Annals of Hematology (2019) 98:723–733
https://doi.org/10.1007/s00277-018-3547-7

ORIGINAL ARTICLE

Copanlisib, a novel phosphoinositide 3-kinase inhibitor, combined with carfilzomib inhibits multiple myeloma cell proliferation

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Received: 5 January 2018 / Accepted: 6 November 2018 / Published online: 15 November 2018

Abstract

Multiple myeloma (MM) is a uniformly fatal disorder of B cells characterized by the accumulation of abnormal plasma cells. Phosphoinositide 3-kinase (PI3K) signaling pathways play a critical regulatory role in MM pathology. Copanlisib, also known as BAY80-6946, is a potent PI3Kα and δ inhibitor. In this study, we investigated the efficacy of copanlisib and a proteasome inhibitor using MM cell lines and primary samples. The p110α and δ catalytic subunits of the class PI3K increased, and carfilzomib activity reduced in the presence of a supernatant from the feeder cell line, HS-5. Phosphorylation of Akt and activation of caspase 3 and poly (ADP-ribose) polymerase (PARP) partially reduced upon carfilzomib treatment in the presence of HS-5. Apoptosis also decreased. Copanlisib treatment for 72 h inhibited growth in MM cell lines and induced apoptosis. Combination treatment of MM cells with carfilzomib and copanlisib caused greater cytotoxicity than that caused by either drug alone and increased apoptosis. Caspase 3 activity increased while that of Akt decreased after combination treatment with copanlisib and carfilzomib. Further, copanlisib inhibited vascular endothelial growth factor (VEGF)-mediated angiogenesis in vitro and in vivo. It also inhibited C-X-C motif chemokine 12 (CXCL12)-mediated chemotaxis. The data suggest that administration of the PI3K inhibitor, copanlisib, may be a powerful strategy against stroma-associated drug resistance of MM cells and can enhance the cytotoxic effects of proteasome inhibitors in such residual MM cells.

Keywords Multiple myeloma · PI3K · p110δ · Proteasome inhibitor · Feeder cell

Introduction

Multiple myeloma (MM) is a hematological malignancy that affects plasma cells [1]. Myeloma cells are found in the bone marrow; however, myeloma can cause many complications such as anemia, kidney failure, infections, and hypercalcemia [2]. The treatment of MM patients has been dramatically changed by new agents such as proteasome inhibitors (e.g., bortezomib and carfilzomib) and immunomodulatory drugs (thalidomide and lenalidomide), high-dose chemotherapy, and autologous stem cell transplantation [3]. These new therapies have prolonged the progression-free survival and overall survival of MM patients [3]. However, MM is commonly diagnosed in elderly patients, and most patients will relapse even if treatment with new agents provides therapeutic advantages [4]. In general, the prognosis of patients who relapse after treatment with novel agents is very poor. Moreover, current therapies are unable to eradicate MM cells in the bone marrow

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00277-018-3547-7) contains supplementary material, which is available to authorized users.

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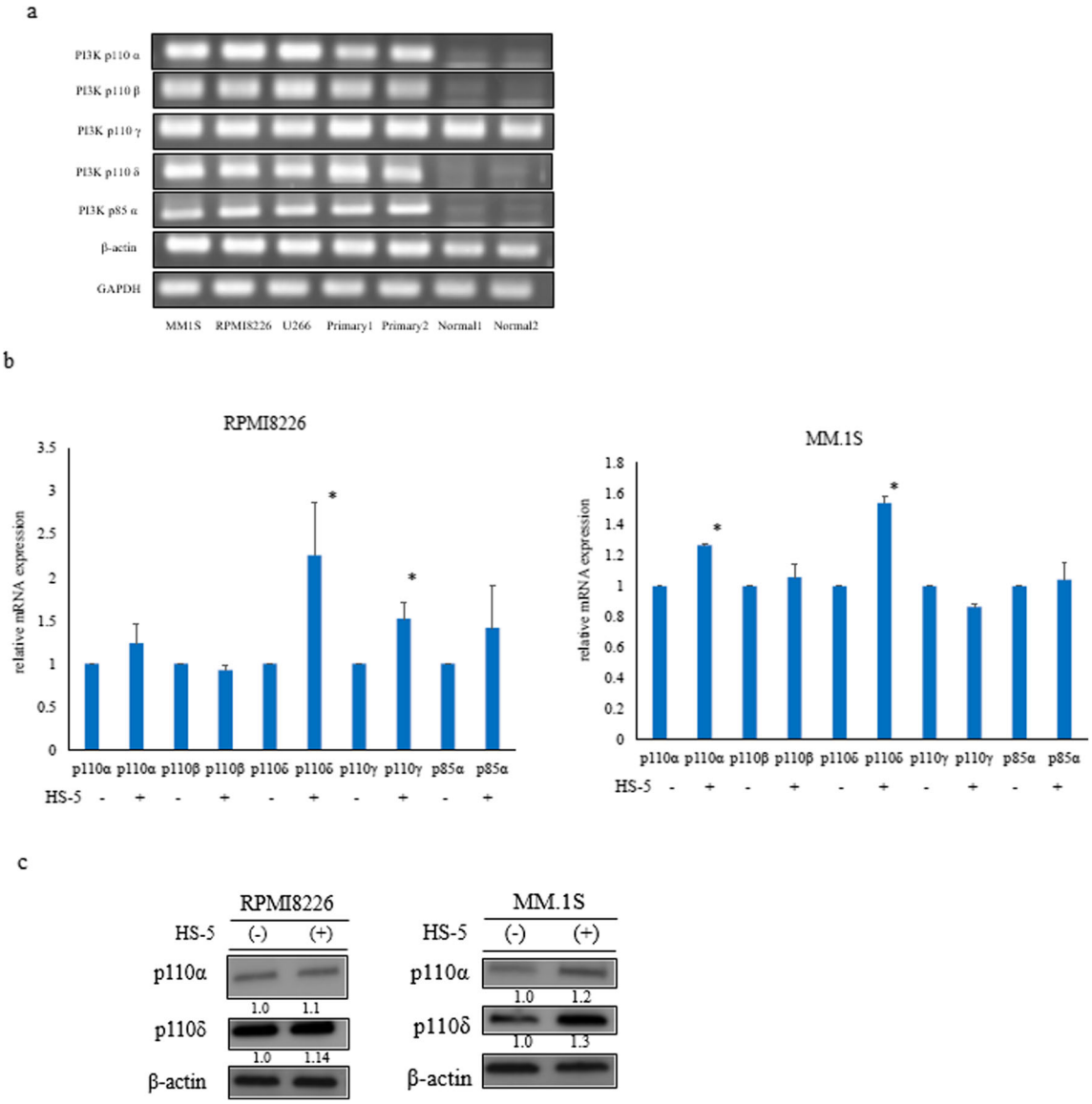


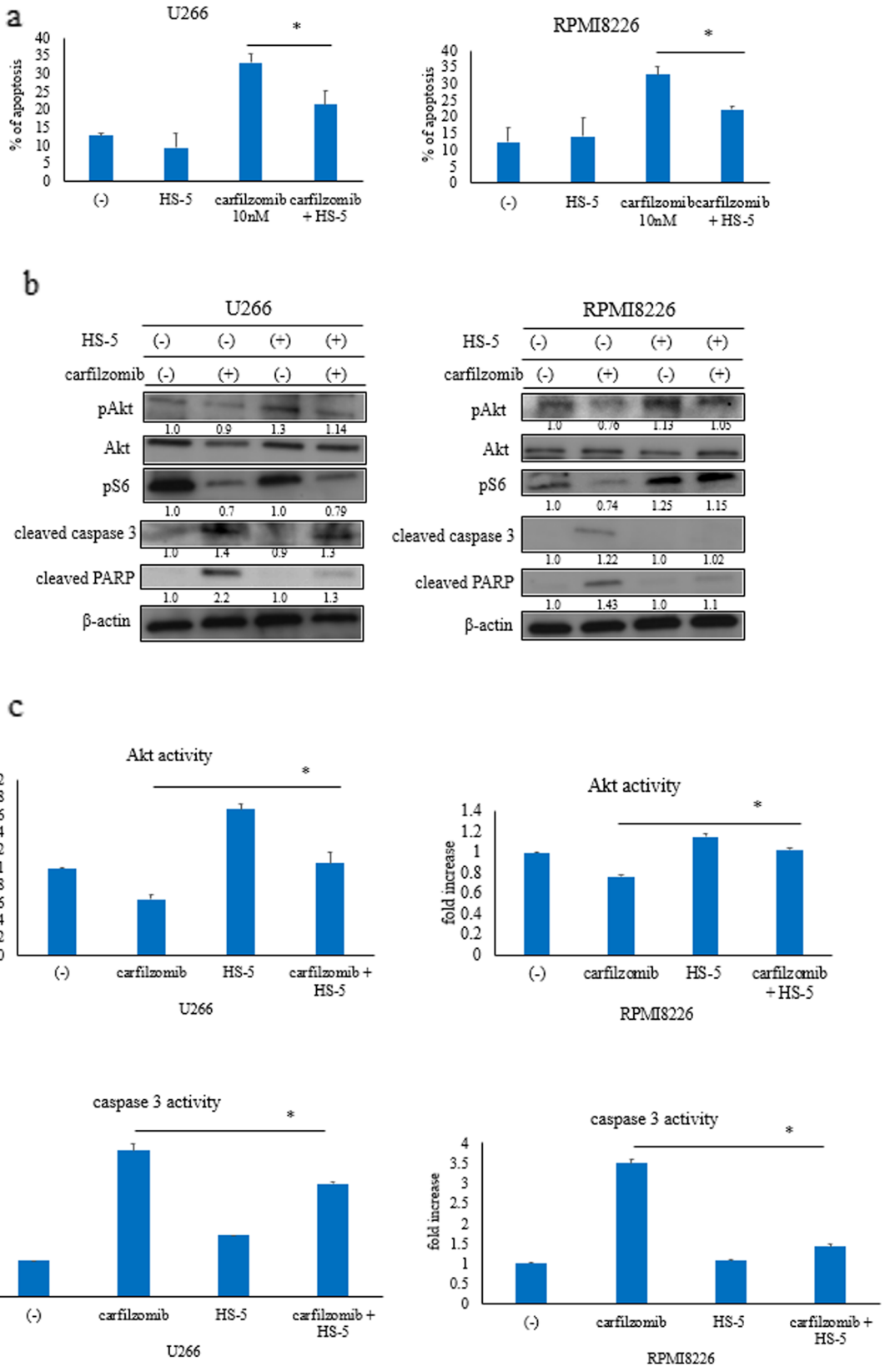
Fig. 1 Expression of PI3K in myeloma cells. **a** The PI3K subunit was examined by quantitative RT-PCR analysis as described in Materials and methods. Results represent three separate experiments. **b** RPMI8226 or MM.1S cells were treated with or without HS-5 culture supernatant for 24 h. The PI3K subunit was examined by quantitative RT-PCR. $^{*}P < 0.05$ compared to cells with HS-5 supernatant treatment. **c** RPMI8226 or MM.1S cells were treated with or without HS-5 culture supernatant or co-cultured with HS-5 for 24 h. p110α and p110δ were examined using immunoblot analysis. Actin was the loading control. Blots were scanned and optical densities were determined using ImageJ software. The results are representative of three separate experiments

microenvironment [5]. To date, MM has been thought to be an incurable disease. Therefore, a new strategy is needed to increase the survival of MM patients [6, 7].

Phosphoinositide 3-kinase (PI3K) plays key regulatory roles in many cellular processes including cell survival [8]. It activates its downstream molecule, Akt, which mediates cell proliferation. Aberrant activation of the PI3K pathway has been implicated in many types of cancers. The PI3K pathway also has a crucial role in drug resistance. Class I of the PI3K family comprises four members, PI3Kα, β, γ, and δ, which are activated in MM cell lines and primary samples [9]. Therefore, the PI3K/Akt pathway is regarded as an ideal target

Fig. 2 Carfilzomib efficacy decreased in the presence of the HS-5 supernatant. **a** U266 and RPMI8226 cells were co-cultured with or without HS-5 and treated with the indicated concentration of carfilzomib for 72 h. The apoptotic cells were analyzed. $^{*}P < 0.05$ compared to control. Results represent three separate experiments. **b** U266 or RPM8226 cells were treated with or without the HS-5 culture supernatant and treated with the indicated concentrations of carfilzomib for 24 h. Phosphorylation of Akt, S6, p110α, and p110δ was examined using immunoblot analysis. Actin was the loading control. Blots were scanned and optical densities were determined using ImageJ software. **c**, **d** U266 cells were co-cultured with or without the HS-5 feeder cell supernatant and treated with carfilzomib at the indicated concentration for 24 h. Akt and caspase 3 activities were determined calculated. $^{*}P < 0.05$ compared to carfilzomib-treated cells. The results shown represent three independent experiments

for MM. A number of PI3K-targeted compounds are being introduced into clinical trials. Copanlisib, also known as BAY80-6946, is a novel pan-class I inhibitor against both PI3Kδ and PI3Kα isoforms [10]. It inhibits the activation of the PI3K signaling pathway in vitro and in vivo and is now being investigated in clinical trials against hematological



malignancies such as non-Hodgkin lymphoma (NHL). In this study, we examined the effects of copanlisib when used singly against MM cells as a clinical candidate for the treatment of MM and/or in combination with cytotoxic agents such as the proteasome inhibitor, carfilzomib.

Materials and methods

Regents

Copanlisib and carfilzomib were purchased from MedKoo Biosciences (Chapel Hill, NC, USA). Stock solutions of carfilzomib were prepared in dimethyl sulfoxide (DMSO). Copanlisib was dissolved in hydrochloric acid and diluted in distilled water according to the manufacturer's protocol. Human CXCL12 and mouse VEGF were purchased from WAKO (Osaka, Japan). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell lines and patient samples

The MM cell lines, U266, RPMI8226, MM.1S, and MM.1R; the human bone marrow stromal cell line, HS-5; and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Mouse fibroblast NIH/3T3 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki Osaka, Japan). These MM cell lines were cultured in RPMI 1640 medium containing 10% or 15% fetal bovine serum (FBS) with 1% penicillin/streptomycin and maintained at 37 °C in a 5% CO_2-humidified atmosphere. HS-5 and NIH/3T3 cells were cultured in DMEM medium containing 10% FBS. HUVECs were cultured in EGM™-2 Media (Lonza, Chiba, Japan). Primary myeloma samples or normal samples were obtained from patients who were previously diagnosed with MM according to standard criteria. This study protocol was approved by the Institutional Review Board of the Tokyo Medical University, and written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Enzyme-linked immunosorbent assay

To investigate caspase 3 and Akt activation, MM cells were cultured with RPMI medium with or without the indicated concentrations of carfilzomib or copanlisib and/or HS-5 supernatant. After 48 h, the cells were harvested and stored at − 80 °C. Phosphorylation of Akt and caspase 3 activity were measured using the AKT Pathway Activation Profile InstantOne™ ELISA Kit (eBioscience, Inc. San Diego, CA), ApoAlert® Caspase-3 Colorimetric Assay Kit (Takara Bio Inc., Shiga, Japan), or Caspase Glo 3/7 assay kit (Promega, Madison, WI, USA). All measurements were performed in triplicate.

Apoptosis assay

U266 or RPMI8226 cells were treated with copanlisib and/or carfilzomib for 48 h using DMSO as a control. Apoptosis was measured by staining cells by using the FITC Annexin-V Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. The cells were analyzed on a flow cytometer (BD Biosciences).

Cell viability assays

The MM cells were treated with the indicated concentrations of copanlisib alone or in combination with carfilzomib. After 72 h, the cells were stained with trypan blue dye or a cell counting kit solution (Dojin, Kumamoto, Japan). This was followed by photometric measurements at an absorbance of 450 nm to determine cell viability. The experiments were performed in triplicate.

Immunoblotting

The immunoblot analysis was performed according to previously described methods [11]. After different treatments, the cells were washed with ice-cold PBS twice and lysed with radioimmunoprecipitation assay lysis buffer. Forty micrograms of total cellular protein was separated on 4–20% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Next, the membranes were probed using primary antibodies of interest at the appropriate dilutions for 1 h at room temperature. The blots were visualized by chemiluminescence using the Amersham ECL chemiluminescence kit (GE Healthcare, Tokyo, Japan). Specific primary antibodies (Abs) including phospho-Akt (Ser473), phospho-S6 ribosomal protein (Ser235/236), p110α, cleaved caspase 3, and poly ADP-ribose polymerase (PARP) were purchased from Cell Signaling (Danvers, MA). Antibodies to p110δ and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Akt1 antibody was purchased from GeneTex, Inc. (Irvine, CA). The experiments were carried out in three separate experimental repeats. Protein band intensity was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from the MM cells and primary samples using the RNAqueous®-4PCR kit (Life Technologies

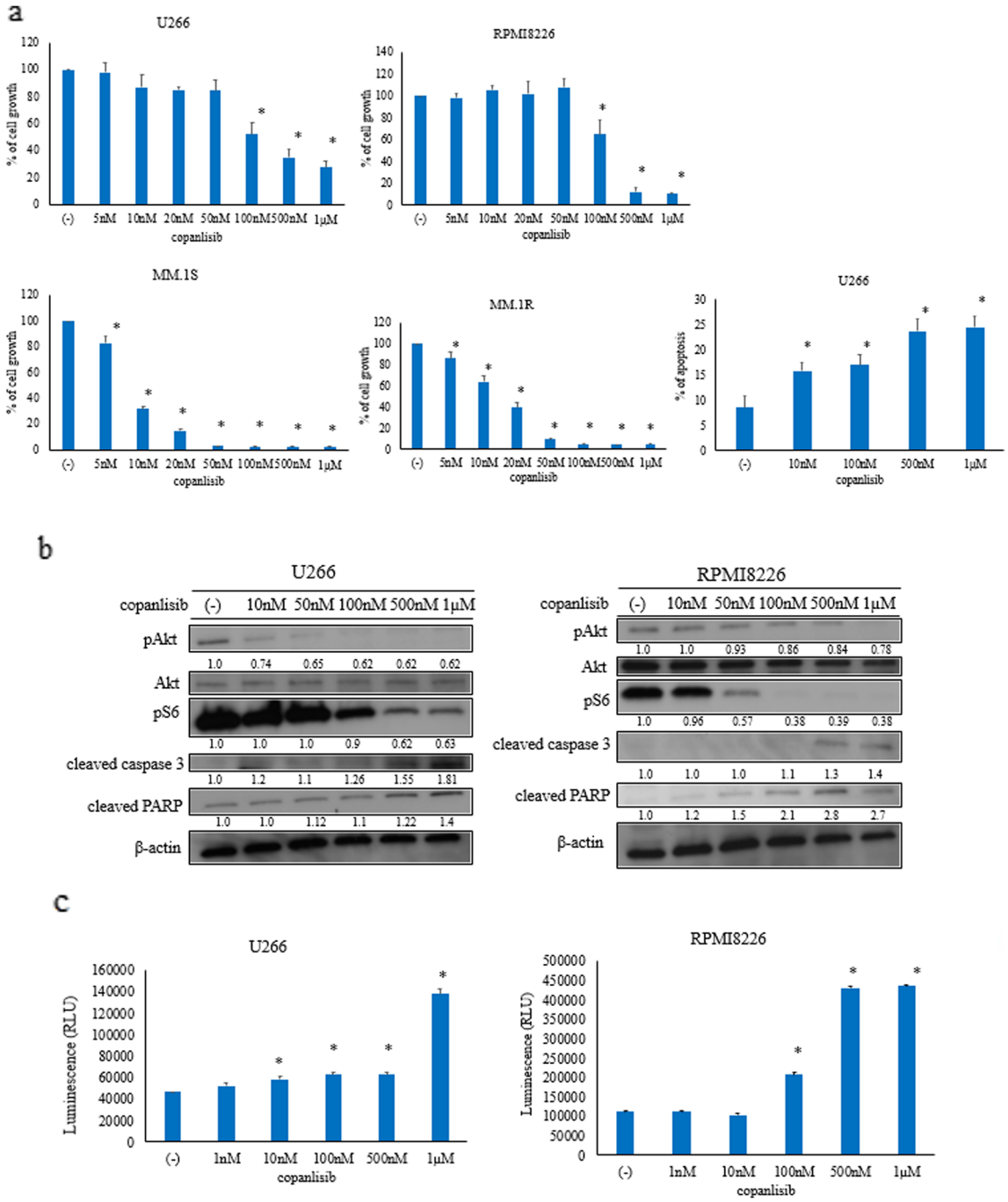


Fig. 3 Effects of copanlisib on myeloma cells. **a** U266, RPMI8226, MM.1S, and MM.1R cells were treated with the indicated concentrations of copanlisib for 72 h. Viable cells and apoptosis were determined. $^{*}P < 0.05$ compared with control. **b** U266 and RPMI8226 cells were treated with copanlisib at the indicated concentrations for 24 h. Total protein extracts were examined by immunoblot analysis using phospho-Akt (Ser473), phospho-S6 ribosomal protein (Ser235/236), cleaved PARP, cleaved caspase 3, and Akt antibodies. Actin was the loading control. Blots were scanned and optical densities were determined using ImageJ software. **c** U266 or RPMI8226 cells were treated with copanlisib for 48 h. Caspase activity was evaluated. $^{*}P < 0.05$, compared with control. These results are representative of three separate experiments

Japan Ltd., Minato-ku, Tokyo, Japan) and reverse transcribed using a first-strand cDNA synthesis kit (OriGene Technologies, Rockville, MD). Real-time PCR was performed using the Roche Light Cyber 2.0 detection system (Roche Diagnostic Gmbh, Minato-ku, Tokyo, Japan). The expression of the human PI3K P110α, β, γ, and δ isoforms, as well as that of p70α, GAPDH, and β-actin was determined using a quantitative SYBER Green PCR kit (Roche) according to the manufacturer's protocol. The specific PCR primers were obtained from Takara Bio Inc. (Otsu, Shiga, Japan).

Chemotaxis assay

HUVECs were cultured in growth medium overnight. The cells were harvested and re-suspended in migration medium (0.2% FBS DMEM) and seeded into the upper chamber of an 8-μM pore size transwell chemotaxis chamber (Corning, NY, USA). In the lower chamber, indicated concentrations of CXCL12 and/or copanlisib were added and incubation was performed at 37 °C for 4 h. Non-migrated cells were removed by a cotton swab, and the migrated cells were stained with hematoxylin and eosin stain. The migrated cells in three random fields (× 100 magnification) were counted.

In vitro wound-healing assay

The wound-healing assay was performed using the CytoSelect™ 24-Well Wound Healing Assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol. NIH/3T3 cells (5×10^4) were seeded in a 24-well chamber and cultured until a monolayer was formed. The cells were treated with the indicated concentrations of copanlisib and monitored for migration into the wound field.

In vivo Matrigel assay

This animal study was approved by the Institutional Review Board of the Tokyo Medical University. Six-week-old female mice were injected subcutaneously with 0.5-ml Corning® Matrigel® (Matrigel: Corning, NY, USA) with or without 30 ng/ml VEGF containing 64 U/ml heparin. After 4 days, the mice were sacrificed and the Matrigel was harvested. Serial sections of Matrigel were stained with hematoxylin and eosin and analyzed.

Statistical analysis

The Student's *t* test was used to determine if the effects on the drug-treated groups were statistically significant compared to those in the control group. $P < 0.05$ or $P < 0.01$ was considered to be statistically significant.

Results

Expression of PI3K isoforms increased in MM cells

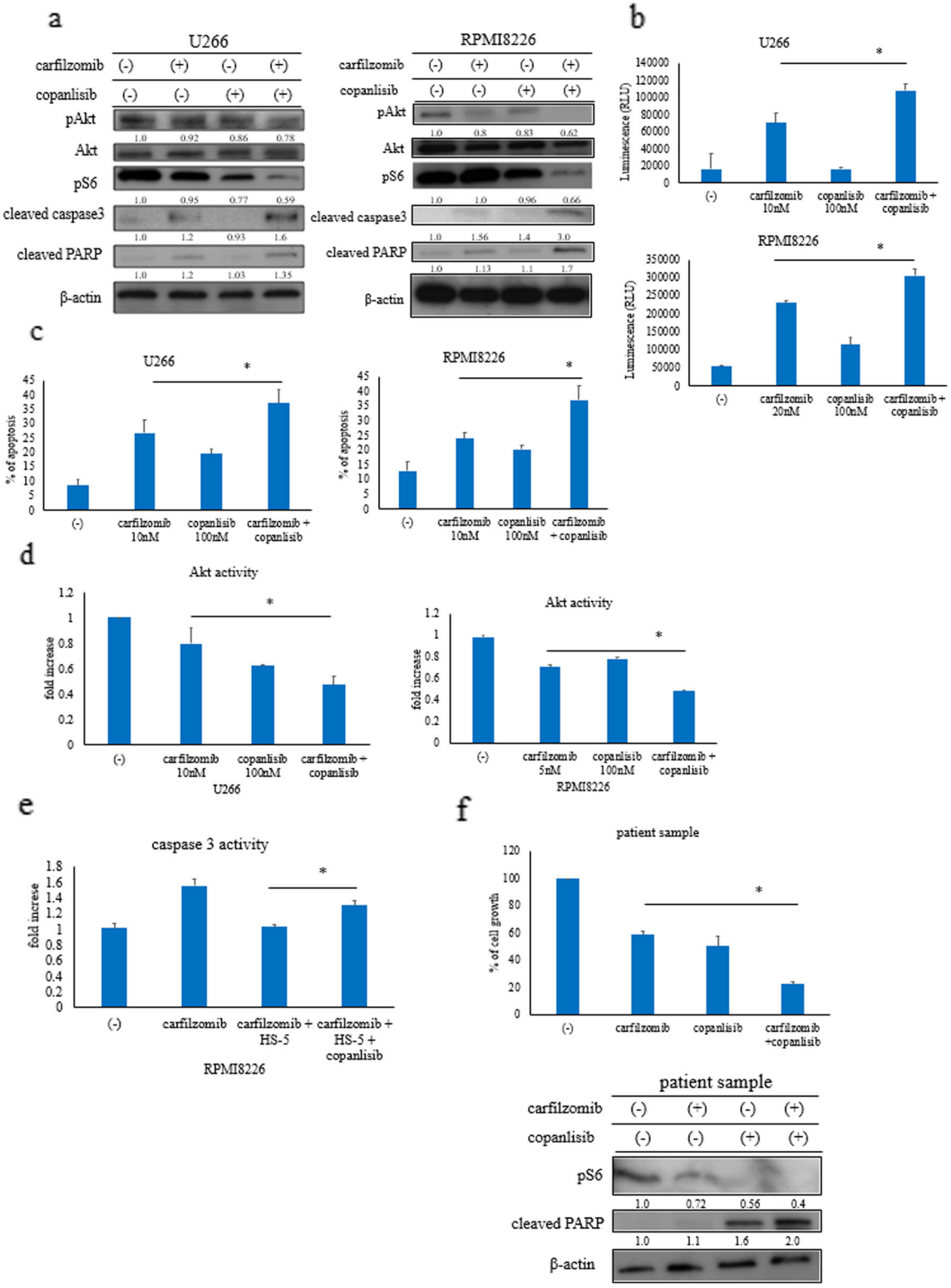
We used RT-PCR to investigate the expression of PI3K isoforms (p110α, β, γ, δ) in five MM cell lines, two MM primary samples, and normal BM plasma samples. Figure 1a shows an increase in the expression of PI3K isoforms (e.g., p110α, β, δ) in MM cells including primary MM samples compared to that in normal plasma cells. In contrast, the expression of the p110γ isoform was equal in MM cells and the normal plasma samples (Fig. 1a). In the bone marrow, myeloma cells were found with bone marrow feeder cells. Therefore, the PI3K isoform whose expression changed in the presence of the feeder cell supernatant was investigated. After incubation with the HS-5 supernatant, we examined PI3K expression by RT-PCR. We found that the expression of the p110α and δ PI3K isoforms increased in the presence of HS-5 supernatant in RPMI8226 and MM.1S cells (Fig. 1b). A subsequent immunoblot analysis confirmed that the protein expression of p110α and δ increased after the HS-5 supernatant treatment (Fig. 1c). These results indicate that the PI3K isoforms (p110α and δ) may be a potential target for MM cells.

Feeder cells protected MM cells from carfilzomib-induced cell death

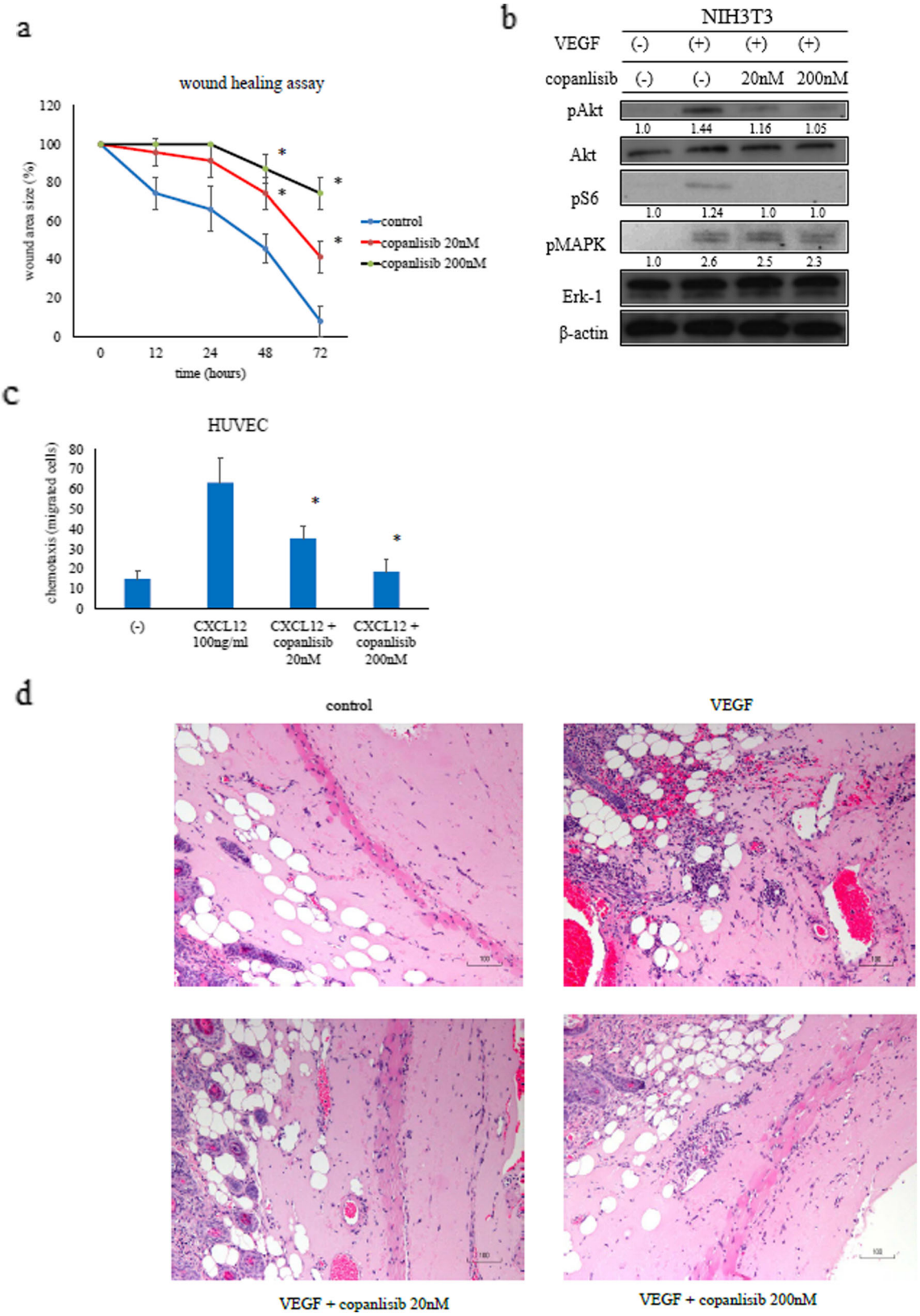
Carfilzomib is a selective proteasome inhibitor and has superior anti-myeloma activity in patients with relapsed and refractory MM compared to that of bortezomib. To determine the role of feeder cells in carfilzomib-induced MM cell death, we examined if the HS-5 supernatant could support MM cells and if it was involved in the activation of carfilzomib. MM cells were incubated with or without the indicated concentrations of carfilzomib and the HS-5 supernatant (20% volume/medium). We found that carfilzomib inhibited MM cell growth and induced apoptosis. In contrast, cell growth inhibition and percent of apoptosis decreased in the presence of the HS-5 supernatant (Fig. 2a and Supplemental Fig. 1a). We next investigated changes in intracellular signaling. Immunoblot analysis revealed that carfilzomib treatment decreased the activity of phosphorylated Akt (Ser473) and the downstream molecule S6 ribosomal protein (Ser235/236) and increased caspase 3 and PARP activities (Fig. 2b). In

Fig. 4 Combination treatment with copanlisib and carfilzomib decreased the proliferation of myeloma cells. **a** U266 or RPMI8226 cells were treated with carfilzomib and/or copanlisib for 24 h. Total extracts were examined by immunoblot analysis using antibodies against phospho-Akt (Ser473), phospho-S6 ribosomal protein (Ser235/236), cleaved PARP, cleaved caspase 3, Akt, and β-actin. Blots were scanned and optical densities were determined using ImageJ software. **b**, **c**, **d** U266 or RPMI8226 cells were treated with carfilzomib and/or copanlisib for 24 h. Caspase 3, apoptosis, and Akt activities were determined. $^{*}P < 0.05$ compared to carfilzomib-treated cells. **e** RPMI8226 cells were treated with carfilzomib and/or copanlisib in the presence of the HS-5 supernatant for 48 h. The relative caspase 3 activity was determined. $^{*}P < 0.05$ compared to cells treated with carfilzomib plus HS-5 supernatant. **f** Primary myeloma cells were treated with carfilzomib and/or copanlisib at the indicated concentrations for 24 h or 72 h. Analysis of relative growth rates and immunoblot analysis were performed. $^{*}P < 0.05$ compared with carfilzomib-treated cells. These results are representative of three separate experiments

contrast, in the presence of the HS-5 supernatant, the activity of phosphorylated Akt and expression of S6 ribosomal protein increased while caspase activity decreased even if the cells were treated with carfilzomib. Akt and caspase 3 activities in MM cells were also investigated. Akt activity reduced upon carfilzomib treatment but increased in the presence of the HS-5 supernatant (Fig. 2c). Akt activity did not decrease after co-treatment with carfilzomib and the HS-5 supernatant compared to that after carfilzomib alone. Caspase 3 activity reduced after



co-treatment with the HS-5 supernatant and carfilzomib compared to that after carfilzomib treatment alone (Fig. 2d). These results indicate that the HS-5 feeder cells support MM cell lines and that carfilzomib activity decreased in the presence of the HS-5 supernatant owing to Akt activation.



Efficacy of copanlisib against MM cells

Copanlisib is a selective PI3Kα and δ inhibitor and is being investigated in clinical trials of hematological malignancies. Therefore, we examined the efficacy of copanlisib against MM cells. The MM cell lines were incubated with the indicated concentrations of copanlisib and the antiproliferative effect of copanlisib was analyzed. Copanlisib treatment resulted in dose-dependent cytotoxicity and apoptosis in the MM cell lines (Fig. 3a). The treatment was effective in MM.1S and MM.1R cells at low concentrations (e.g., 5 nM). In contrast, in RPMI8226 and U266 cells, copanlisib was effective at a concentration of 100 nM, suggesting that copanlisib's efficacy differs in the MM cell lines. We next examined intracellular signaling. Copanlisib treatment reduced the activity of phosphorylated Akt and expression of the downstream molecule S6 ribosomal protein. In contrast, it increased caspase 3 and PARP activities in a dose-dependent manner (Fig. 3b). We also investigated if copanlisib induced apoptosis in MM cell lines and found that copanlisib induced caspase 3/7 activation in a dose-dependent manner (Fig. 3c). These results indicate that copanlisib was effective against MM cell lines.

Copanlisib enhanced carfilzomib efficacy in MM cell lines

We examined whether carfilzomib and copanlisib could synergistically inhibit cell growth in MM cells. After culturing in the presence of copanlisib and/or carfilzomib for 72 h, U266 cells were harvested and the cell proliferation assay was performed. The growth of U266 or RPMI8226 cells reduced to a greater extent after combination treatment with carfilzomib or bortezomib and copanlisib compared to that after treatment with either drug alone (Supplemental Fig. 2a, b). We next investigated the intracellular signaling. Combination treatment with carfilzomib and copanlisib increased Akt phosphorylation, S6 ribosomal protein expression, and caspase 3 and PARP activities (Fig. 4a). We also examined Akt and caspase 3 activities. As shown in Fig. 4b–d, caspase 3 activity and apoptosis increased after combination treatment with carfilzomib and copanlisib while Akt activity decreased. We next examined whether the combined effects of carfilzomib and other PI3K inhibitors (pictilisib, alpelisib, and idelalisib) enhanced MM cell inhibition. We found that combination treatment with carfilzomib and the pan-PI3K inhibitor pictilisib inhibited cell growth, in contrast to the effects of each drug alone (Supplemental Fig. 3). The specific PI3Kα inhibitor, alpelisib, or the PI3Kδ inhibitor, idelalisib, showed lower efficacy than that shown by pictilisib. Moreover, combination treatment with carfilzomib and alpelisib plus idelalisib increased cell growth inhibition, suggesting that dual inhibition of PI3Kα and δ enhances carfilzomib activity. As carfilzomib activity decreased in the presence of feeder cells, we next examined if copanlisib-induced MM cell death was inhibited by feeder cells. We found that combination treatment with carfilzomib and copanlisib increased caspase 3 activity in the presence of the HS-5 supernatant (Fig. 4e). These results indicate that combination treatment with carfilzomib and copanlisib increased the cytotoxic effect against MM cells and could overcome the protection afforded by feeder cells.

Efficacy of carfilzomib and copanlisib against MM primary cells

As our data suggested that copanlisib and carfilzomib are promising therapeutic agents in MM cell lines, we evaluated the efficacy of copanlisib in primary MM samples. We found that combination treatment with copanlisib and carfilzomib enhanced cytotoxicity in primary MM samples. Moreover, phosphorylation of the S6 ribosomal protein decreased and PARP activity increased after the combination treatment (Fig. 4f).

Copanlisib inhibited the migration of fibroblasts

As endothelial cell migration is essential for tumor angiogenesis, we examined whether PI3K signaling was involved in cell migration. To study cell migration and cell interactions, we investigated cell migration using NIH/3T3 cells. In the wound-healing assay, NIH/3T3 cells migrated in a time-dependent manner (Fig. 5a). In contrast, cell migration reduced in the presence of copanlisib in a dose-dependent manner. In the immunoblot analysis, phosphorylation of Akt, S6 ribosomal protein expression, and mitogen-activated protein kinase (MAPK) activity increased after incubation of NIH/3T3 cells with VEGF. In contrast, phosphorylation of Akt, S6 ribosomal protein expression, and MAPK activity reduced upon pretreatment with copanlisib (Fig. 5b). In the chemotaxis analysis in HUVECs, CXCL12-induced chemotaxis was inhibited by copanlisib in a dose-dependent manner (Fig. 5c). Moreover, in the in vivo angiogenesis assay, copanlisib inhibited VEGF-mediated angiogenesis (Fig. 5d).

◀ **Fig. 5** Inhibitory effects of copanlisib on tumor angiogenesis. **a** The wound-healing assay was performed as described in Material and methods. $^{*}P < 0.05$ compared with control. The results shown represent three independent experiments. **b** NIH/3T3 cells were treated with VEGF and/or copanlisib for 5 min. Total extracts were examined by immunoblot analysis using antibodies against phospho-Akt (Ser473), phospho-S6 ribosomal protein (Ser235/236), phospho-MAPK, Akt, Erk1, and β-actin. Blots were scanned and optical densities were determined using ImageJ software. **c** Chemotaxis analysis of HUVECs was performed as described in Material and methods. $^{*}P < 0.05$ compared with CXCL12-treated cells. **d** In vivo Matrigel assay was performed as described in Material and methods. Matrigel samples were analyzed histologically via hematoxylin and eosin staining. The results shown represent two independent experiments

Discussion

Carfilzomib is an effective anticancer agent against MM cells, and its effect is regulated by the NF-κβ signaling pathways. However, our data demonstrated that carfilzomib activity reduced in the presence of the feeder cell supernatant. Bone marrow stromal cells (BMSCs) activate MM cells via a multitude of signaling pathways. Several cytokines and growth factors are secreted by BMSCs [12, 13] and these cytokines activate PI3K/Akt signaling. In this study, we used the HS-5 feeder cell line as a model of BMSCs. The HS-5 feeder cell line produces cytokines such as granulocyte colony-stimulating factor, granulocyte-macrophage-CSF, interleukin-6, and leukemia inhibitory factor [14]. These cytokines may support MM cells and protect them from carfilzomib cytotoxicity.

Angiogenesis plays an important role in the biology of MM and disease progression [15]. VEGF is an inducer of angiogenesis and secreted by several MM cells [16]. In this study, we demonstrated that VEGF stimulation increased Akt and MAPK phosphorylation in NIH/3T3 cells. Our wound-healing assay also demonstrated that copanlisib inhibited the migration of NIH/3T3 cells. This finding was supported by those of the in vivo Matrigel assay, where copanlisib inhibited VEGF-induced vessel formation in mice, suggesting the involvement of PI3K in angiogenesis, and copanlisib could inhibit both in vitro and in vivo angiogenesis in our models.

The PI3K/Akt signaling pathway plays a major role in cancer proliferation and chemoresistance [8]. Thus, PI3K may be a target for MM treatment and several studies have demonstrated the antitumor effects of PI3K and downstream molecules in hematological malignancies resulting from PI3K signaling inhibition. There have been several reports of PI3K and downstream molecule inhibitors. Spencer et al. reported the effects of the oral Akt inhibitor, afuresertib, in hematological malignancies including MM [17] where clinical activity was observed in afuresertib-treated MM patients. Ghobrial et al. reported the effects of the oral dual TORC1/2 inhibitor, TAK-228 (formerly MLN0128), in MM patients [18]. Further, Miura et al. reported that a novel allosteric Akt inhibitor, TAS-117, enhanced the cytotoxicity of proteasome inhibitors in a preclinical study [19]. Sahin et al. reported that the PI3K inhibitor, buparlisib, decreased the proliferation of MM plasma cells and Waldenstrom macroglobulinemia cells [20]. Zhu et al. reported that PIK-C98 inhibited all class I PI3K isoforms at nano- or low micromolar concentrations and specifically inhibited the PI3K signaling pathway in MM samples [21]. Copanlisib is now being investigated against indolent B cell NHL and a phase 3 study is ongoing. Copanlisib is a pan-class I PI3K inhibitor with an IC_{50} of 0.5, 3.7, 6.4, and 0.7 nM for PI3Kα/β/γ/δ. We evaluated the cytotoxicity induced by 100 nM copanlisib in RPMI8226 and U266 cells. However, the plasma concentration of copanlisib was found to reach up to 800 nM in a clinical trial [22]; these are reflected clinically relevant concentrations. Moreover, our study showed that treatment with carfilzomib and alpelisib, PI3Kα inhibitor, plus idelalisib, PI3Kδ inhibitor, was more cytotoxic compared to treatment with PI3K isoform-specific inhibitors. Isoform-specific PI3K inhibitors may be associated with fewer side effects than those associated with pan-PI3K inhibitors in clinical settings. Although the PI3Kα- specific inhibitor, alpelisib, or the PI3Kδ-specific inhibitor, idelalisib, could not enhance carfilzomib activity, the combination of the PI3Kα- and δ-specific inhibitors enhanced carfilzomib activity in vitro, indicate that targeting both PI3Kα and δ may improve outcomes for myeloma patients. The present study also demonstrated that copanlisib could enhance the cytotoxicity of carfilzomib and suppress PI3K/Akt activation and induce MM cell apoptosis. Moreover, it enhanced carfilzomib's effect and the combination treatment could overcome feeder cell protection and reduce angiogenesis.

A previous study [23] presented a novel insight into the effects of combination treatment with a proteasome inhibitor, carfilzomib, and a PI3K inhibitor, copanlisib. Therefore, these findings together support the therapeutic potential of copanlisib and provide a rationale for further development of novel PI3K inhibitors that may afford novel therapeutic strategies for MM patients in the near future.

Acknowledgments We thank the Tokyo Medical University Research Center for providing technical support.

Authors' contributions Conception and design: S Okabe, Y Tanaka, T Tauchi, and K Ohyashiki. Development of methodology: S Okabe, Y Tanaka, T Tauchi, and K Ohyashiki. Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S Okabe, Y Tanaka, and T Tauchi. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S Okabe, Y Tanaka, and T Tauchi. Writing, review, and/or revision of the manuscript: S Okabe, Y Tanaka, T Tauchi, and K Ohyashiki. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S Okabe, Y Tanaka, T Tauchi, and K Ohyashiki.

Funding information This work was supported by a High-Tech Research Center Project for private universities, a matching fund subsidy from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), and by the University-Industry Joint Research Project for private universities (another matching fund subsidy from MEXT). This work was also supported by grants-in-aid for Scientific Research from MEXT and Supporting Positive Activities for Female Researchers. In addition, the study was supported by a grant from the Bristol-Myers Squibb Company.

Compliance with ethical standards

This study protocol was approved by the Institutional Review Board of the Tokyo Medical University (No. 1974, H-28026).

Conflict of interest The authors declare that they have no conflicts of interest.

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