

Antimyeloma activity of NK012, a micelle-forming macromolecular prodrug of SN-38, in an orthotopic model

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NK012 is a micelle-forming macromolecular prodrug of 7-ethyl-10-hydroxy camptothecin (SN-38), an active metabolite of irinotecan. It is accumulated and retained in tumor tissues and gradually releases SN-38 in an enzyme-independent manner. NK012 was previously demonstrated to have stronger antitumor activity than irinotecan in a broad range of human solid-tumor xenograft models. In our study, we used an orthotopic multiple myeloma (MM) model created by injecting CD138-positive U266B1, a myeloma cell line that produces human IgE lambda light chain (monoclonal protein, M protein), into immunodeficient NOD/Shi-scid, IL-2R γ ^{null} mice. This model shows typical bone marrow infiltration by the human myeloma cells. We evaluated the antimyeloma activity of intravenously administered NK012 in this model and showed that it suppressed the M protein concentration in the plasma and proliferation of myeloma cells in the bone marrow in a dose-dependent manner. NK012 suppressed the progression of hind-leg paralysis and prolonged the survival time of the mice compared to the untreated control group. In combination with bortezomib (BTZ), NK012 increased the median survival time compared to that with BTZ alone. In conclusion, these results suggest that NK012 is a potential candidate for use—alone and in combination—in the treatment of MM in humans.

The proteasome inhibitor bortezomib (BTZ) and immunomodulatory drugs such as thalidomide and lenalidomide have been validated for their efficacy in the treatment of multiple myeloma (MM) patients in the last decade.^{1–3} These agents have led to improved survival. However, these treatments are not curative, even when used in combination. Most patients eventually fail to respond and die. Thus, the therapeutic effects of these drugs remain unsatisfactory, and more effective treatments are needed to prolong the survival of MM patients.

The topoisomerase I inhibitor irinotecan is a prodrug that is enzymatically hydrolyzed by carboxylesterase to become

the active metabolite, 7-ethyl-10-hydroxy camptothecin (SN-38). Irinotecan is commonly used to treat patients with various cancers such as colorectal cancer, small cell lung cancer and non-small cell lung cancer. NK012 is a micelle-forming macromolecular prodrug of SN-38 (the active metabolite of irinotecan). NK012 is obtained by binding SN-38 to an amphiphilic block copolymer.⁴ The chemical bonds between SN-38 and the block copolymer are gradually hydrolyzed under neutral conditions.⁴ Although irinotecan must be enzymatically hydrolyzed by carboxylesterases to release SN-38,^{5–8} NK012 does not. Therefore, NK012 will show antitumor activity regardless of a patient's enzyme level.

NK012 shows a unique pharmacokinetic profile based on the enhanced permeability and retention (EPR) effect in tumor tissues.^{4,9} The blood vessel in newly vascularized tumor tissues are permeable to macromolecules such as micelles, whereas normal tissues are not.¹⁰ Koizumi *et al.* showed that NK012 accumulates in tumor tissues, where it is retained longer than irinotecan and releases SN-38 continuously.⁴ As SN-38-induced apoptosis is time-dependent,¹¹ these profiles are advantages of NK012. NK012 exhibits superior antitumor activity to irinotecan in various xenograft models.^{9,12} Moreover, in a preclinical study, NK012 caused little mucosal damage to the small intestinal or diarrhea, adverse effects that are characteristic of irinotecan.¹³ In phase I clinical studies of NK012 in patients with refractory solid tumors conducted in Japan and the USA, the principal dose-limiting toxicity was controllable myelosuppression.^{14,15} Diarrhea, which is seen with irinotecan, was mild, *i.e.*, within

Key words: NK012, multiple myeloma, SN-38, orthotopic model
Abbreviations: BMD: bone mineral density; BTZ: bortezomib; ELISA: enzyme-linked immunosorbent assay; EPR: enhanced permeability and retention; H&E: hematoxylin–eosin; IV: intravenous; MM: multiple myeloma; MST: median survival time; MTD: maximum tolerated dose; SN-38: 7-ethyl-10-hydroxy camptothecin
Additional Supporting Information may be found in the online version of this article.

Conflict of interest: O.M., K.S., N.N., E.I., K.E., D.N., T.O., K.O. and T.M. are employees of Nippon Kayaku Co., Ltd

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What's new?

The prodrug NK012 has been shown to have stronger anti-tumor activity than irinotecan in a broad range of human solid-tumor xenograft models. In this study, the authors evaluated intravenous NK012 in a mouse model of human multiple myeloma (MM). They found that NK012 suppressed tumor-cell proliferation and prolonged survival time, both alone and in combination with bortezomib. NK012 thus shows promise as a therapeutic candidate for the treatment of MM.

grade 2, in the Japanese phase I study.¹⁴ The recommended phase II dosage of NK012 was 28 mg/m² as a single intravenous (IV) infusion, with an interval of at least 3 weeks between cycles. To date, phase II clinical studies have been conducted in patients with triple-negative breast cancer and relapsed small cell lung cancer in the USA.¹⁶

In our study, we used an orthotopic model to investigate the antimyeloma activity of NK012. Our results show that NK012 significantly prolonged the median survival time (MST) of the mice. Furthermore, NK012 in combination with BTZ further increased the MST compared to BTZ alone. Taken together, these data suggest that NK012 is a potential candidate for use in the treatment of MM.

Material and Methods**Cell culture and compounds**

Human U266B1 myeloma cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, ATCC code TIB-196) and were maintained in RPMI1640 (Mediatech, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (Moregate Biotech, Bulimba, Australia). BTZ was purchased from Janssen Pharmaceutical K.K. (Tokyo, Japan). NK012 was synthesized in-house, as described elsewhere.⁴ At the time of use, BTZ was dissolved in 0.9% saline, whereas NK012 was dissolved in 5% glucose.

U266B1 orthotopic xenograft

A human myeloma orthotopic model was prepared as described previously.¹⁷ A suspension of 2×10^6 CD138-positive U266B1 cells, which produce human IgE lambda light chain (monoclonal protein, M protein), was injected into immunodeficient NOD/Shi-scid, IL-2R γ_c^{null} (NOG) mice¹⁸ (female mice; 8 weeks old; the Central Institute for Experimental Animals, Kanagawa, Japan) *via* the tail vein. Approximately 4 weeks after inoculation (day -2, Fig. 1a), human M protein was assayed in the plasma of the transplanted mice, which were then allocated into groups so as to have equal M protein distribution. All the described animal studies were conducted after approval by the in-house experimental animal committee, which had reviewed all protocols according to the Guidelines for Proper Conduct issued by the Japanese Association for Laboratory Animal Science.

Evaluation of NK012 in the U266B1 orthotopic xenograft

The effects of NK012 on myeloma cell growth in bone marrow, human M protein production and osteolysis were

assessed in the orthotopic model. NK012 was administered IV at 1/2 the maximum tolerated dose (MTD),⁴ 1/4 MTD and 1/8 MTD (*i.e.*, 15, 7.5 and 3.75 mg/kg/day, respectively) on days 1, 5 and 9, respectively. The dosing schedule of NK012 was decided with reference to the schedule previously reported to exhibit antitumor activity against solid tumors in xenograft models. As a positive control, BTZ was administered IV at 1 mg/kg/day (MTD) on days 1, 4, 8 and 11. Mice were euthanized on day 20 to measure the human M protein level in the plasma and to detect human myeloma cells in the bone marrow of the femur. Mice were sacrificed on day 35 for bone radiography and bone mineral density (BMD) analysis (Fig. 1a).

To examine the effect of NK012 on survival time, the myeloma-transplanted mice were IV injected with NK012 at 9.4 mg/kg/day on days 1, 8, 15, 22, 29 and 36. To take the clinical dosing schedule into account, we extended the dosing interval from the widely used q4d $\times$ 3 schedule to 7-day intervals, which a preliminary experiment showed was necessary for hematological recovery in the case of treatment using the MTD. BTZ was administered IV at 0.5 mg/kg/day on days 1, 4, 8, 11, 22, 25, 29 and 32. The dosages of NK012 and BTZ were decided with reference to their recommended clinical dosages. NK012 9.4 mg/kg/day corresponds to 28.2 mg/m²/day, which is almost the same as its recommended clinical dosage of 28 mg/m²/day.¹⁴ BTZ 0.5 mg/kg/day corresponds to 1.5 mg/m²/day, which is $\sim$ 115% of its recommended clinical dosage on a mg/m² basis. To examine the combination effect of NK012 and BTZ on the survival time, mice were treated with 9.4 mg/kg/day NK012 (days 1, 8 and 15, IV) and 0.5 mg/kg/day BTZ (days 1, 4, 8 and 11, IV). The animals were observed for clinical signs and weighed twice weekly. The MST was determined.

Enzyme-linked immunosorbent assay for human M protein

Blood was collected from each mouse, and the plasma was separated by centrifugation. The human M protein, human lambda light chain, was measured by enzyme-linked immunosorbent assay (ELISA; Bethyl Laboratories, Montgomery, TX), according to the manufacturer's instructions.

Detection of U266B1 human myeloma cells in mouse bone marrow

Myeloma cells in the bone marrow of femora were determined using phycoerythrin-conjugated anti-human CD138 antibody (Bay Bioscience, Tokyo, Japan) and an LSR II flow

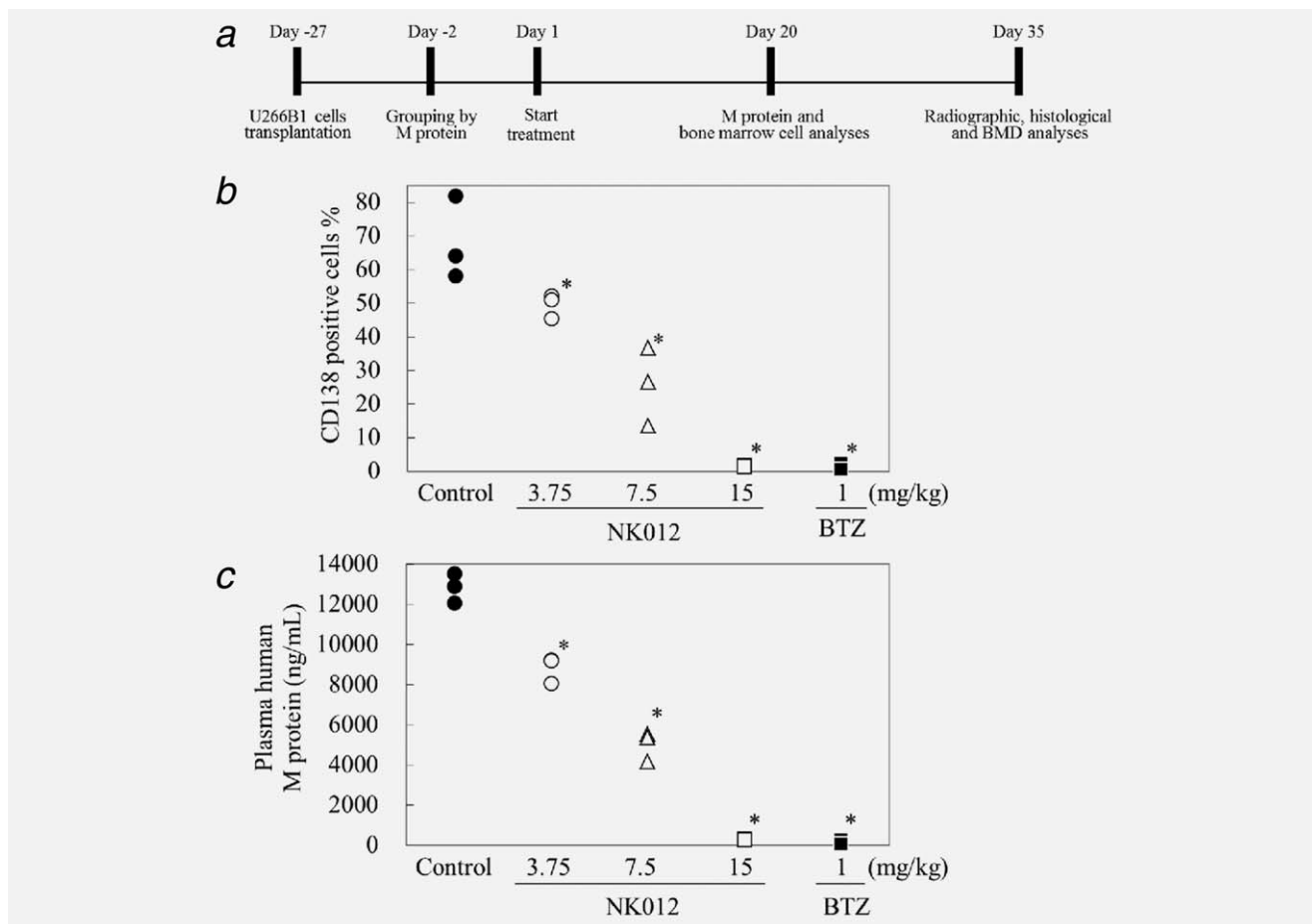


Figure 1. NK012 inhibits myeloma cell growth in the bone marrow and reduces human M protein in the plasma. Mice injected with 2×10^6 U266B1 cells were treated with NK012 or BTZ. NK012 was administered by IV injection at 3.75, 7.5 or 15 mg/kg/day on days 1, 5 and 9, respectively. BTZ was administered by IV injection at 1 mg/kg/day on days 1, 4, 8 and 11. Animals were sacrificed on day 20. (a) The experimental time line shows the timing of each procedure, beginning with the U266B1 cell transplantation. (b) Myeloma (CD138-positive) cell mean percentages were determined by harvesting cells from the femur and analyzing for human CD138 by flow cytometer. (c) Human M protein (lambda light chain) in the plasma was measured by ELISA. Significant differences from the untreated control group are indicated with asterisks ($p < 0.05$). NK012 suppressed myeloma cell growth in the bone marrow and reduced the plasma human M protein level, both in a dose-dependent manner.

cytometer (Becton-Dickinson, Tokyo, Japan). The data from 10,000 events were analyzed. The mean percentage of human CD138-positive U266B1 cells among all bone marrow cells was determined using FACSDiva™ software (Becton-Dickinson).

Radiographic and BMD analyses

The tibiae of sacrificed mice were dissected and fixed with 10% formalin in neutral buffer solution. Each specimen was radiographed with a soft X-ray apparatus (TRS-1005, SOFRON, Tokyo, Japan) to evaluate osteolytic lesions. The BMD of the distal tibia metaphysis was measured by peripheral quantitative computed tomography (XCT Research SA+, Stratec Medizintechnik, Birkenfeld GmbH, Pforzheim, Germany). The distal site of the tibia was defined as 1.2 mm away from the growth plate.

Histological analysis

For histological analysis, tibial specimens were embedded in paraffin after 10% formalin fixation. Paraffin-embedded blocks were thin-sliced and stained with hematoxylin-eosin (H&E).

Statistical analysis

The data for the CD138-positive cell counts in the bone marrow, human M protein levels in the plasma and BMD analysis were examined for statistical significance by Dunnett's test for multiple comparisons with the untreated control using SAS software. The overall survival time of mice was defined as the number of days from the start of treatment until death and compared between groups using Kaplan-Meier analysis. The p values were calculated using a log-rank test. In all analyses, $p < 0.05$ was considered statistically significant.

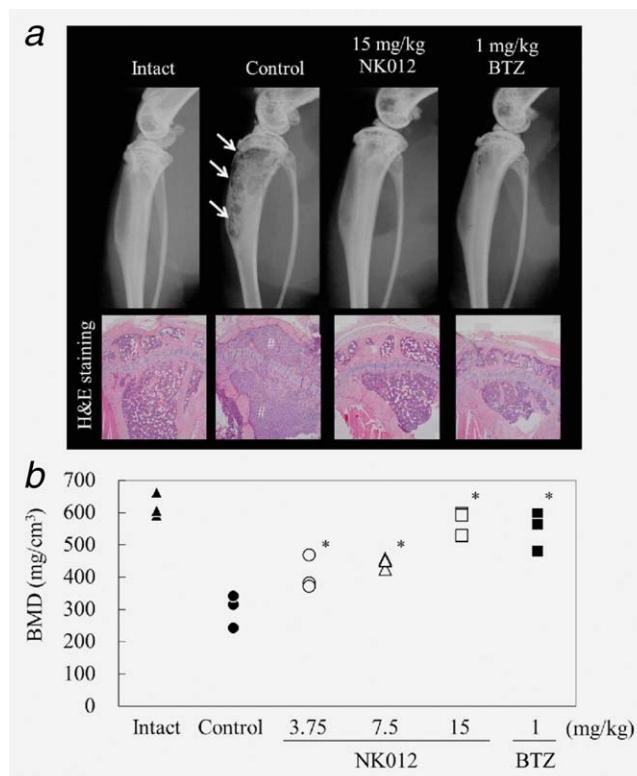


Figure 2. NK012 significantly decreases bone destruction. Mice transplanted with human U266B1 myeloma cells were treated with NK012 or BTZ and then sacrificed on day 35. (a) Representative radiographs and results of H&E staining of tibiae are shown. U266B1 cells caused typical bone destruction in the untreated control group, as indicated by the arrows. NK012-treated mice showed reduced bone destruction at the same site. The histological findings correlated well with the radiographic images. Bone marrow was replaced by numerous plasma cells in the untreated control mice (#). NK012-treated mice had fewer plasma cells in the bone marrow. Original magnification, $\times 5$. Micrographs were obtained with an Fx A light microscope (Nikon) using a DS-R1 digital camera and NIA-Elements software (Nikon). (b) The BMD was measured by peripheral quantitative computed tomography analysis at a site 1.2 mm distal from the growth plate of the tibia. NK012-treated mice showed significantly less decrease in the BMD of the tibiae compared to the untreated control group (* $p < 0.05$).

Results

NK012 clearly reduced major manifestations of MM

To assess the effects of NK012 on myeloma cell growth *in vivo*, the mean percentage of CD138-positive cells in the bone marrow in each treatment group at day 20 was analyzed and compared to the untreated control group. The mean percentage of CD138-positive cells in the untreated control group was 68.2% (Fig. 1b). NK012 reduced that percentage in a dose-dependent manner, and in the 15 mg/kg/day group it fell below 5% (Fig. 1b). The plasma M protein level on day 20 in the untreated control group was significantly elevated, whereas that in the untransplanted intact mice was below the limit of detection (data not shown). NK012 suppressed the elevation of M protein in a dose-dependent manner, and in the 15 mg/kg/day group it was suppressed to

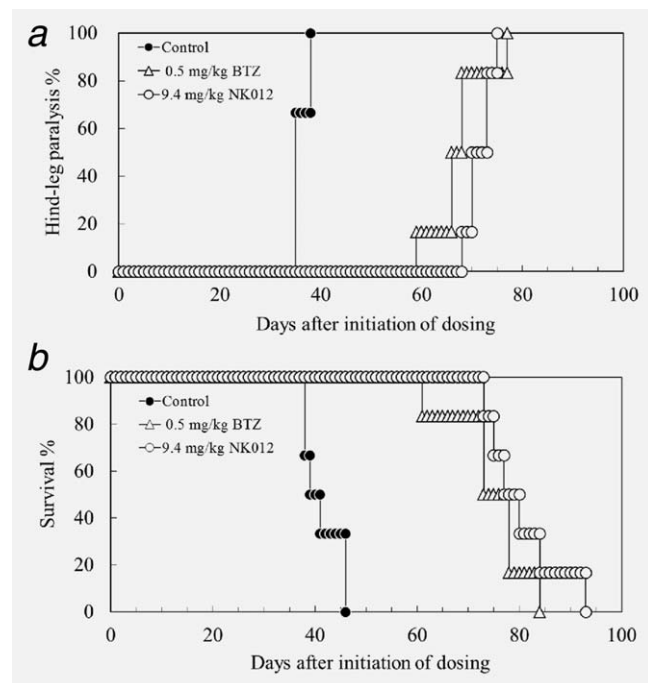


Figure 3. NK012 suppresses paralysis and significantly prolongs survival in the orthotopic MM model. NK012 was administered by IV injection at 9.4 mg/kg/day on days 1, 8, 15, 22, 29 and 36. BTZ was administered by IV injection at 0.5 mg/kg/day on days 1, 4, 8, 11, 22, 25, 29 and 32. Each group consisted of six mice. NK012-treated mice did not show hind-leg paralysis until day 69 (a). The survival time was defined as the number of days from the first treatment until death. NK012 and BTZ each significantly enhanced the MST of mice compared to the untreated control ($p = 0.0007$ and $p = 0.0007$, respectively). The MST in the untreated control group was 40 days, whereas in the NK012-treated group it was 78.5 days (b).

the same level as in the BTZ group (Fig. 1c). The mean percentage of M protein reduction was 97.7 and 98.8%, respectively, in the NK012 15 mg/kg/day group and the BTZ group compared to the untreated control group. These results are consistent with the decrease in CD138-positive cells in the NK012-treated groups.

To investigate for an effect of NK012 on bone destruction by human myeloma cells, the tibia of U266B1-transplanted mice were assessed by radiographic analysis. The radiography of untreated control mice showed increased bone destruction compared to untransplanted intact mice (Fig. 2a). The radiographic images correlated well with the H&E findings. Replacement of healthy bone marrow cells by numerous plasma cells was detected in the untreated control mice. Mice treated with NK012 at 15 mg/kg/day and the BTZ group showed similar radiographic findings. Both drugs significantly decreased bone destruction (Fig. 2a). To further confirm the effect of NK012 on bone destruction, the tibia BMD was measured using peripheral quantitative computed tomography. The BMD of untreated control mice (U266B1-transplanted mice) decreased to $\sim 50\%$ of that of untransplanted intact mice. NK012 suppressed that decrease in a dose-dependent manner.

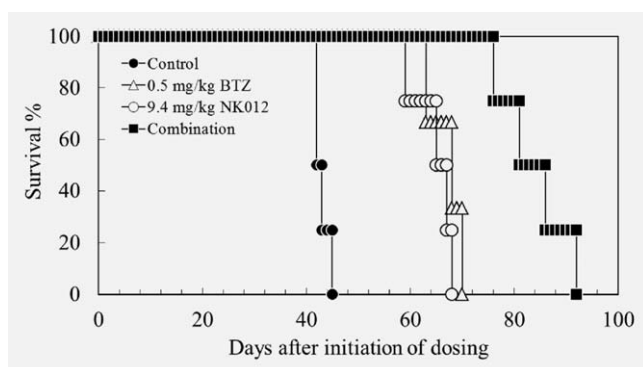


Figure 4. Combination of NK012 with BTZ prolongs the MST in the orthotopic MM model. Myeloma-transplanted mice were treated with 9.4 mg/kg/day of NK012 (days 1, 8 and 15) alone, 0.5 mg/kg/day of BTZ (days 1, 4, 8 and 11) alone or combination of drugs (NK012 + BTZ). Each group consisted of three or four mice. Kaplan–Meier analysis was used to determine the effect on the MST. The MST with NK012 monotherapy and BTZ monotherapy was 66 and 68 days, respectively, and was prolonged compared to the untreated control (MST of 42.5 days). The MST with NK012 and BTZ combination therapy was 83.5 days, which was significantly ($p < 0.05$) prolonged compared to BTZ monotherapy (68 days). None of the mice showed treatment-related body weight loss or toxic death.

In particular, the 15 mg/kg/day NK012 group showed almost the same BMD as the untransplanted intact group (Fig. 2b).

NK012 suppressed paralysis and prolonged survival

We next assessed whether treatment with NK012 could suppress paralysis and prolong survival in the orthotopic MM model. The mice were treated with NK012 or BTZ from day 1 and were observed for survival, as described in Material and Methods section. Mice transplanted with human U266B1 cells exhibit hind-limb paralysis.¹⁹ In our study, hind-limb paralysis was observed in the untreated control group beginning from around day 36, but it was not seen in NK012-treated mice until day 69 (Fig. 3a). The video data on day 40 are shown in Supporting Information. Moreover, the NK012-treated group showed significantly prolonged survival compared to the untreated control group (MST: NK012 78.5 days vs. control 40 days, $p < 0.05$) (Fig. 3b). That MST with NK012 was almost the same as with BTZ (75.5 days).

Combination therapy with NK012 and BTZ further prolonged the MST

We also investigated the effect of combination therapy with BTZ and NK012 on survival prolongation in the orthotopic MM model. The untreated control group MST was 42.5 days (Fig. 4), which was prolonged by each of NK012 and BTZ monotherapies (MSTs of 66 and 68 days, respectively). Combination therapy using both NK012 and BTZ clearly prolonged the MST to 83.5 days (Fig. 4, $p < 0.05$). The combination therapy was well tolerated: no toxic signs were observed during administration or the following observation period (data not shown).

Discussion

Topoisomerase I is essential for cell growth and important for cellular functions, especially chromatin processes.²⁰ The level of this enzyme is also elevated in many cancer cells such as colon carcinoma cells, lung cancer cells and lymphoma. Therefore, topoisomerase I is an attractive target for cancer chemotherapy. In fact, topoisomerase I inhibitors such as irinotecan and topotecan are effective in cancer patients.

Plasma cells isolated from the bone marrow of MM patients and human myeloma cell lines express topoisomerase I.^{21,22} Topoisomerase I inhibitors are able to inhibit myeloma cell growth. Irinotecan significantly reduced the tumor volume and the level of serum M protein in xenograft models of MM.²¹ In fact, NK012 exhibited antitumor activity against both human myeloma RPMI8226 and KMS-11 in subcutaneous xenograft models (Supporting Information figure). Furthermore, a phase II study of topotecan showed an overall response rate of 16% in refractory myeloma patients.²³ Even more interesting, the median progression-free survival of all patients in that study was 13 months. These findings suggest that topoisomerase I is an attractive therapeutic target for the treatment of MM.

For our study, we used an orthotopic MM model, created in immunodeficient NOG mice, to evaluate NK012. In this model, myeloma cells grow site-specifically in the bone marrow and induce elevation of human M protein in the plasma, bone destruction and neuroparalysis, similar to as seen in MM patients.¹⁷ We found that NK012 inhibited the growth of CD138-positive myeloma cells in the bone marrow and ameliorated plasma M protein elevation, bone destruction and paralysis. NK012's inhibition of myeloma cell growth in the bone marrow was dose-dependent, which was consistent with the suppression of M protein elevation. Such efficacy may be primarily dependent on NK012's cytotoxic effect on CD138-positive myeloma cells. On the other hand, it is known that the bone marrow microenvironment is important for the growth and survival of MM cells.^{24,25} Various cytokines—such as interleukin-6—are known to be key molecules for myeloma cell growth. SN-38 inhibited secretion of interleukin-6 and vascular endothelial growth factor by bone marrow stroma cells.²⁶ *In vivo* studies of cytokine depletion in the bone marrow microenvironment may enable further elucidation of the mechanisms underlying NK012's effects.

Koizumi *et al.* showed that NK012 preferentially accumulates in solid-tumor tissues.⁴ This phenomenon is based on the EPR effect, which is derived from the pathophysiologic characteristics of tumor tissues, such as neovascularization and absence of lymphatic drainage.¹⁰ Neovascularization was also reported to be abundant in the bone marrow of MM patients,^{27,28} suggesting the possibility that NK012 may accumulate in bone marrow containing myeloma cells—again owing to the EPR effect—as in solid tumors and then exhibit antimyeloma activity. Further evaluation of the pharmacokinetics of NK012 is needed to clarify this hypothesis.

Therapy for MM has been greatly improved by BTZ and thalidomide in the past decade. However, the effects of these drugs are not sufficient to effect a cure, and most patients eventually become insensitive to them. New medicines that have different profiles—such as in terms of their mechanism and toxicity—from the approved drugs are needed. NK012 has distinctly different profiles from the approved drugs, such as its favorable pharmacokinetics owing to the EPR effect and its improved safety owing to less peripheral neuropathy.¹⁴ Therefore, NK012 may potentially be a new treatment option for MM patients. We also evaluated the antitumor activity of NK012 in combination with BTZ, because combination of BTZ with DNA-damaging agents was demonstrated to show synergistic antitumor effects.^{29,30} The NK012-BTZ combination significantly prolonged the

MST compared to BTZ monotherapy, suggesting that NK012 may be a good candidate for combined use with BTZ.

In conclusion, we showed that NK012, a micelle-forming macromolecular prodrug of SN-38, reduced plasma M protein level and bone destruction and prolonged the survival time in an orthotopic MM model. We believe that NK012 has the potential to become a new treatment option for MM patients. Accordingly, NK012 is currently being evaluated in phase I/II clinical studies in relapsed or refractory MM patients in Japan.

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Supplementary Figure: Antitumor activity of NK012 against RPMI8226 and KMS-11 human myeloma bearing mice.

To evaluate the antimyeloma activity of NK012 using subcutaneous tumor xenograft models, human myeloma cell line RPMI8226 or KMS-11 was inoculated subcutaneously to the dorsal skin of NOD/SCID mice. When tumors reached a volume of approximately 300 mm³, mice were randomly assigned to the treatment groups (n = 5~6 mice/group). NK012 was administered by IV bolus at 12.5 mg/kg/day on days 1, 8 and 15. That NK012 dosage was 1/4 of the maximum tolerated bolus dosage. BTZ was administered by IV injection at 1 mg/kg/day on days 1, 4, 8 and 11. That BTZ dosage was the maximum tolerated bolus dosage.

The antimyeloma activities of NK012 and BTZ were evaluated on the basis of the mean relative tumor volume. The relative tumor volume was calculated as follows. The large diameter (L) and small diameter (S) of each tumor were measured to calculate the tumor volume $((L) \times (S)^2/2)$, and the relative tumor volume was calculated using the tumor volume on the first day of treatment as 1. Differences between the treated groups and the control group were examined for significance using Welch's test. A P-value of <0.05 was required for statistical significance (*).

NK012 exhibited antimyeloma activity against both RPMI8226 and KMS-11 in the subcutaneous tumor xenograft models. In particular, NK012 achieved elimination of KMS-11 tumors by day 34 of treatment (#). NK012 was well tolerated by the mice, with no body weight loss.

