Title: Acute suppression of translation by hyperthermia enhances anti-myeloma activity of carfilzomib

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Abstract

Hyperthermia is a unique treatment option for cancers. Multiple myeloma (MM) remains incurable and innovative therapeutic options are needed. We investigated the efficacy of hyperthermia and carfilzomib in combination against MM cells. Although MM cell lines exhibited different susceptibilities to pulsatile carfilzomib treatment, mild hyperthermia at 43℃ induced MM cell death in all cell lines in a time-dependent manner. Hyperthermia and carfilzomib cooperatively induced MM cell death even under suboptimal conditions. The pro-survival mediators PIM2 and NRF2 accumulated in MM cells due to inhibition of their proteasomal degradation by carfilzomib; however, hyperthermia acutely suppressed translation in parallel with phosphorylation of eIF2α to reduce these proteins in MM cells. Recovery of β5 subunit enzymatic activity from its immediate inhibition by carfilzomib was observed at 24 hours in carfilzomib-insusceptible KMS-11, OPM-2, and RPMI8226 cells, but not in carfilzomib-sensitive MM.1S cells. However, heat treatment suppressed the recovery of β5 subunit activity in these carfilzomib-insusceptible cells. Therefore, hyperthermia re-sensitized MM cells to carfilzomib. Our results support the treatment of MM with hyperthermia in combination with carfilzomib. Further research is warranted on hyperthermia for drug-resistant extramedullary plasmacytoma.

Introduction

The recent clinical implementation of newly developed therapeutic agents has been improving the overall survival of patients with multiple myeloma (MM). However, MM eventually relapses and remains incurable. Novel therapeutic options with different modes of action need to be developed in order to overcome drug resistance in refractory MM. Hyperthermia is a unique treatment option for cancers. We developed novel superparamagnetic nanoparticles (SPIONs), which accumulate in extramedullary tumors in mouse plasmacytoma models and extirpate MM cells by heat locally generated with alternative magnetic currency [1]. SPIONs may be utilized as a contrast agent for the detection of extramedullary tumors by magnetic resonance imaging. Therefore, this method is sufficiently sophisticated to identify extramedullary tumors to be targeted and selectively delivers heat to them by controlling alternative magnetic currency [1-3]. We also demonstrated that hyperthermia targeted drug-resistant clonogenic MM cells or cancer stem cell-like cells, which are resistant to proteasome inhibitors (PIs), and showed that hyperthermia and PIs cooperatively enhanced their anti-MM effects [4]. We confirmed that hyperthermia at 43°C was effective and enhanced anti-MM effects in combination with bortezomib [4]. Based on this observation, we set 43°C as the condition of hypothermia in the present study.

We reported the critical role of PIM2 in MM cell growth and survival, and demonstrated PIM2 as an important therapeutic target for MM [5-7]. PIM2 has been demonstrated to be robustly produced in MM cells and rapidly degraded by proteasomes [7, 8]. Reactive oxygen species (ROS) play an important role in exerting cytotoxic activities of anti-cancer agents; and NRF2 is a vital transcription factor for various cytoprotective genes against oxidative stress by anti-cancer agents. NRF2 is bound by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, and quickly degraded by the ubiquitin-proteasome pathway [9]. PIs have been reported to robustly increase Nrf2 protein levels in mouse hepatoma cells [9]. Therefore, we hypothesized that PIM2 and NRF2 remained after PI treatment may compromised the therapeutic efficacy of PIs.

Therefore, the present study investigated the mechanisms underlying MM cell resistance to PIs and the therapeutic potential of hyperthermia to overcome PI resistance in MM cells. The results obtained demonstrated that hyperthermia killed MM cells irrespective of their resistance to PIs, while normal quiescent cells appeared to be less susceptible to the cytotoxicity of hyperthermia. Mild hyperthermia at 43℃ for 20 minutes acutely suppressed translation, sensitized MM cells to carfilzomib (CFZ) with reductions in PIM2 and NRF2 protein levels, and suppressed the recovery of β5 subunit activity in CFZ-resistant MM cells. The present results provide a rationale for hyperthermia

combined with CFZ, which may overcome MM cell resistance to PIs.

Materials and Methods

Reagents

The following reagents were purchased from the indicated manufacturers: rabbit antibodies against PIM2 (#4730), eukaryotic initiation factor 2α (eIF2α) (#9722), phosphorylated eIF2α (p-eIF2α) (#119A11), MYC (#5605), IRF4 (#4948), MCL1 (#94296) and Sp1 (#5931), horseradish peroxidase (HRP)-anti-rabbit IgG (#7074S), and anti-mouse IgG (#7076S) from Cell Signaling Technology (Beverly, MA, USA); a rabbit NRF2 antibody (#GTX103322) from Gene Tex (Los Angeles, USA); a mouse anti-β-actin antibody (#A5441) from Sigma-Aldrich (MO, USA); an anti-puromycin antibody (#EQ0001) from Kerafast, Inc. (Boston, MA); CFZ from Chemie Tek (Indianapolis, USA); puromycin from Sigma-Aldrich; AMG9810 and SB366791 from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Cells and cultures

The MM cell lines MM.1S, RPMI 8226, and U266B1 were obtained from the American Type Culture Collection (Rockville, MD, USA). The OPM-2 cell line was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The KMS-11 cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham MA, USA), penicillin G at 50 μg/mL, and streptomycin at 50 μg/mL. Peripheral blood mononuclear cells (PBMCs) were isolated from normal subjects as previously described [10]. All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki. The present study was approved by the Institutional Review Board of Tokushima University (permission number 3842-3).

Cell viability

Culture tubes with MM cell lines were tightly closed and immersed in a water bath at 43℃ for 20, 40, and 60 minutes. After the heat treatment, MM cells were washed and incubated at 37℃ for 24 hours in 5% CO_2. Bolus CFZ was administered to MM cell lines for 1 hour, and after the CFZ pulsatile treatment, MM cells were washed and cultured at 37℃ for 24 hours in 5% CO_2. Viable cell numbers were measured by a cell proliferation assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-

tetrazolium (WST-8; Kishida Chemical, Osaka, Japan).

Western blot analysis

Whole-cell extracts were lysed in RIPA lysis buffer (Santa Cruz). The cell lysates obtained were supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Sigma-Aldrich). Cell lysates were then subjected to SDS-PAGE on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 3% bovine serum albumin (FUJIFILM Wako Chemicals) or 5% nonfat dry milk in tris (hydroxymethyl) aminomethane-buffered saline with 0.01% Tween 20 at room temperature for 1 hour and then incubated at 4℃ overnight with primary antibodies. After washing, a secondary HRP-conjugated antibody was added for 1 hour. Protein bands were visualized with an Enhanced Chemiluminescence Plus Western Blotting Detection System (Vilber GmbH, France).

Quantitative real-time PCR analysis

RNA was obtained using TRI REAGENT (Molecular Research Center, Inc., OH, USA). Reverse transcription for the synthesis of complementary DNA (cDNA) was performed using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan). Quantitative real-time PCR measurements were conducted using a Quant Studio 3 Real-Time PCR System (Thermo Fisher Scientific) with the following temperature protocol: at 95℃ for 10 minutes, followed by 40 cycles at 95℃ for 30 seconds and 60℃ for 1 minute. In real-time PCR, each cDNA sample was amplified using 10 μL SYBR® qPCR mixα (NIPPON GENE CO., LTD., Japan), a forward (Fwd) primer, reverse (Rev) primer, template cDNA, and nuclease-free water. The total volume was 20 μL/reaction. The following primers were used: *GAPDH*; Fwd: TGTCTTCACCACCATGGAGAAGG, Rev: GTGGATGCAGGGATGATGTTCTG, *NFE2L2*; Fwd: GTTGCCCACATTCCCAAATC, Rev: TGACTGAAACGTAGCCGAAGAA,
PSMB5 Fwd: TGCTTGCCAACATGGTGTATC, Rev: TGAAATCCGGTTCCCTTCAC and, *PIM2*; Fwd: CCATTCCCGTGGAGTTGT, Rev: GAAGCAGGGCACCAGAAC and, *TRPV1*; Fwd: AACTGGACCACCTGGAACAC, Rev: GCCTGAAACTCTGCTTGACC.

Proteasome activity

MM cells were collected and washed using ice-cold phosphate-buffered saline. Pellets were suspended in proteolysis buffer (50 mM Tris HCl, 25 mM KCl, 10 mM NaCl, 1 mM

$MgCl_2$, and 0.1 mM 1,4-dithiothreitol) and subjected to a 5-minutes freeze/5-minutes thaw cycle 3 times. The freeze/thaw cycle was performed by alternately placing samples in dry ice and then in a room temperature water bath three times. Samples were centrifuged at 13,000 rpm for 10 minutes and the supernatant was collected for protein quantification. Protein concentrations were examined using a BCA assay (Bio-Rad). Different amounts of cell proteins from samples were loaded into 96-well black plates, with proteolysis buffer and 20 mM of an AMC substrate. The AMC substrate was used to measure the chymotrypsin-like activity of β5 subunits (Enzo Biochem, Inc., NY, USA), the caspase-like activity of β1 subunit and the trypsin-like activity of β2 subunit (PEPTIDE INSTITUTE, Inc., Osaka, Japan). Plates were sealed with a plastic cover, and fluorescence emission was measured for 1 hour (intervals of 2.5 minutes) using the SpectraMax i3 microplate reader ($\lambda_{excitation} = 355$ nm, $\lambda_{emission} = 444$ nm). Each sample was examined three times. The protein concentration, AMC substrate concentration, and incubation time were varied according to the experimental design, as previously reported [11] .

Statistical analysis

Statistical analyses were performed using the Student's t-test. All data were analyzed using JMP13.0 (SAS Institute Inc.). All recorded P values were two-sided, and $P < 0.05$ was considered to be significant.

Results

Hyperthermia induces MM cell death and sensitizes them to CFZ.

The PK/PD profiles of PIs showed a high C_{max} with short $T_{1/2}$ in patients with MM after the administration of PIs. In the present study, we followed a previous study with experiments modeling the anticipated *in vivo* pharmacokinetics of drug exposure in patients with MM [12]. We added CFZ at high concentrations to MM cell lines transiently for 1 hour. Cell viability under the pulsatile CFZ treatment markedly varied among the MM cell lines tested; MM.1S cells were the most susceptible to the CFZ treatment, while KMS-11 cells were the most resistant (Fig.1A). However, the heat treatment at 43℃ induced MM cell death in a time-dependent manner in all of the MM cell lines tested, whereas PBMCs from normal donors were less sensitive (Fig.1B). The heat treatment at 43℃ for 20 minutes appeared to weakly induce cell death in U266B1, OPM-2, and RPMI8226 cells. Although these MM cell lines were also resistant to the pulsatile CFZ treatment at 250 nM, the heat treatment at 43℃ for 20 minutes induced cell death in

combination with the suboptimal CFZ treatment (Fig.1C). KMS-11 cells, the most resistant MM cell line to both CFZ and the heat treatment among the MM cell lines tested, became susceptible to the combination of CFZ and heat treatment. These results suggest that MM cells are vulnerable to hyperthermia, which, in turn, increases their susceptibility to CFZ.

Hyperthermia acutely and profoundly suppresses translation in MM cells.

Heat is a typical stress that induces the integrated stress response in cells. The integrated stress response is initiated with the phosphorylation of eIF2α, a critical regulator of translation in cells [13]. Therefore, we examined the acute effects of the heat treatment on protein translation in MM cells resistant to the pulsatile CFZ treatment. The incorporation of puromycin in proteins directly reflects the rate of mRNA translation into proteins. The present results showed that the incorporation of puromycin was mostly suppressed in MM cells immediately after the heat treatment at 43℃ for 20 minutes, but not after the pulsatile CFZ treatment at 250 nM (Fig.2A). Consistent with the inhibition of translation, the phosphorylation of eIF2α was induced by the heat treatment (Fig.2B). However, the heat treatment at 43℃ for 20 minutes did not affect the incorporation of puromycin in PBMCs from normal subjects (Fig.2C). The heat treatment induced the phosphorylation of eIF2α in PBMCs to the levels weaker than that in MM cells (Supplementary Figure 1). Because eIF2α phosphorylation is generally accepted to have the significant impact on translation, the phosphorylation levels of eIF2α may cause the difference in protein translation between MM cells and normal PBMCs as indicated with the puromycin incorporation. These results suggest that the heat treatment acutely suppressed protein translation in MM cells.

Hyperthermia mitigates NRF2 and PIM2 protein accumulation in MM cells by CFZ.

The serine/threonine kinase PIM2 is highly expressed in MM cells and acts as a pro-survival mediator [5]. We previously reported that a continuous treatment with bortezomib, CFZ, and ixazomib at low concentrations time-dependently suppressed the proteasomal degradation of the PIM2 protein in MM cells without affecting *PIM2* mRNA expression [7]. The transcription factor NRF2 is a key regulator of antioxidant defense mechanisms and is overexpressed in cancer cells to compromise ROS-mediated cytotoxicity by anti-cancer agents. NRF2 is also subject to proteasomal degradation [14]. Consistent with previous findings [7, 8], PIM2 protein levels were increased in MM cells upon the CFZ treatment at low concentrations for 6 hours (Fig.3A). NRF2 was concomitantly increased at the protein level in these MM cells. The pulsatile CFZ

treatment at 250 and 500 nM for 1 hour markedly increased NRF2 and PIM2 protein levels in MM cells (Fig.3A). However, the heat treatment at 43℃ for 20 minutes strongly attenuated the CFZ-induced increases in these proteins in MM cells (Fig.3B). Furthermore, the heat treatment did not affect the expression of *NFE2L2* or *PIM2* mRNA (Fig.3C). Together with the inhibition of translation (Fig.2), these results suggest that hyperthermia rapidly and markedly inhibited the synthesis of NRF2 and PIM2 proteins at the translation, but not transcription level, thereby reducing the accumulation of these proteins through the suppression of their proteasomal degradation by CFZ.

Hyperthermia suppresses the recovery of proteasome β5 subunit activity in MM cells after the pulsatile CFZ treatment.

To further elucidate the mechanisms underlying the cooperative cytotoxic activity of hyperthermia and the pulsatile CFZ treatment, we investigated proteasome β5 subunit activity after the pulsatile CFZ treatment with or without hyperthermia. The pulsatile CFZ treatment at 250 nM slightly induced cell death in KMS-11, OPM-2, and RPMI8226 cells, and killed the majority of MM.1S cells (Fig.1A). The pulsatile CFZ treatment immediately suppressed β5 subunit activity in all MM cells (Fig.4A). However, β5 subunit activity recovered in CFZ-resistant MM cells, namely, KMS-11, OPM-2, and RPMI8226 cells, but not in CFZ-susceptible MM.1S cells at 24 hours. The combination of the heat treatment at 43℃ for 20 minutes minimized the recovery of proteasome β5 subunit activity. The pulsatile CFZ treatment increased *PSMB5* mRNA levels in MM cells (Fig.4B), which is in accordance with a phenomenon called ‘‘the bounce-back response’’[15, 16]. However, *PSMB5* mRNA expression in MM cells was not affected by the heat treatment (Fig.4C). Since CFZ irreversibly inhibited proteasome β5 subunit activity, the recovery of β5 subunit activity after 24 hours may be attributed to the synthesis of a new β5 subunit in MM cells. Based on the immediate and prompt suppression of translation by the heat treatment, it appears to inhibit the synthesis of new β5 subunits, thereby mitigating the recovery of β5 subunit activity or prolonging the inhibition of β5 subunit activity by the pulsatile CFZ treatment.

Transient receptor potential vanilloid 1 antagonists enhance the cytotoxicity of hyperthermia in MM cells.

Transient receptor potential vanilloid 1 (TRPV1) is a cation channel that transduces pain, heat, and osmotic stimuli as well as acid/basic pH changes [17, 18]. As a heat sensor, TRPV1 is activated at temperatures of 42℃ and higher [19]. TRPV1 is expressed in various types of cells, including cancer cells [20-22]. Its expression was previously shown

to be higher in MM cell lines and primary MM cells than in normal plasma cells [23]. MM cells constitutively expressed *TRPV1* mRNA, and its expression increased after the heat treatment at 43℃ for 20 minutes (Fig.5A). However, the pulsatile CFZ treatment did not affect *TRPV1* mRNA expression in MM cells (Supplementary Figure 2). The treatment with the TRPV1 antagonists, AMG9810 and SB366791, enhanced the cytotoxic effects of the heat treatment on MM cells (Figs.5B and 5C, respectively).

Discussion

Hyperthermia has been approved as an adjuvant local anti-cancer treatment in Japan and can be applied in combination with radiotherapy or chemotherapy for the treatment of cancers. Different types of hyperthermia delivery equipment are currently available and under investigation with better controlled and more effective forms of heat delivery.

PIs are widely used to treat patients with new and relapsed/refractory MM. However, the acquisition of resistance to PIs is a major obstacle to the treatment of MM [24]. The molecular mechanisms contributing to the acquisition of resistance to PIs include the up-regulation of constitutive proteasome subunits, point mutations in the *PSMB5* gene, and the aberrant activation of pro-survival pathways in MM cells [25, 26]. Nevertheless, hyperthermia is suggested to kill MM cells irrespective of their resistance to PIs, while normal quiescent cells appear to be less susceptible to cytotoxicity by hyperthermia (Fig.1B). Mild hyperthermia at 43℃ for 20 minutes only weakly induced cell death in MM cell lines; however, hyperthermia killed MM cells in combination with CFZ, even at suboptimal concentrations (Fig.1C). We previously reported that hyperthermia eradicated side population fractions with MM progenitors with a self-renewal capacity and enhanced MM cell death in combination with bortezomib [4]. We also reported that hyperthermia induced endoplasmic reticulum (ER) stress in MM cells through prompt phosphorylation of eIF2α besides perturbation of protein structure and function [4]. Furthermore, hyperthermia has been shown to induce the activation of the intrinsic apoptotic pathway with the accumulation of the proapoptotic BH3-only protein Noxa in MM cells [27, 28], which is also triggered by PIs [29-31], suggesting the cooperation of hyperthermia with PIs.

However, the mechanisms by which hyperthermia overcomes drug resistance in MM cells have yet to be elucidated. MM cells appeared to be more susceptible to the suppression of translation initiation by the heat treatment than normal PBMCs, as demonstrated using puromycin incorporation assays (Fig.2C). The incorporation of puromycin was almost completely abolished in parallel with the phosphorylation of eIF2α in MM cells immediately after mild hyperthermia at 43℃ for 20 minutes, but not by the

pulsatile treatment with a high dose of CFZ. This prompt and potent suppression of translation by heating may reduce the levels of proteins with a short half-life. We previously reported that PIM2 was highly expressed and played a critical role in the growth and survival of MM cells [5, 6]. Furthermore, NRF2 is a major cellular defense factor against oxidative stress induced by external insults, including exposure to chemotherapeutic agents [32]. PIM2 and NRF2 proteins accumulated in MM cells immediately after exposure to CFZ for 1 hour, suggesting the rapid proteasomal degradation of these proteins. However, concomitant hyperthermia ameliorated the accumulation of these proteins in MM cells and decreased their levels upon the treatment with CFZ. Therefore, the accumulation of PIM2 and NRF2 proteins has been suggested to contribute to MM cell resistance to PIs. Since the majority of proteins are subjected to proteasomal degraduation in cells, PIs induce the accumulation of various factors that are critical for MM cell growth and survival and their drug resistance other than PIM2 and NRF2, which may be suppressed with hyperthermia. Although we focused on PIM2 and NRF2 proteins in the present study, therefore, proteins other than PIM2 and NRF2 proteins may also be involved in the resistance against PIs. We tentatively checked certain proteins relevant to MM cell growth and survival, including MYC, IRF4, MCL1, and Sp1 under treatment with heat or CFZ alone or both in combination. We found the reduction of MYC after the heat treatment as shown in Supplementary Figure 3; however, the levels of the other proteins were not clearly changed. To screen such factors appears to be important for development of novel therapeutic options.

The recovery of β5 subunit activity appears to be another mechanism of MM cell resistance to CFZ. Proteasome expression and activity are generally accepted to be up-regulated in MM cells because these cells robustly synthesize proteins, including the M protein. Moreover, β5 subunit synthesis increased after a treatment with PIs, the so-called "bounce-back response". Although CFZ is an irreversible PI, the synthesis of new β5 subunit is considered to compromise the anti-MM effects of CFZ. However, a concomitant treatment with heat may promptly suppress the synthesis of new β5 subunits at the translational level, and, thus, may mitigate the recovery of β5 subunit activity in order to prolong the inhibition of β5 subunit activity after the pulsatile CFZ treatment. In addition, we further analyzed β1 and β2 subunit activity in MM cells under heat treatment at 43˚C for 20 minutes or pulsatile treatment with CFZ at 500 nM for 1 hour. However, β1 and β2 subunit activity was not significantly changed in our experimental conditions with heat treatment or pulsatile treatment with CFZ (Supplementary Figure 4).

TRPV1 is a sensor of ambient pH and temperature [17, 18, 33]. We previously reported the predominant role of TRPV1 in the acid-induced activation of the PI3K-Akt

pathway in MM cells [34]. The PI3K-Akt survival pathway is activated in MM cells under acidic conditions, which further up-regulates the expression of TRPV1 to facilitate acid sensing, thereby forming a positive feedback loop between acid sensing and the activation of PI3K-Akt survival signaling in MM cells. TRPV1 also senses heat. TRPV1 antagonists enhanced the cytotoxic effects of hyperthermia, which is consistent with our previous findings showing that the acid-induced activation of TRPV1 promoted MM cell survival. TRPV1 also senses noxious pain in sensory nerves and, thus, the inhibition of TRPV1 not only enhances the therapeutic effects of hyperthermia, but may also desensitize or alleviate heat-associated pain, discomfort with heat sensation, and hyperhidrosis in patients undergoing hyperthermia.

Heat shock proteins (HSPs) are members of the chaperone family of proteins to protect various proteins from their degradation. Hyperthermia promptly induces translocation of heat shock transcription factor 1 (HSF-1) into the nucleus and thereby the transcription of HSPs. We previously reported that heat treatment at 43˚C upregulated the protein levels of HSP70 and HSP60 in MM cells [4]. Therefore, MM cell-specific inhibition of HSPs is expected to enhance the therapeutic efficacy of hypothermia against MM cells.

Hyperthermia exerts multifaceted effects on cancer cells. The therapeutic efficacy of hyperthermia is expected to be augmented in combination with treatment options with different mode of action. However, combinatory effects of hyperthermia with currently available therapeutic options, including therapeutic monoclonal antibodies, are largely unknown. Further studies on the efficacy of combinatory treatment of hyperthermia with currently available therapeutic options are warranted.

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Figure legends

Fig. 1. Hyperthermia in combination with the pulsatile carfilzomib treatment cooperatively induces MM cell death.

A. KMS-11, U266B1, RPMI8226, OPM-2, and MM.1S cells were treated with CFZ at the indicated concentration for 1 hour. After the treatment, cells were washed and cultured at 37℃ for 24 hours. B. MM cell lines (left) and PBMCs from normal donors (right) were cultured at 43℃ for the indicated periods. After the treatment, cells were washed and cultured at 37℃ for 24 hours. C. KMS-11, OPM-2, and RPMI8226 cells were cultured at 37 or 43℃ for 20 minutes with or without the pulsatile CFZ treatment at 250 nM. After the treatment, cells were washed and cultured at 37℃ for 24 hours. Cell viability was analyzed using the WST-8 assay (n=3). **p <0.01.

Fig. 2. The heat treatment induces the immediate blockade of translation in MM cells.

KMS-11, OPM-2, RPMI8226, and U266B1 cells were cultured at 37 or 43℃ for 20 minutes with or without the pulsatile CFZ treatment at the indicated concentration for 1 hour. A. Puromycin was added at 1 μM for the last 15 minutes of the treatment. Cells were harvested, and the incorporation of puromycin was assessed by a Western blot analysis. β-actin was blotted as a loading control. B. Cells were harvested, and the protein levels of p-eIF2α and e-IF2α were analyzed by a Western blot analysis. The relative levels of each protein were normalized to the loading control. C. PBMCs from normal donors were cultured at 37 or 43℃ for 20 minutes. The incorporation of puromycin was examined by a Western blot analysis.

Fig. 3. The heat treatment rapidly reduces NRF2 and PIM2 protein levels and marginally affects *NRF2* and *PIM2* mRNA expression.

A. KMS-11, U266B1, MM.1S, OPM-2, and RPMI8226 cells were treated with or without CFZ at the indicated concentrations for 6 hours (continuous) or 1 hour (pulse) and were then cultured at 37℃ for 5 hours. B. KMS-11 and OPM-2 cells were cultured at 37 or 43℃ for 20 minutes (heat treatment) with or without the pulsatile CFZ treatment. Cell lysates were harvested and the protein levels of NRF2 and PIM2 were analyzed by a Western blot analysis. β-actin was blotted as a loading control. The relative expression levels of each protein were normalized to the loading control. C. KMS-11, OPM-2, U266B1, RPMI8226, and MM.1S cells were treated with or without heating at 43℃ for 20 minutes. *NFE2L2* and *PIM2* mRNA expression was analyzed by real-time PCR. *GAPDH* was used as an internal control.

Fig. 4. The heat treatment inhibits the recovery of β5 proteasome subunit activity in MM cells in the presence of CFZ.

A. KMS-11, OPM2, RPMI8226, and MM.1S cells were cultured at 37 or 43℃ for 20 minutes with or without CFZ for 1 hour. Cells were harvested immediately or 24 hours after the treatment (n=3). β5 proteasome subunit activity was measured using specific fluorogenic substrates and relative changes from the baseline are shown. B. KMS-11, OPM2, RPMI8226, MM.1S, and U266B1 cells were treated with or without CFZ for 1 hour at the indicated concentration. Cells were washed, cultured at 37℃, and then harvested 6 hours after the treatment. *PSMB5* mRNA expression was assessed by real-time PCR. *GAPDH* was used as an internal control. C. KMS-11, OPM2, RPMI8226, MM.1S, and U266B1 cells were treated with or without heating at 43℃ for 20 minutes. MM cells were cultured for 6 hours, and *PSMB5* mRNA expression was then assessed by real-time PCR. *GAPDH* was used as an internal control. *p <0.05, **p <0.01, N.S. not significant.

Fig. 5. TRPV1 inhibition enhances MM cell death in combination with the heat treatment.

A. KMS-11, OPM-2, and MM.1S cells were treated with or without heating at 43℃ for 20 minutes. Cells were then cultured at 37℃ for 3 hours. *TRPV1* mRNA expression was assessed by real-time PCR. *GAPDH* was used as an internal control. KMS-11, OPM-2, and MM.1S cells were cultured at 43℃ for 20 minutes with or without the TRPV1 antagonists AMG9810 (B) and SB366791 (C) at 10 μM. Cells were washed and cultured at 37℃ for 24 hours. Cell viability was analyzed at 24 hours by the WST-8 assay (n=3). **p <0.01.

Figure

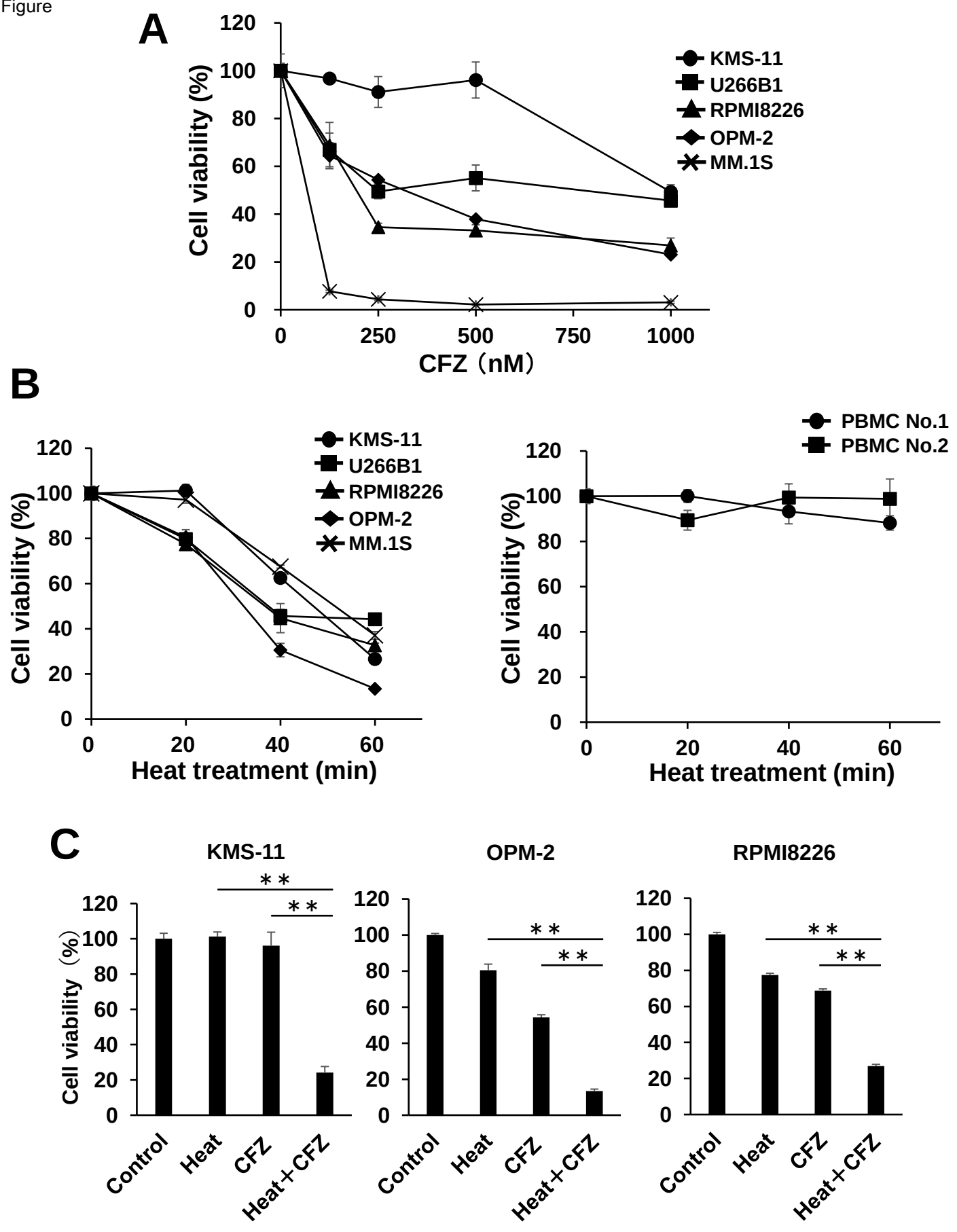
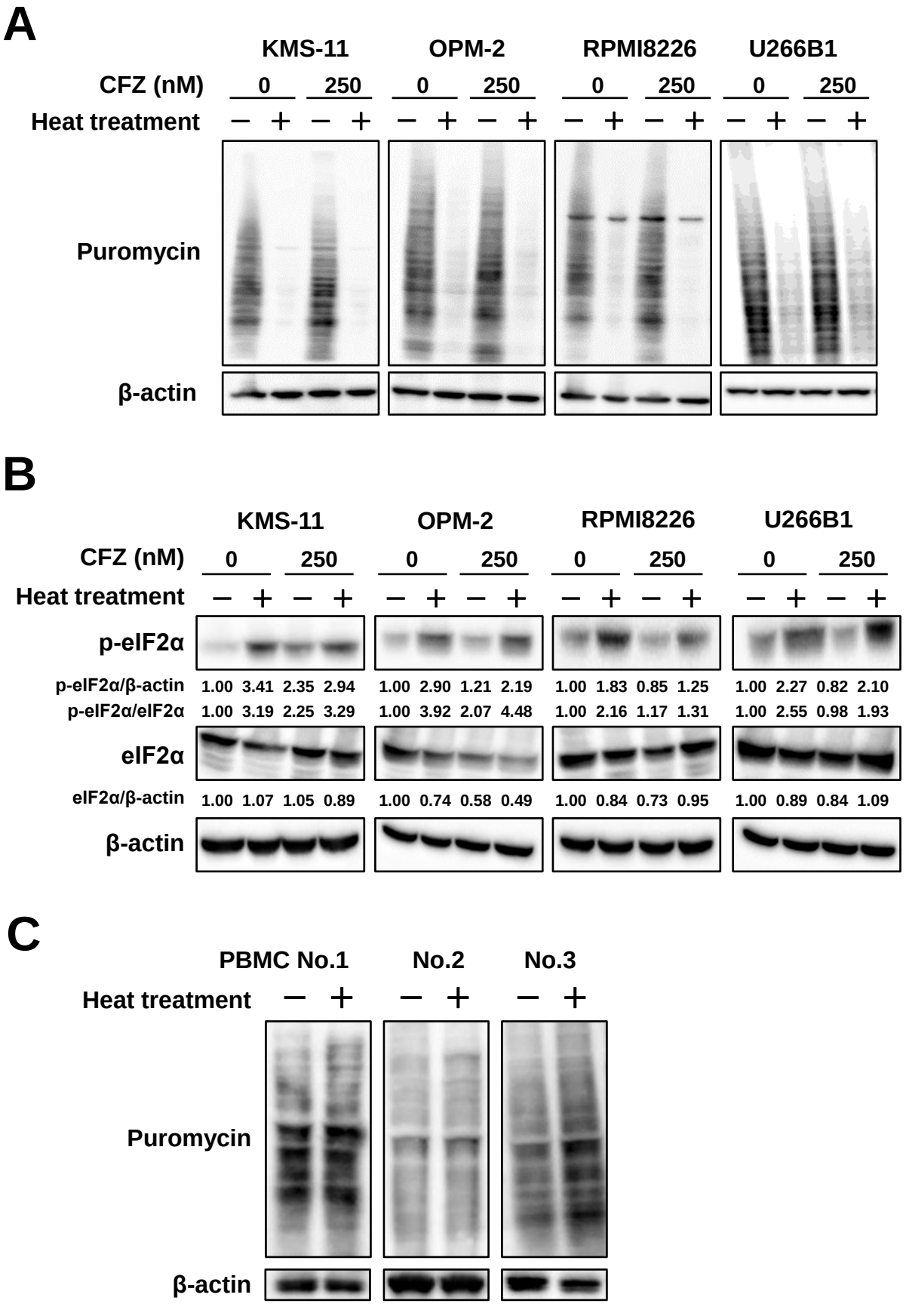


Figure 1


A
KMS-11
OPM-2
RPMI8226
U266B1
CFZ (nM)
0
250
Heat treatment
−
+
Puromycin
β-actin
B
KMS-11
OPM-2
RPMI8226
U266B1
CFZ (nM)
Heat treatment
p-eIF2α
p-eIF2α/β-actin 1.00 3.41 2.35 2.94 1.00 2.90 1.21 2.19 1.00 1.83 0.85 1.25 1.00 2.27 0.82 2.10
p-eIF2α/eIF2α 1.00 3.19 2.25 3.29 1.00 3.92 2.07 4.48 1.00 2.16 1.17 1.31 1.00 2.55 0.98 1.93
eIF2α
eIF2α/β-actin 1.00 1.07 1.05 0.89 1.00 0.74 0.58 0.49 1.00 0.84 0.73 0.95 1.00 0.89 0.84 1.09
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C
PBMC No.1
No.2
No.3
Heat treatment
Puromycin
β-actin

Figure 2

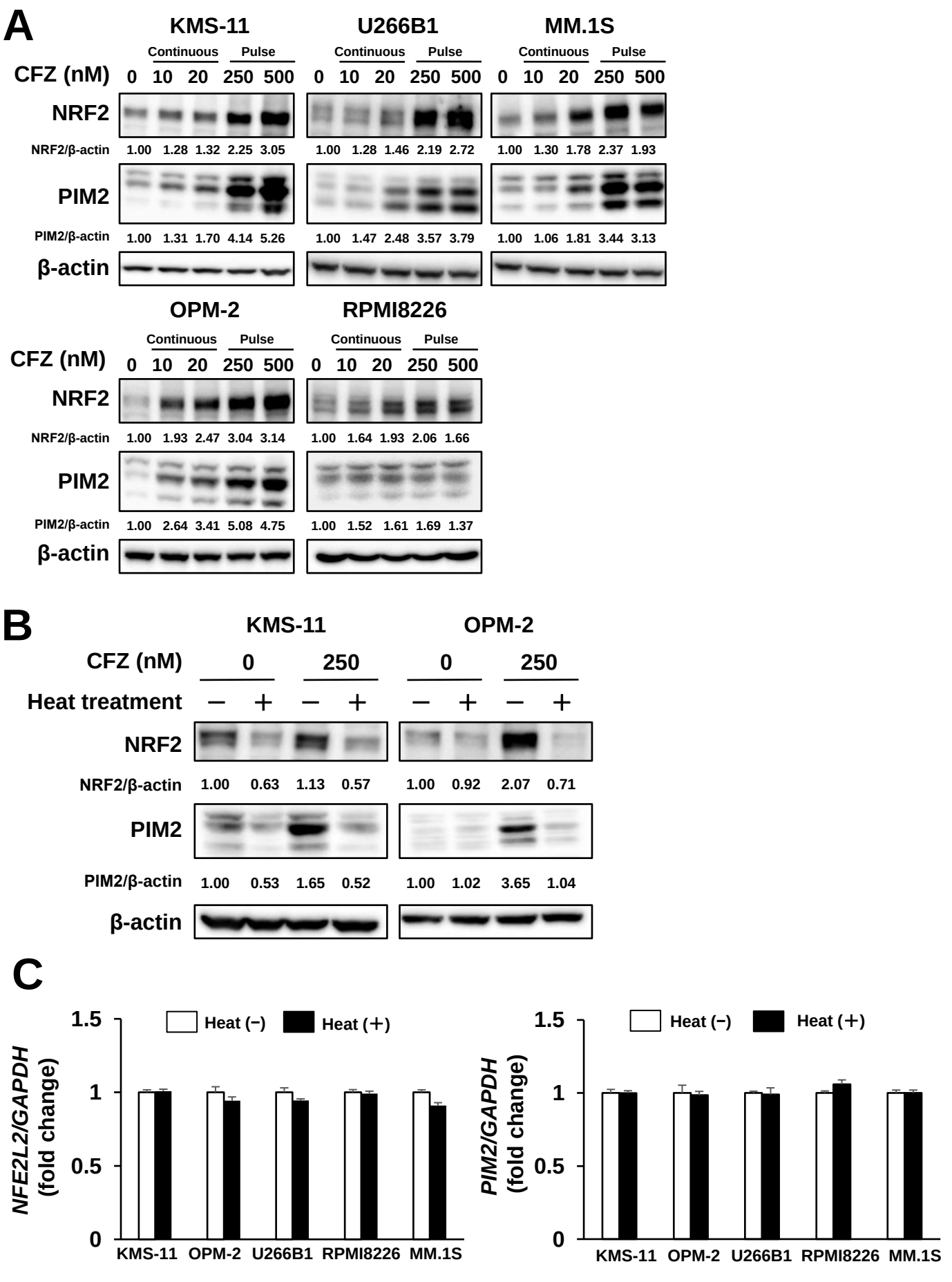
A
KMS-11
U266B1
MM.1S
Continuous
Pulse
CFZ (nM)
0 10 20 250 500
NRF2
NRF2/β-actin
1.00 1.28 1.32 2.25 3.05
1.00 1.28 1.46 2.19 2.72
1.00 1.30 1.78 2.37 1.93
PIM2
PIM2/β-actin
1.00 1.31 1.70 4.14 5.26
1.00 1.47 2.48 3.57 3.79
1.00 1.06 1.81 3.44 3.13
β-actin
OPM-2
RPMI8226
1.00 1.93 2.47 3.04 3.14
1.00 1.64 1.93 2.06 1.66
1.00 2.64 3.41 5.08 4.75
1.00 1.52 1.61 1.69 1.37
B
KMS-11
OPM-2
CFZ (nM)
0
250
Heat treatment
– + – + – + – +
NRF2
NRF2/β-actin
1.00 0.63 1.13 0.57
1.00 0.92 2.07 0.71
PIM2
PIM2/β-actin
1.00 0.53 1.65 0.52
1.00 1.02 3.65 1.04
β-actin
C
Heat (–)
Heat (+)
NFE2L2/GAPDH (fold change)
PIM2/GAPDH (fold change)
1.5
1
0.5
0
KMS-11 OPM-2 U266B1 RPMI8226 MM.1S

Figure 3

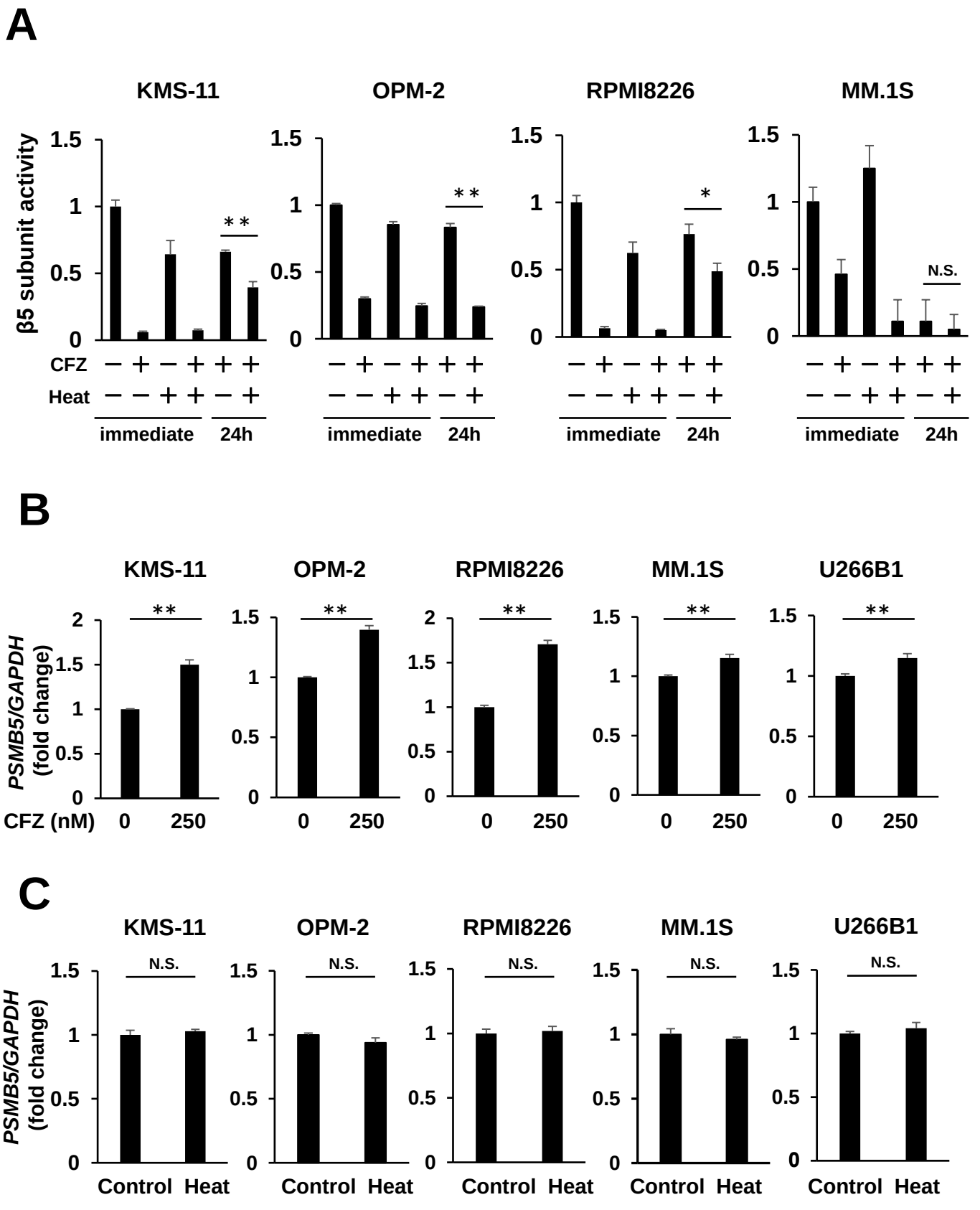
A
KMS-11
OPM-2
RPMI8226
MM.1S
β5 subunit activity
1.5
1
0.5
0
**
**
*
N.S.
CFZ
Heat
immediate
24h
B
KMS-11
OPM-2
RPMI8226
MM.1S
U266B1
PSMB5/GAPDH
(fold change)
2
1.5
1
0.5
0
**
CFZ (nM)
0
250
C
KMS-11
OPM-2
RPMI8226
MM.1S
U266B1
PSMB5/GAPDH
(fold change)
N.S.
Control
Heat

Figure 4

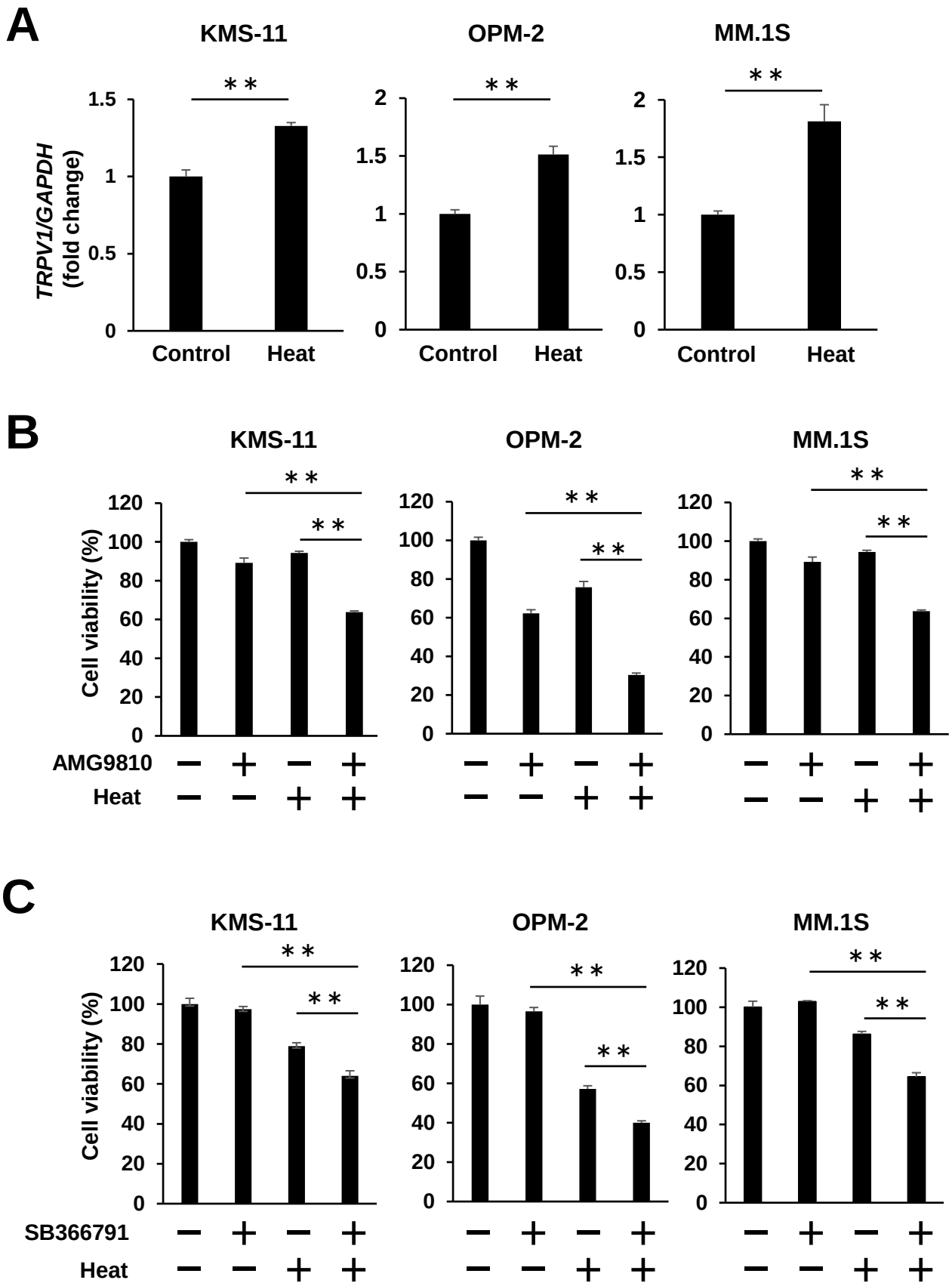
A
KMS-11
OPM-2
MM.1S
TRPV1/GAPDH (fold change)
Control
Heat
B
Cell viability (%)
AMG9810
Heat
C
SB366791
Heat

Figure 5

Akifumi Takaori-Kondo MD, PhD
Editor-in-Chief, International Journal of Hematology

Dear Professor Akifumi Takaori-Kondo,

Re. Manuscript ID: IJHM-D-23-00468

Title: Acute suppression of translation by hyperthermia reinforces anti-myeloma activity of carfilzomib

We would like to thank the reviewers and Academic Editor of *International Journal of Hematology* for their thoughtful review of our paper referenced above. We have attempted to address all concerns raised by the reviewers and revised our paper. All changes to the paper are indicated in red in the revised manuscript to make it easier for the reviewers to follow our changes. We believe that the revised manuscript has been improved significantly and is now acceptable for publication in *International Journal of Hematology*.

All the authors have reviewed the manuscript, agreed its contents and approved its re-submission to *International Journal of Hematology* for publication consideration.

Thank you very much for your kind consideration of our manuscript.

Sincerely,

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