cancer therapy. Cancer Lett 283: 125–134. doi: 10.1016/j.canlet.2009.01.022 PMID: 19217204
20. Chang-Yew Leow C, Gerondakis S, Spencer A (2013) MEK inhibitors as a chemotherapeutic intervention in multiple myeloma. Blood Cancer J 3: e105. doi: 10.1038/bcj.2013.1 PMID: 23524590
21. Yasui H, Hideshima T, Richardson PG, Anderson KC (2006) Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. Br J Haematol 132: 385–397. PMID: 16412014
22. Hideshima T, Anderson KC (2011) Novel therapies in MM: from the aspect of preclinical studies. Int J Hematol 94: 344–354. doi: 10.1007/s12185-011-0917-5 PMID: 21881879
23. Chng WJ, Huang GF, Chung TH, Ng SB, Gonzalez-Paz N, Troska-Price T, et al. (2011) Clinical and biological implications of MYC activation: a common difference between MGUS and newly diagnosed multiple myeloma. Leukemia 25: 1026–1035. doi: 10.1038/leu.2011.53 PMID: 21468039
24. Chng WJ, Gonzalez-Paz N, Price-Troska T, Jacobus S, Rajkumar SV, Oken MM, et al. (2008) Clinical and biological significance of RAS mutations in multiple myeloma. Leukemia 22: 2280–2284. doi: 10.1038/leu.2008.142 PMID: 18528420
25. Mulligan G, Lichter DI, Di Bacco A, Blakemore SJ, Berger A, Koenig E, et al. (2014) Mutation of NRAS but not KRAS significantly reduces myeloma sensitivity to single-agent bortezomib therapy. Blood 123: 632–639. doi: 10.1182/blood-2013-05-504340 PMID: 24335104

26. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. (2014) Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. N Engl J Med 371: 1877–1888. doi: 10.1056/NEJMoa1406037 PMID: 25265492

27. Johnson DB, Flaherty KT, Weber JS, Infante JR, Kim KB, Kefford RF, et al. (2014) Combined BRAF (Dabrafenib) and MEK inhibition (Trametinib) in patients with BRAFV600-mutant melanoma experiencing progression with single-agent BRAF inhibitor. J Clin Oncol 32: 3697–3704. doi: 10.1200/JCO.2014.57.3535 PMID: 25287827

28. Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N (2010) RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature 464: 427–430. doi: 10.1038/nature08902 PMID: 20179705

29. Spagnolo F, Ghiorzo P, Queirolo P (2014) Overcoming resistance to BRAF inhibition in BRAF-mutated metastatic melanoma. Oncotarget 5: 10206–10221. PMID: 25344914

30. Shi H, Hugo W, Kong X, Hong A, Koya RC, Moriceau G, et al. (2014) Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. Cancer Discov 4: 80–93. doi: 10.1158/2159-8290.CD-13-0642 PMID: 24265155

31. Acquaviva J, Smith DL, Sang J, Friedland JC, He S, Sequeira M, et al. (2012) Targeting KRAS-mutant non-small cell lung cancer with the Hsp90 inhibitor ganetespib. Mol Cancer Ther 11: 2633–2643. doi: 10.1158/1535-7163.MCT-12-0615 PMID: 23012248

32. Hideshima T, Catley L, Yasui H, Ishitsuka K, Raje N, Mitsiades C, et al. (2006) Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. Blood 107: 4053–4062. PMID: 16418332

33. Hideshima T, Neri P, Tassone P, Yasui H, Ishitsuka K, Raje N, et al. (2006) MLN120B, a novel IkappaB kinase beta inhibitor, blocks multiple myeloma cell growth in vitro and in vivo. Clin Cancer Res 12: 5887–5894. PMID: 17020997

34. Cirstea D, Hideshima T, Rodig S, Santo L, Pozzi S, Vallet S, et al. (2010) Dual inhibition of akt/mammalian target of rapamycin pathway by nanoparticle albumin-bound-rapamycin and perifosine induces antitumor activity in multiple myeloma. Mol Cancer Ther 9: 963–975. doi: 10.1158/1535-7163.MCT-09-0763 PMID: 20371718

35. Yasui H, Hideshima T, Raje N, Roccaro AM, Shiraishi N, Kumar S, et al. (2005) FTY720 induces apoptosis in multiple myeloma cells and overcomes drug resistance. Cancer Res 65: 7478–7484. PMID: 16103102

36. Meloche S, Pouyssegur J (2007) The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene 26: 3227–3239. PMID: 17496918

37. Suzuki T, J KT, Ajima R, Nakamura T, Yoshida Y, Yamamoto T (2002) Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. Genes Dev 16: 1356–1370. PMID: 12050114

38. Mirza AM, Gysin S, Malek N, Nakayama K, Roberts JM, McMahon M (2004) Cooperative regulation of the cell division cycle by the protein kinases RAF and AKT. Mol Cell Biol 24: 10868–10881. PMID: 15572689

39. Hoang B, Zhu L, Shi Y, Frost P, Yan H, Sharma S, et al. (2006) Oncogenic RAS mutations in myeloma cells selectively induce cox-2 expression, which participates in enhanced adhesion to fibronectin and chemoresistance. Blood 107: 4484–4490. PMID: 16497971

40. Lito P, Rosen N, Solit DB (2013) Tumor adaptation and resistance to RAF inhibitors. Nat Med 19: 1401–1409. doi: 10.1038/nm.3392 PMID: 24202393

41. Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, et al. (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 140: 209–221. doi: 10.1016/j.cell.2009.12.040 PMID: 20141835

42. Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, et al. (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 464: 431–435. doi: 10.1038/nature08833 PMID: 20130576

43. Ding H, McDonald JS, Yun S, Schneider PA, Peterson KL, Flatten KS, et al. (2014) Farnesyltransferase inhibitor tipifarnib inhibits Rheb prenylation and stabilizes Bax in acute myelogenous leukemia cells. Haematologica 99: 60–69. doi: 10.3324/haematol.2013.087734 PMID: 23996484

44. Tai YT, Fulciniti M, Hideshima T, Song W, Leiba M, Li XF, et al. (2007) Targeting MEK induces myeloma-cell cytotoxicity and inhibits osteoclastogenesis. Blood 110: 1656–1663. PMID: 17510321

45. Zhao Y, Adjei AA (2014) The clinical development of MEK inhibitors. Nat Rev Clin Oncol 11: 385–400. doi: 10.1038/nrclinonc.2014.83 PMID: 24840079

46. Martz CA, Ottina KA, Singleton KR, Jasper JS, Wardell SE, Peraza-Penton A, et al. (2014) Systematic identification of signaling pathways with potential to confer anticancer drug resistance. Sci Signal 7: ra121. doi: 10.1126/scisignal.aaa1877 PMID: 25538079

47. Hata AN, Yeo A, Faber AC, Lifshits E, Chen Z, Cheng KA, et al. (2014) Failure to induce apoptosis via BCL-2 family proteins underlies lack of efficacy of combined MEK and PI3K inhibitors for KRAS-mutant lung cancers. Cancer Res 74: 3146–3156. doi: 10.1158/0008-5472.CAN-13-3728 PMID: 24675361

48. Nakadate Y, Kodera Y, Kitamura Y, Shirasawa S, Tachibana T, Tamura T, et al. (2014) KRAS mutation confers resistance to antibody-dependent cellular cytotoxicity of cetuximab against human colorectal cancer cells. Int J Cancer 134: 2146–2155. PMID: 24136682

49. Peeters M, Douillard JY, Van Cutsem E, Siena S, Zhang K, Williams R, et al. (2013) Mutant KRAS codon 12 and 13 alleles in patients with metastatic colorectal cancer: assessment as prognostic and predictive biomarkers of response to panitumumab. J Clin Oncol 31: 759–765. doi: 10.1200/JCO.2012.45.1492 PMID: 23182985

50. Gollob JA, Wilhelm S, Carter C, Kelley SL (2006) Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduction pathway. Semin Oncol 33: 392–406. PMID: 16890795

51. Lin L, Sabnis AJ, Chan E, Olivas V, Cade L, Pazarentzos E, et al. (2015) The Hippo effector YAP promotes resistance to RAF- and MEK-targeted cancer therapies. Nat Genet.

52. Andrulis M, Lehners N, Capper D, Penzel R, Heining C, Huellein J, et al. (2013) Targeting the BRAF V600E mutation in multiple myeloma. Cancer Discov 3: 862–869. doi: 10.1158/2159-8290.CD-13-0014 PMID: 23612012

53. Sharman JP, Chmielecki J, Morosini D, Palmer GA, Ross JS, Stephens PJ, et al. (2014) Vemurafenib response in 2 patients with posttransplant refractory BRAF V600E-mutated multiple myeloma. Clin Lymphoma Myeloma Leuk 14: e161–163. doi: 10.1016/j.clml.2014.06.004 PMID: 24997557

54. Lohr JG, Stojanov P, Carter SL, Cruz-Gordillo P, Lawrence MS, Auclair D, et al. (2014) Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. Cancer Cell 25: 91–101. doi: 10.1016/j.ccr.2013.12.015 PMID: 24434212