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ORIGINAL ARTICLE

Activity of TAS4464, a novel NEDD8 activating enzyme E1 inhibitor, against multiple myeloma via inactivation of nuclear factor κB pathways

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Abstract

The ubiquitin proteasome pathway is essential for the proliferation and survival of multiple myeloma (MM) cells. TAS4464, a novel highly potent inhibitor of NEDD8 activating enzyme, selectively inactivates cullin-RING ubiquitin E3 ligases, resulting in accumulation of their substrates. Here, we examined 14 MM cell lines treated with TAS4464. TAS4464 induced growth arrest and cell death in the MM cell lines even in the presence of bone marrow stromal cells. It also induced the accumulation of phospho-inhibitor of κBα and phospho-p100, impaired the activities of nuclear factor κB (NF-κB) transcription factors p65 and RelB, and decreased the expression of NF-κB target genes, suggesting that TAS4464 inhibits both the canonical and non–canonical NF-κB pathways. TAS4464 had similar effects in an in vivo human-MM xenograft mouse model in which it was also observed to have strong antitumor effects. TAS4464 synergistically enhanced the antitumor activities of the standard MM chemotherapies bortezomib, lenalidomide/dexamethasone, daratumumab and elotuzumab. Together, these results suggest that the anti–MM activity of TAS4464 occurs via inhibition of the NF-κB pathways, and that treatment with TAS4464 is a potential approach for treating MM by single and combination therapies.

KEYWORDS
molecular targeted therapy, multiple myeloma, NEDD8 activating enzyme, nuclear factor κB, ubiquitin-like protein NEDD8

1 | INTRODUCTION

Multiple myeloma (MM) is a type of blood cancer in which malignant plasma cells accumulate in the bone marrow and produce an abnormal antibody called M protein. In the past decade, several molecular-targeted agents and antibodies such as proteasome inhibitors (bortezomib, carfilzomib and ixazomib), immunomodulatory imide drugs (thalidomide and lenalidomide) and monoclonal antibodies (daratumumab and elotuzumab) have been approved for the treatment of relapsed MM.[1,2] Although

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most patients respond to those treatments initially, almost all patients who survive initial treatment eventually relapse and require further therapy. Therefore, more effective treatment options are urgently needed for the treatment of refractory MM.

Nuclear factor κB (NF-κB) is constitutively activated in MM cells and is a trigger for the progression of MM.[3-6] NF-κB activity is controlled by canonical and non–canonical pathways. The NF-κB family of transcription factors comprises p65 (RelA), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2). These transcription factors form heterodimers such as p65–p50, which is involved in the canonical pathway, and p52–RelB, which is involved in the non–canonical pathway.[7] Although these dimers are constitutively present in the cytosol and nucleus, p65–p50 and p52–RelB are inactivated in the cytosol by inhibitor of κBα (IκBα) and p100–RelB, respectively; p100 is the precursor of p52. Treatment of MM cells with proteasome inhibitors accumulates unfolded proteins in the endoplasmic reticulum and cytosol.[8] This accumulation triggers several pro–apoptotic factors and cell stress accumulation, leading to apoptosis.[9] Inactivation of NF-κB signaling activated in MM via accumulation of phospho-IκBα (p-IκBα) by blocking proteasomal degradation has been reported as another mechanism of action of a proteasome inhibitor in MM.[10]

Conjugation of the ubiquitin-like protein NEDD8 to its target proteins (neddylation) occurs via a process very similar to ubiquitination, although the process requires different E1 and E2 proteins for ubiquitin activation and conjugation, respectively. NEDD8 activating enzyme E1 (NAE, a heterodimer comprising APPBP1 and UBA3 subunits), the first enzyme in the neddylation pathway, activates cullin-RING E3 ubiquitin ligases (CRL) thorough the neddylation of cullin proteins. Activated CRL conjugate ubiquitin to their substrates and promote ubiquitin-dependent degradation of their substrates by proteasomes.[11] CRL substrates include many key proteins essential for a diverse range of cellular processes, including cell cycle progression, DNA replication and signal transduction. Neddylation is considered an essential process for cancer progression because overexpression of NEDD8 and NAE have been reported in various cancers.[12-15] Furthermore, elevated levels of NEDD8 transcripts are correlated with poor prognosis in bortezomib-treated MM patients.[16] Studies with the NAE inhibitor MLN4924 (pevonedistat) suggest that NAE is an attractive target for cancer intervention.[17] MLN4924 is reported to induce apoptosis in MM cells through suppression of PI3K/mTOR signaling via REDD1 accumulation.[18]

TAS4464 is one of the most selective and potent NAE inhibitors reported to date both in vitro and in vivo.[19] TAS4464 exhibits widespread antiproliferative activity in various tumor cell lines and is especially active against most hematologic malignancy cell lines, including myeloma-derived cell lines.[19]

In this study, we investigated the antitumor properties and mechanism of action of NAE inhibition by TAS4464 in MM cells, focusing on the perturbation of the NF-κB signaling that is activated in MM cells.

2 | MATERIALS AND METHODS

2.1 | Chemical compounds

TAS4464 was synthesized at Taiho Pharmaceutical. Bortezomib was purchased from LKT Laboratories. Lenalidomide was purchased from MedChemExpress. Dexamethasone was purchased from Kyoritsu Seiyaku. Daratumumab was purchased from Janssen-Cilag. Elotuzumab was purchased from Bristol-Myers Squibb GmbH & Co. KGaA.

2.2 | Cell lines and cell culture

Fourteen MM cell lines and HS-5 bone marrow stromal cells (BMSC) were used. EJM (ACC-560), LP-1 (ACC-41) and OPM-2 (ACC-50) were purchased from DSMZ. KMS-11 (JCRB1179) was obtained from the Health Science Research Resources Bank. KMS-12-BM (JCRB0429), KMS-12-PE (JCRB0430), KMS-21-BM (JCRB1185), KMS-26 (JCRB1187), KMS-28-BM (JCRB1192) and KMS-34 (JCRB1195) were obtained from the Japanese Collection of Research Bioresources Cell Bank. HS-5 (CRL-11882), MM.1S (CRL-2974), MM.1R (CRL-2975), NCI-H929 (CRL-9068) and U266B1 (TIB-196) were obtained from the American Type Culture Collection. EMJ was cultured in Iscove's modified Dulbecco's medium supplemented with 20% FBS. HS-5 was cultured in DMEM supplemented with 10% FBS. NCI-H929 was cultured in RPMI 1640 medium supplemented with 10% FBS and 0.05 mmol/L 2-mercaptoethanol. U266B1 was cultured in RPMI 1640 medium supplemented with 15% FBS. The other cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS. All cell lines used were authenticated by means of short tandem repeat-based DNA profiling.

2.3 | Growth inhibition assay

Cells were treated with TAS4464 for 72 hours. Then, cell viability was measured by using the CellTiter-Glo 2.0 Assay (Promega). To assess the cytotoxicity of TAS4464, growth inhibition curves were generated in which the percentage of growth was determined with the following formula: $100 \times (T - T_0)/(C - T_0)$, where T = mean luminescence measured with the CellTiter-Glo 2.0 Assay after 3 days of treatment; T_0 = mean luminescence measured with the CellTiter-Glo 2.0 Assay on the day the treatment was started; and C = mean luminescence measured with the CellTiter-Glo 2.0 Assay after 3 days without TAS4464 treatment. If T was less than T_0, the formula was modified as follows: $100 \times (T - T_0)/T_0$.

2.4 | Co–culture of myeloma and stromal cell lines

The human multiple myeloma cell line MM.1S was plated on pre–seeded monolayers of the human stromal cell line HS-5. After 24 hours of co–culture of MM.1S cells with HS-5 cells at 37°C in a humidified atmosphere containing 5% CO_2, cells were treated with TAS4464 for 48 hours. Then, MM.1S and HS-5 cell viability were

measured by using a Vi-CELL XR Cell Viability Analyzer (Beckman Coulter).

2.5 | Flow cytometric analysis

Cells were treated with TAS4464 and then fixed and stained by using a BD Cycletest Plus DNA Reagent Kit (BD Biosciences). Stained cells were analyzed by using a fluorescence-activated cell sorter (BD FACSVerse, BD Biosciences).

2.6 | Antibodies and western blotting

Anti–NEDD8 (ab81264), anti–NRF2 (ab62352) and anti–α-tubulin (ab4074) were purchased from Abcam PLC. Anti–CDT1 (#8064), anti–p-IκBα (#2859 or #9246) anti–p-p100 (#4810), anti–p21 (#2947), anti–cleaved PARP (#9541), anti–cleaved caspase-3 (#9664) and anti–cleaved caspase-8 (#9496) were purchased from Cell Signaling Technology. Anti–GADD 34 (sc-8327) and anti–PTTG (sc-56207) were purchased from Santa Cruz Biotechnology. Cells were treated with TAS4464, washed in PBS, and lysed in lysis buffer (M-PER Mammalian Protein Extraction Reagent [Thermo Fisher Scientific] supplemented with cOmplete, Mini Protease Inhibitor Cocktail [Roche Applied Science] and PhosSTOP Phosphatase Inhibitor Cocktail [Roche Applied Science] as recommended by the manufacturer, and 2 mmol/L of 1,10-phenanthroline [Tokyo Chemical Industry]). Lysates were then centrifuged at 17 800 *g* for 10 minutes, and the supernatants were collected. Proteins were separated by means of SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories). Membranes were blocked with Blocking One or Blocking One P blocking reagent (Nacalai Tesque), then probed with the appropriate primary antibodies, which were diluted using 5% (v/v) Blocking One or Blocking One P in TBS supplemented with 0.05% (v/v) Tween 20 (TBS-T). The membranes were then incubated with HRP-linked secondary antibodies (Cell Signaling Technology) that were diluted using 5% (v/v) Blocking One or Blocking One P in TBS-T. Proteins were visualized by means of luminol-based enhanced chemiluminescence (Thermo Fisher Scientific). Luminescent images were captured with an LAS-3000 imaging system (Fuji Photo Film) or Amersham Imager 600 (GE Healthcare Japan).

2.7 | Quantitative RT-PCR

TaqMan Gene Expression Assays (gene symbols and assay IDs are shown in Table S1) were purchased from Thermo Fisher Scientific. Cells and tumors were treated with TAS4464 for the indicated times. Then, DNase-treated RNA was isolated from cells by using an RNeasy Plus Mini Kit (Qiagen). For the extraction of RNA from tumors, the excised tumor was soaked in ISOGEN (Nippon Gene) and then homogenized with Pellet Mixer for Microtubes 1.5 mL (Nolato Treff). DNase-treated RNA was isolated by using an RNeasy Plus Mini Kit. cDNA was synthesized by using SuperScript III First-Strand Synthesis SuperMix for quantitative RT-PCR (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed by using a 7900HT Fast Real-Time PCR System (Applied Biosystems) or a QuantStudio 7 Flex System (Thermo Fisher Scientific). Relative mRNA expression was calculated by using the comparative Ct method with the following formulae:

$$\Delta Ct = (\text{Ct of target gene in sample}) - (\text{Ct of 18S gene in sample})$$

$$\Delta\Delta Ct = (\Delta Ct \text{ of target gene in sample}) - (\text{mean } \Delta Ct \text{ of target gene in control}).$$

2.8 | Quantification of nuclear factor κB binding activation

Cells were treated with TAS4464 for 4 hours, and then nuclear extracts were prepared by using a Nuclear Extract Kit (Active Motif). NF-κB binding to its consensus DNA site was quantified by using a TransAM NFκB Family Kit (Active Motif). Statistical significance was calculated by using Student's *t* test to assess the difference in NF-κB binding between the control and TAS4464-treated groups. $P < 0.05$ was considered statistically significant.

2.9 | In vivo efficacy studies

Multiple myeloma cells were subcutaneously implanted into male SCID mice (Charles River Laboratories Japan) and allowed to grow to over 100 mm^3. Six animals were assigned to each group for each experiment, and TAS4464 formulated in 5% (w/v) glucose was administered intravenously twice a week at 50 or 100 mg/kg. Bortezomib formulated in 2% (v/v) ethanol and 0.98% (w/v) ascorbic acid in saline was administered intravenously twice a week at 1 mg/kg. Lenalidomide formulated in 0.25% (v/v) Tween 20 and 0.5% (w/v) carboxymethylcellulose in sterile purified water was orally administered daily at 30 mg/kg. Dexamethasone was diluted in D-PBS (−) and administered orally at 1 mg/kg for 4 consecutive days followed by 4 days' rest. Daratumumab was diluted in saline and administered intraperitoneally once a week at 8 mg/kg. Elotuzumab was diluted in saline and administered intraperitoneally once a week at 10 mg/kg. The individual therapies were performed for 3 weeks. Combinations of therapies followed the same administration schedules as the individual therapies for 3 weeks. Tumor volume was calculated with the following formula: $[\text{length} \times (\text{width})^2]/2$. The vehicle for TAS4464 (5% [w/v] glucose) was used as the control for all treatments. Statistical significance was calculated by using Dunnett's test to assess the difference in tumor volumes between the control (vehicle treated) and agent-treated groups. The Aspin-Welch's *t* test was used to assess the difference in tumor volumes between the single agent-treated groups and the combination therapy-treated groups. $P < 0.05$ was considered statistically significant. For pharmacodynamic analysis, tumors were harvested after administration of TAS4464. For evaluation of the amounts and phosphorylation status of proteins, the excised tumors were homogenized in lysing matrix D (MP Biomedicals) containing lysis buffer. Lysates were then centrifuged at 17 800 *g* for 10 minutes and the supernatants were collected. The amounts

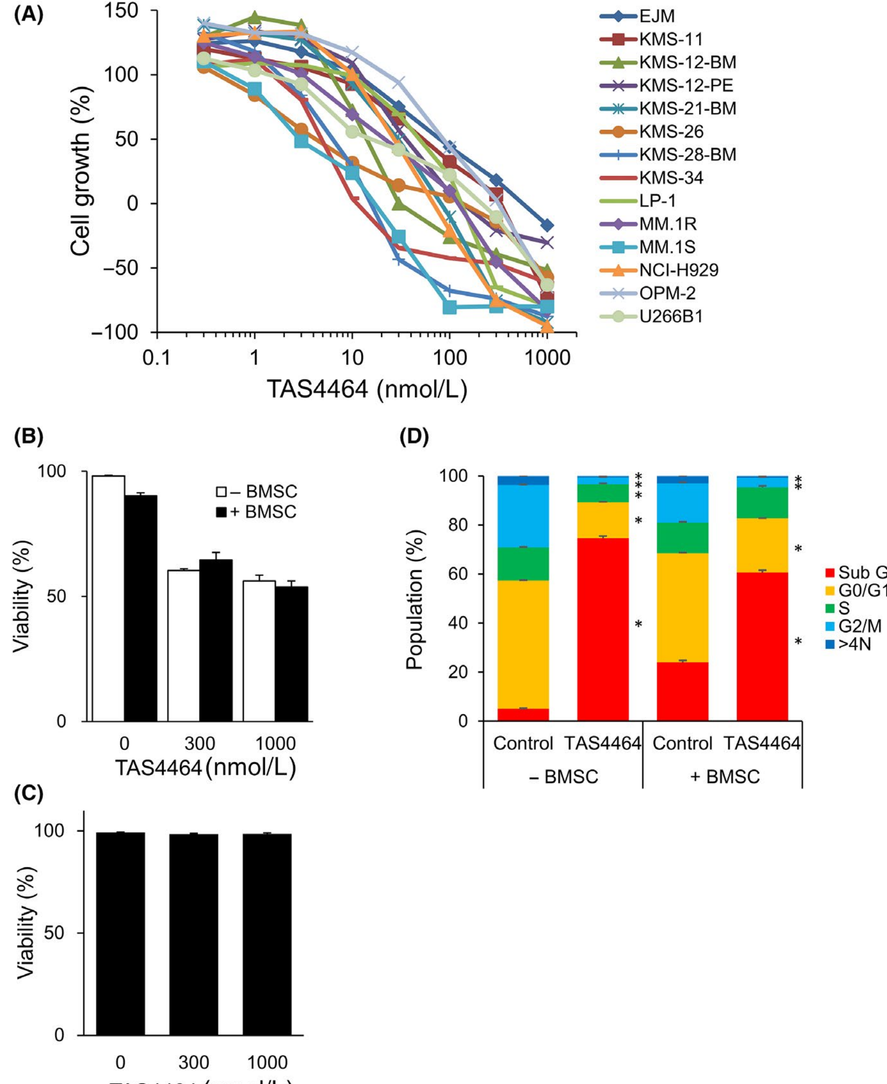


FIGURE 1 Growth inhibitory effects of TAS4464 on multiple myeloma (MM) cells and the effects of microenvironmental survival signaling on the efficacy of TAS4464 in MM.1S cells. A, Growth inhibitory effects of TAS4464 on MM cells. Cells were treated with TAS4464 (0.3-1000 nmol/L) for 72 h. Cytotoxicity was assessed by means of the CellTiter-Glo 2.0 Assay. Percentage of growth was determined as described under Section 2. B, Effects of TAS4464 on the viability of MM.1S cells in the presence or absence of bone marrow stromal cells (BMSC). C, Effect of TAS4464 on the viability of BMSC alone. MM.1S cells and BMSC were treated with 300 or 1000 nmol/L TAS4464 for 48 h. Viability was measured by means of trypan blue staining. D, Effects of TAS4464 on the cell cycle progression of MM.1S cells in the presence or absence of BMSC. DNA profiles were determined by using propidium iodide staining and a fluorescence-activated cell sorter. Percentages of cells in the sub G1, G0/G1, S, G2/M and >4N of cell cycle under each condition were calculated. Data are presented as mean ± SE (n = 3). *$P < 0.05$ vs control group in each BMSC condition by Student's t test

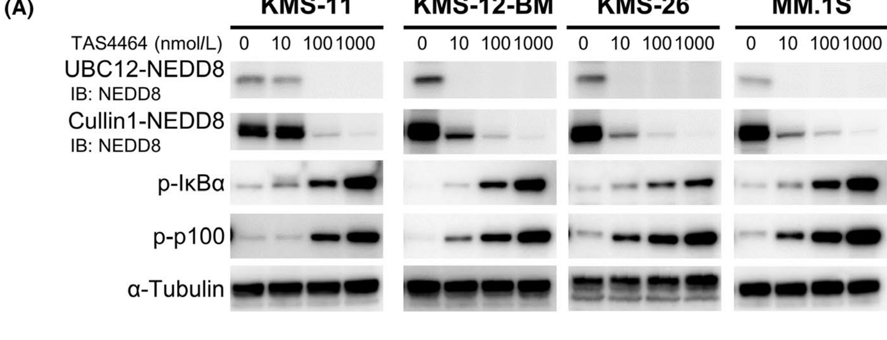
and phosphorylation status of proteins were evaluated by means of western blotting with the appropriate antibodies. For quantification of NF-κB target mRNA, the excised tumors were homogenized and then total RNA was isolated by using ISOGEN and RNeasy Plus Mini Kit. RNA was transcribed and the expression of mRNA was evaluated by means of qPCR as described above. All animal experiments were performed with the approval of the institutional animal care and use committee of Taiho Pharmaceutical and carried out according to the guidelines for animal experiments of Taiho Pharmaceutical.

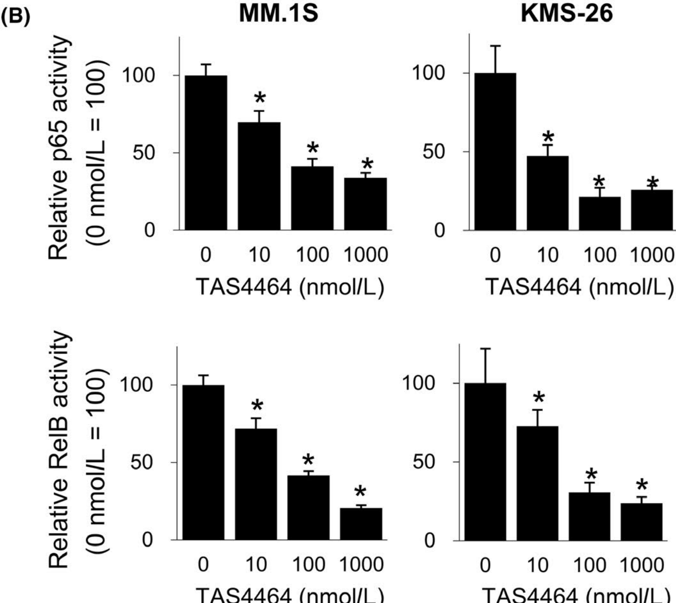
3 | RESULTS

3.1 | TAS4464 inhibits proliferation and induces apoptosis of multiple myeloma cells

To assess the growth inhibitory effect of TAS4464 in MM cells, 14 MM cell lines were treated with TAS4464 for 72 hours. TAS4464 inhibited proliferation of all of the cell lines, with the half-maximal growth inhibitory concentration lowest (3.62 nmol/L) in MM.1S cells and highest (149 nmol/L) in OPM-2 cells (Figure 1A and Table S2). In addition, at concentrations ≥ 100 nmol/L, TAS4464 increased the proportion of cells in the sub-G1 phase (Figure S1). These results indicate that TAS4464 inhibits cell growth and induces apoptotic or mitotic cell death in MM cell lines.

It has been reported that the bone marrow microenvironment is important for the growth of MM cells and their resistance to many chemotherapies.[20-22] Therefore, we assessed the impact of stromal interactions on the sensitivity of MM cells to TAS4464. MM.1S cells were co–cultured with HS-5 BMSC and then treated with 0-1000 nmol/L TAS4464 for 48 hours. The effects of TAS4464 on the viability of MM.1S cells were not attenuated by co–culture with BMSC (Figure 1B), even though TAS4464 had no effect on the viability of the HS-5 cells (Figure 1C). TAS4464 again increased the proportion of MM.1S cells in the sub-G1 phase, even in the presence of BMSC (Figure 1D; representative flow cytometry patterns are in Figure S2). Although adhesion of MM cells to BMSC upregulates the secretion of growth factors such as interleukin 6 and insulin-like growth factor 1 from BMSC,[23-26] neither of these growth factors attenuated the antiproliferative activity of TAS4464 in MM.1S cells (Figure S3).





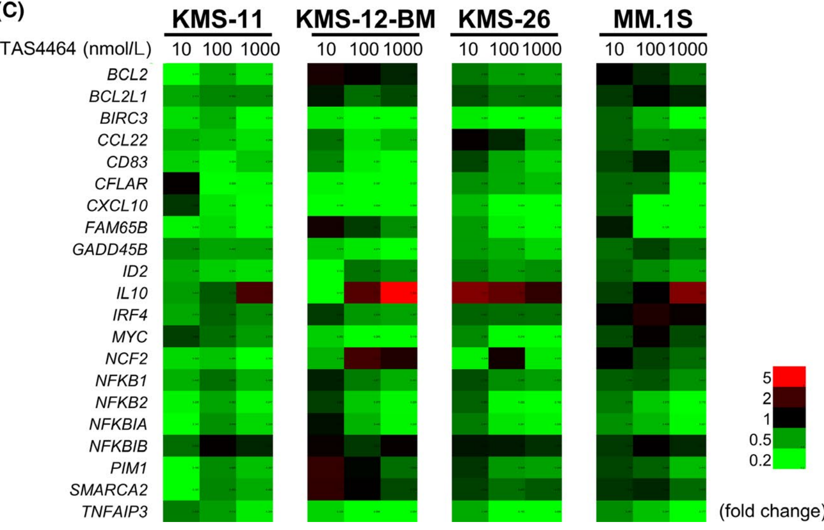


FIGURE 2 Inactivation of the nuclear factor κB (NF-κB) pathways via inhibition of cullin neddylation in multiple myeloma (MM) cells. KMS-11 (A, C), KMS-12-BM (A, C), KMS-26 (A–C) and MM.1S (A–C) cells were treated with 0-1000 nmol/L TAS4464 for 4 h. A, Effect of TAS4464 on the neddylation of cullin and the amount of p-p100 and p-IκBα in MM cell lines. Twenty micrograms of protein extracted from cells were subjected to western blot analysis. The protein levels of neddylated UBC12 and cullin1 (immunoblotted [IB] with an anti–NEDD8 antibody), p-IκBα and p-p100 were detected. The protein level of α-tubulin was used as the loading control. B, Effect of TAS4464 on the DNA binding activities of NF-κB family proteins in MM.1S and KMS-26 cells. A nuclear fraction was prepared and subjected to enzyme-linked immunosorbent assay-based quantitation of p65 (canonical pathway) or RelB (non–canonical pathway) levels. Nuclear extract used: 20 μg for p65 activity; 2 μg (MM.1S) or 10 μg (KMS-26) for RelB activity. Data are presented as mean ± SE (n = 3). *$P < 0.05$ vs 0 nmol/L group by Student's *t* test. C, Changes in NF-κB-targeted gene transcription in MM cell lines exposed to TAS4464. Fifty nanograms of total RNA from cells were subjected to quantitative reverse transcription PCR analysis and the levels of NF-κB-targeted gene transcripts were measured

3.2 | TAS4464 downregulates both the canonical and non–canonical nuclear factor κB pathways in multiple myeloma cells

We have reported that TAS4464 inhibits the neddylation pathway via inhibition of NAE, which leads to accumulation of CRL substrates in several solid and hematologic tumor cell lines.[19] In the present study, TAS4464 also reduced the levels of neddylated UBC12 (an E2 for NEDD8) and neddylated cullin1 (a component of CRL) and selectively induced the accumulation of the CRL substrates p-IκBα, CDT1, NRF2 and p21, but not substrates of non–CRL E3s (GADD34 and PTTG), in MM.1S cells in a dose-dependent and time-dependent manner (Figure S4).

We then examined whether TAS4464 could affect the NF-κB pathways activated in MM cells. Four MM cell lines were exposed

to 0-1000 nmol/L TAS4464 for 4 hours (Figure 2A). Treatment with 10 nmol/L (KMS-12-BM, KMS-26 and MM.1S cells) or 100 nmol/L (KMS-11) TAS4464 led to reduced levels of neddylated UBC12 and cullin1, but increased levels of the NF-κB regulators p-IκBα and p-p100. Therefore, we hypothesized that accumulation of p-IκBα and p-p100 by TAS4464 treatment could inhibit the NF-κB pathways. NF-κB activity was assessed using the DNA binding activity of p65 and RelB and the transcription levels of NF-κB-targeted genes. Treatment with TAS4464 for 4 hours significantly inhibited the DNA binding activity of p65 (canonical pathway) and RelB (non–canonical pathway) in MM.1S and KMS-26 cells in a dose-dependent manner ($P < 0.05$, Student's *t* test; Figure 2B), thereby downregulating both NF-κB pathways. Furthermore, under the same conditions, the transcription of NF-κB-targeted genes was downregulated in all four tested MM cell lines (Figure 2C).

3.3 | TAS4464 downregulates the nuclear factor κB pathways and has antitumor activity in human-multiple myeloma xenograft mouse models

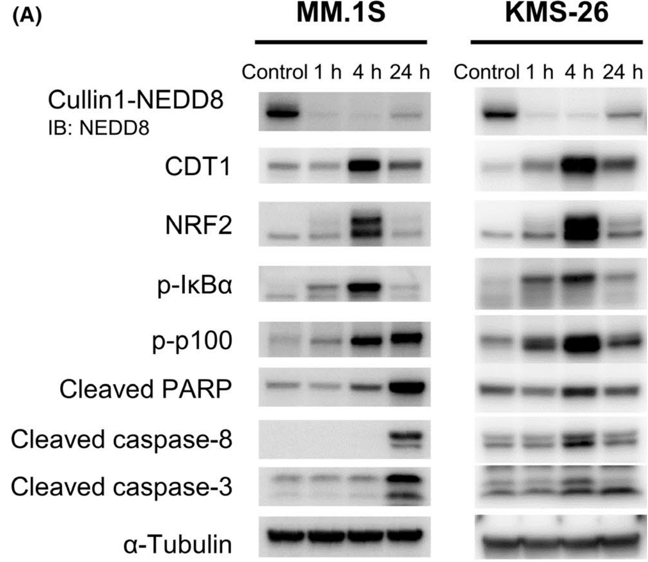
To evaluate the pharmacodynamic response to TAS4464 and the expression of NF-κB-targeted genes in vivo, MM.1S and KMS-26 tumors were harvested from human-MM xenograft mouse models at 1, 4 or 24 hours after administration of TAS4464 at 100 mg/kg (Figure 3). In both models, TAS4464 decreased the level of neddylated cullin1 as early as 1 hour after administration. In addition, at 4 hours after administration, the levels of CRL substrates were increased, indicating that TAS4464 inhibited NAE in the MM tumors. The levels of apoptosis-related factors (cleaved forms of caspase-8, caspase-3 and PARP) were also increased (Figure 3A). In addition, TAS4464 treatment downregulated NF-κB-targeted genes, suggesting downregulation of the NF-κB pathways following the accumulation of p-IκBα and p-p100 in tumors (Figure 3B).

In accordance with the inhibition of the NF-κB pathways in tumors, intravenous administration of TAS4464 at 50 or 100 mg/kg twice a week significantly inhibited tumor growth (Figure 4 and Figure S5) without remarkable body weight loss (Figure S6), whereas standard MM chemotherapies (ie, bortezomib, lenalidomide, dexamethasone, daratumumab and elotuzumab) showed weak tumor growth inhibition.

Next, we evaluated whether TAS4464 can be combined with the key MM chemotherapies bortezomib, lenalidomide/dexamethasone, daratumumab and elotuzumab. All combination groups demonstrated significantly greater tumor growth inhibition than each therapy alone (Figure 4). In addition, there were no deaths or decreases in body weight of 15% or more in any of the groups, suggesting that all treatments were tolerable (Figure S6). Together, these results indicate that TAS4464 shows marked anti–MM activity alone and enhanced anti–MM activity in combination with key MM chemotherapies.

4 | DISCUSSION

In this study, we evaluated the anti–MM activity of a highly potent and selective NAE inhibitor, TAS4464, and examined how TAS4464 affects MM cells. We found that TAS4464 inhibited both the canonical and non–canonical NF-κB pathways, which led to strong antitumor activity in human-MM xenograft mouse models.



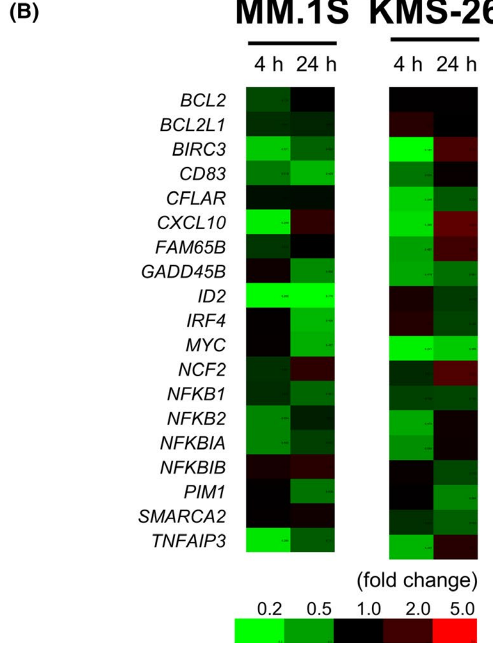
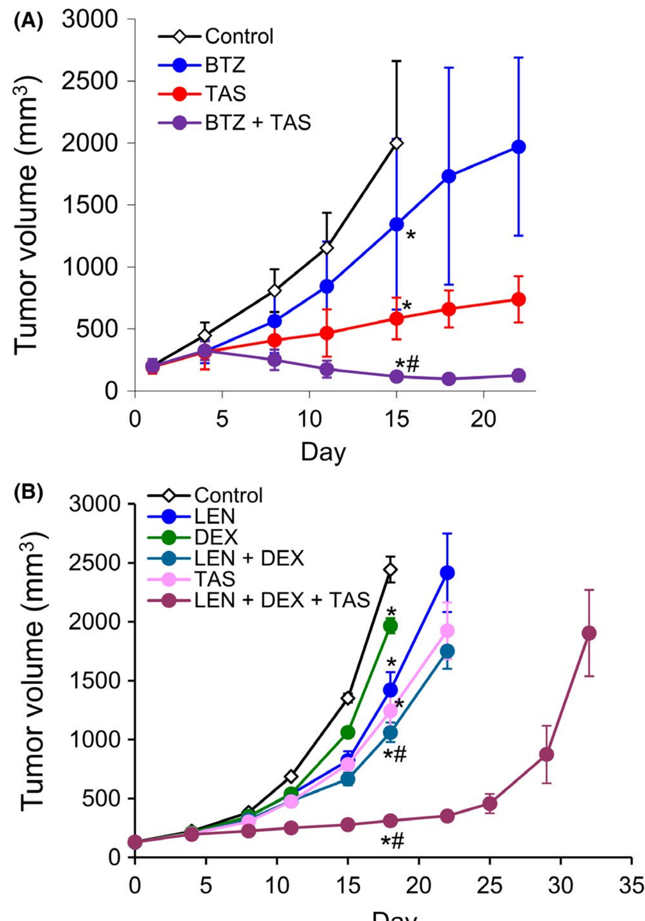


FIGURE 3 In vivo pharmacodynamic activity of TAS4464 in human multiple myeloma xenograft mouse models. Pharmacodynamic activity of TAS4464 against MM.1S and KMS-26 xenograft models. SCID mice bearing MM.1S or KMS-26 (n = 3 mice per sampling point) were intravenously administered 100 mg/kg TAS4464. At 1, 4 or 24 h after administration, the mice were killed and the tumors were harvested. Mice in the control group were untreated, and the tumors were harvested at the same time as those in the 1-h treatment group. A, Protein extracts (MM.1S, 20 μg; KMS-26, 30 μg) from tumors were subjected to western blot analysis. B, Total RNA (50 ng) from tumors were subjected to quantitative RT-PCR analysis and the levels of NF-κB-targeted gene transcripts were measured



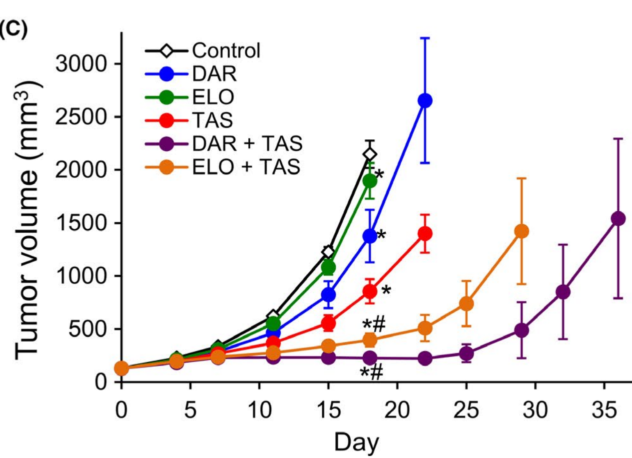


FIGURE 4 Antitumor activity of TAS4464 alone and in combination with standard multiple myeloma (MM) chemotherapies in human-MM xenograft mouse models. Antitumor activity of TAS4464 alone and in combination with standard therapies in human-MM KMS-26 (A) and MM.1S (B, C) xenograft models. SCID mice bearing KMS-26 or MM.1S were administered the chemotherapies as follows: TAS4464 (TAS), intravenously twice a week; bortezomib (BTZ), intravenously twice a week; lenalidomide (LEN), daily oral; dexamethasone (DEX), orally 4-days on/4-days off; daratumumab (DAR) and elotuzumab (ELO), intraperitoneally once a week. The individual therapies were performed for 3 weeks. Combinations of therapies followed the same administration schedules as the individual therapies for 3 weeks. Data are presented as mean ± SD (n = 6 mice per group). Body weight changes of the mice are shown in Figure S6. $^{*}P < 0.05$ vs control group by Dunnett's *t* test. $^{\#}P < 0.05$ vs control group by Aspin-Welch's *t* test

In MM cells, activation of the NF-κB pathways is caused by several genetic aberrations and extracellular signals,[3-6,27] with the canonical pathway activated mostly by extracellular signals, such as B-cell antigen receptor signaling and tumor necrosis factor receptor signaling, and the non–canonical pathway activated by cytokines.[28,29] Although simultaneous inhibition of both the canonical and non–canonical pathways seems to be required to fully inactivate NF-κB function in MM cells,[30] the complexity of the many different molecular mechanisms involved in NF-κB activation makes this difficult. According to previous reports,[31,32] the proteasome inhibitor bortezomib exerts its anti–MM activity through inhibition of the NF-κB pathways; however, it has been shown that bortezomib does not completely inhibit the NF-κB pathways in nonclinical and clinical samples.[33-35] For example, bortezomib is reported to inactivate the non–canonical NF-κB pathway, which inhibits the conversion of p100 to p52, and to enhance the activity of the canonical NF-κB pathway through downregulation of p-IκBα,[33] suggesting that bortezomib does not fully inactivate NF-κB function in MM cells. Indeed, we found that treatment with the maximum tolerated dose of bortezomib (1 mg/kg) determined by our evaluation did not completely inhibit NF-κB activity and had limited efficacy in KMS-26 tumors despite inhibiting proteasome activity (Figure S7). In contrast to bortezomib, we found in the present study that TAS4464 stabilizes the intrinsic NF-κB inhibitors p-IκBα and p-p100 through inactivation of their ubiquitin E3 ligase CRL, and inhibits NF-κB activity with strong anti–MM activity both in vitro and in vivo. This suggests that TAS4464 exerts its antitumor activity through inhibition of both the canonical and non–canonical NF-κB pathways in MM cells. However, this is different to the reported mechanism of action of another NAE inhibitor, MLN4924, which exerts its antitumor activity in MM cells through suppression of PI3K/mTOR signaling via REDD1 accumulation.[18] One possible explanation for this discrepancy is that suppression of the NF-κB pathways and of PI3K/mTOR signaling via NAE inhibition is time-dependent and/or dose-dependent. In four MM cell lines, we found that treatment with MLN4924 or TAS4464 for 4 hours reduced the levels of neddylated UBC12 and neddylated cullin1, and induced the accumulation of p-IκBα and p-p100, in a dose-dependent manner (Figure S8A). Both compounds also dose-dependently induced the expression of REDD1 protein, which is a key regulator of the PI3K/mTOR activity[18]; however, the induction of REDD1 protein was slightly weaker than that of p-IκBα and p-p100, except in U266B1 cells. In all four cell lines, MLN4924 was almost 10-fold less active than TAS4464, based on the reduction of neddylated UBC12 and neddylated cullin1; that is, 1000 nmol/L of MLN4924, but only 100 nmol/L of TAS4464, was needed for almost complete inhibition of NAE activity in the cell lines. When the four cell lines were treated with 1000 nmol/L

MLN4924 or 100 nmol/L TAS4464, the time course of PI3K/mTOR signaling suppression, determined as the level of phospho-p70S6K and -4E-BP1 expression, was found to vary in the different cell lines (Figure S8B). Although suppression of phospho-p70S6K and phospho-4E-BP1 expression was observed at 4 hours after treatment with MLN4924 or TAS4464 in U266B1 cells, similar suppression was not observed until 24 hours after treatment in KMS-26 and MM.1R cells. Similarly, we found that NF-κB inhibition occurred earlier than PI3K/mTOR signaling suppression upon NAE inhibition; that is, the transcription of NF-κB-targeted genes was downregulated after 4 hours of treatment with TAS4464 in cell lines KMS-11, KMS-12-BM, KMS-26 and MM.1S (Figure 2C). Further studies are needed to examine the extent to which the inhibition of each pathway is involved in the anti–MM activities of these compounds; however, MLN4924 rapidly increased the levels of p-IκBα and p-p100 in MM cells (Figure S8) and is reported to inhibit the NF-κB pathway in other hematological malignancies such as diffuse large B-cell lymphoma and B-cell chronic lymphocytic leukemia.[36-40]

Interactions of MM cells with other cells and the extracellular matrix in the bone marrow microenvironment are important for the progression and survival of MM cells.[41] In addition, these interactions play a crucial role in the drug resistance of MM cells. Indeed, the anti–MM activities of the conventional chemotherapies dexamethasone and doxorubicin have been shown to be suppressed by co–culture with BMSC, although that of bortezomib is reported to be unaffected under such conditions.[22] In the present study, we show that the effects of TAS4464 on MM cells were not weakened under conditions that mimic the bone marrow microenvironment (ie, under co–culture with BMSC or in the presence of cytokines), suggesting that TAS4464 has the potential to overcome the growth stimulatory effects triggered by microenvironmental signaling.

Because MM is a heterogeneous hematologic malignancy, MM patients usually receive combination therapy with two or more drugs that target different molecules.[42,43] Importantly, we found that TAS4464 significantly inhibited tumor growth without remarkable toxicities not only as a single agent but also when it was administered in combination with the current standard therapies bortezomib, lenalidomide/dexamethasone, daratumumab and elotuzumab in human-MM xenograft mouse models. Because each standard therapy has a different mechanism of action, it is plausible that TAS4464 enhances the apoptosis induced by these other drugs through NF-κB inhibition. Although the molecular mechanisms by which TAS4464 enhanced these different types of drugs remain to be elucidated, TAS4464 has the potential to be used to treat MM in the clinic as a drug that can be combined with current standard chemotherapies.

In summary, TAS4464 showed antitumor activity in human-MM mouse models by inhibiting NF-κB activation in MM cells. With TAS4464 affecting both the canonical and non–canonical NF-κB pathways, it demonstrated the potential to be used for the treatment of MM regardless of genetic background. TAS4464 may also overcome the growth stimulatory and anti–apoptotic effects triggered by microenvironmental signaling. We also found in human-MM xenograft mouse models that TAS4464 enhances the antitumor activities of current standard MM chemotherapies without remarkable toxicities. Together, these specific properties indicate that TAS4464 has the potential to be a valuable option in the treatment of MM either as a standalone or combination therapy, and that it may also improve the treatment of other NF-κB-activated cancers.

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DISCLOSURE

All authors are employees of Taiho Pharmaceutical.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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