

**Figure 4.** Cellular miR-92a in CD8<sup>+</sup> or CD4<sup>+</sup> lymphocytes in MM and normal subjects. (a) The CD4<sup>+</sup> miR-92a ( $P = 0.0178$ ) and CD8<sup>+</sup> miR-92a ( $P = 0.0092$ ) are significantly downregulated. (b) Comparison between miR-92a levels in CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in normal subjects (closed circles), MM at diagnosis (open squares) and myeloma in CR/VGPR (open triangles), showing a positive correlation. Comparison between plasma miR-92a and the cellular miR-92a of CD4<sup>+</sup> T lymphocytes (c) or CD8<sup>+</sup> T lymphocytes (d) in identical subjects. Myeloma patients with low levels of miR-92a in CD4<sup>+</sup> T lymphocytes cluster at low levels of plasma miR-92a, and some MM patients with CR/VGPR show normalization of miR-92a levels in plasma, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes.

correlated with the miR-92a level in T lymphocytes, especially CD8<sup>+</sup> lymphocytes. Some MM patients in CR/VGPR showed normalization of the plasma miR-92a level, in combination with upregulation of T-cell miR-92a. A recent study reported that the miR-17-92a cluster was critical for optimal T-lymphocyte co-stimulation via CD28 and was thereby essential for regulatory T function under certain conditions.<sup>35</sup> Moreover, it is reported that the type-1-skewing tumor microenvironment induces downregulation of miR-17-92a expression, with decreased levels of miR-17-92a noted in both CD4<sup>+</sup> and CD8<sup>+</sup> cells in tumor-bearing mice and in CD4<sup>+</sup> cells in patients with glioblastoma multiforme.<sup>36</sup> Together, these findings suggest that the low level of miR-92a in T lymphocytes may represent dysregulation of immunity and the downregulated plasma miR-92a level in MM patients may reflect the dysfunction of T lymphocytes *in vivo*. In addition to miR-17-92a downregulation, the TaqMan low-density array screening revealed downregulation of miR-223, a regulator of neutrophil proliferation and activation.<sup>37</sup> The ultimate downregulation of plasma miR-223 in MM patients might also be related with granulocyte dysfunction.

In conclusion, the plasma miR-92a level may serve as a biomarker for monitoring therapeutic response in MM patients and disease progression in asymptomatic MM patients. The

downregulated plasma miR-92a level may be linked to the expression level of T-cell-derived miR-92a. Although we examined a small number of patients, especially MM patients who had been treated, these initial findings suggest that plasma miR-92a levels in monoclonal gammopathy patients may be a promising parameter not only for determining disease status but also whether further treatment is required.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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#### AUTHOR CONTRIBUTIONS

SY, KO and JHO designed the study, analyzed the data, interpreted the data and wrote the manuscript; TU and MO performed quantitative PCR analysis of plasma miRNA; KO performed statistical analysis; SY, KO, KS, AI, and SI provided patient samples.

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# Risk of Japanese carriers of hyperphosphorylated paratarg-7, the first autosomal-dominantly inherited risk factor for hematological neoplasms, to develop monoclonal gammopathy of undetermined significance and multiple myeloma

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Hyperphosphorylated paratarg-7 (pP-7) is a frequent target of paraproteins in German patients with monoclonal gammopathy of undetermined significance (MGUS)/multiple myeloma (MM). The frequency of MGUS/MM is lower in Japan than in Europe. As pP-7, the first molecularly defined autosomal-dominant risk factor for any hematological neoplasm, is inherited in a dominant fashion, we determined the incidence of the pP-7 carrier state in a Japanese population, and compared the frequency of pP-7-specific paraproteins and the pP-7 carrier state in Japanese and German patients with MGUS/MM. Peripheral blood from 111 Japanese patients with MGUS/MM and 278 healthy blood donors was analyzed for the pP-7 carrier state by isoelectric focusing and for pP-7-specific antibodies by ELISA. The Japanese group was compared with 252 German MGUS/MM patients and 200 healthy controls. Five of 111 (4.5%) Japanese and 35/252 (13.9%) German IgA/IgG MGUS/MM patients had a pP-7-specific paraprotein ( $P = 0.009$ ). The prevalence of healthy pP-7 carriers in the Japanese study group was 1/278 (0.36%), whereas it was 4/200 in the German group ( $P = 0.166$ ). The relative risk for pP-7 carriers developing MGUS/MM had an odds ratio of 13.1 in the Japanese and 7.9 in the German group. In conclusion, the fraction of pP-7 carriers with a pP-7-specific paraprotein is lower among Japanese than in German patients with MGUS/MM, but pP-7 carriers in both ethnic groups have a high risk of developing MGUS/MM. (*Cancer Sci* 2011; 102: 565–568)

**M**ultiple myeloma (MM) is a B-lymphocyte-derived malignancy characterized by a monoclonal proliferation of plasma cells that produce a clonal immunoglobulin. Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic precursor condition commonly preceding MM.<sup>(1)</sup> Recent studies show that family members of MGUS and MM patients have a two to threefold higher risk of developing MGUS/MM.<sup>(2,3)</sup> Environmental influences, chance occurrence, and inherited factors might all contribute to familial clusters. Also, the incidence of MM and the prevalence of MGUS are reportedly two to threefold higher among Black people than White people,<sup>(4–6)</sup> and lower in Asians.<sup>(6,7)</sup> Until now, the pathogenesis of MGUS/MM has remained obscure. A causal relationship between MGUS/MM and chronic antigenic stimulation has been suggested by the results of several studies,<sup>(8–12)</sup> hence the identification of the antigenic stimuli of B-cell neoplasms might be of considerable importance. Antigenic targets of paraproteins

were discovered accidentally due to clinical symptoms caused by the paraprotein (e.g. chronic cold agglutinin disease or cryoglobulinemia<sup>(13)</sup> or bleeding disorders<sup>(14)</sup>), because of interference of the paraprotein with laboratory tests ordered for the clinical work-up of the patient (e.g. HIV-1 p24 antigen in an HIV-infected patient with myeloma),<sup>(15)</sup> and/or by screening paraproteins against pre-defined antigens (e.g. anti-streptolysin, anti-DNA, or anti-IgG<sup>(13)</sup>). The first systematic studies covering a broad spectrum of potential antigens used serological identification of antigens by expression cloning (SEREX), which allows for the systematic screening of putative antibody-antigen interactions, even if neither the antigen nor the antibody are known.<sup>(16)</sup> cDNA libraries derived from human testis, lung, and breast cancer, bovine and porcine muscle, and wheat germ were expressed in *Escherichia coli* and investigated by SEREX for reactivity with paraproteins from the sera of 114 patients with MGUS or MM. More than  $6 \times 10^8$  paraprotein-antigen interactions were probed, resulting in the identification of only four antigens, each recognized by the paraprotein of only one patient.<sup>(17,18)</sup> In a complementary approach using a human fetal brain-derived macroarray and IgA or IgG paraprotein-containing sera, the paraproteins of 29 (15.1%) consecutive MGUS and MM patients reacted with paratarg-7 (P-7).<sup>(19)</sup> Paratarg-7 is identical to STOML2 (stomatin [EPB72]-like), also known as HSPC108 or stomatin-like protein and SLP-2,<sup>(20)</sup> that has also been reported to be expressed in all human tissues and overexpressed<sup>(21,22)</sup> in several cancers.<sup>(23)</sup> Other investigators have reported that P-7 modulates T cell activation,<sup>(24)</sup> and in a recent publication it was claimed that SLP-2 is required for stress-induced mitochondrial hyperfusion.<sup>(25)</sup> In an extension of our earlier study, the high frequency of P-7-specific paraproteins in the sera of MGUS/MM patients (35/252; 14%) was confirmed in a subsequent study.<sup>(26)</sup> Moreover, it was shown that all patients with P-7-specific paraproteins were carriers of a hyperphosphorylated version of the protein (pP-7) and that this hyperphosphorylation is inherited in a dominant fashion.<sup>(26,27)</sup> As only 2% of healthy Germans are carriers of pP-7, the pP-7 carrier state is associated with an increased risk (odds ratio, 7.9) to develop MGUS/MM. Thus, pP-7 is the first molecularly defined inherited risk factor known for any hematological neoplasm. Because of the autosomal-dominant inheritance of pP-7, it is of interest to determine the prevalence of the pP-7

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carrier state and the frequency of pP-7-specific paraproteins in other ethnic groups. As the incidence of MGUS and MM is lower in Asians than in Europeans,<sup>6,7)</sup> it was the aim of this study to compare a German population with a Japanese population with respect to the prevalence of the pP-7 carrier state and the incidence of P-7-specific paraproteins in MGUS and MM.

## Materials and Methods

**Patients and controls.** This study was approved by the local German ethical review board (Ethikkommission der Ärztekammer des Saarlandes, Saarbrücken, Germany) and the internal review board of Nagoya City University and Nagoya City Midori Municipal Hospital (Nagoya, Japan). Serum samples were taken from 111 Japanese patients with MGUS/MM treated at Nagoya City University Hospital or Nagoya City Midori Municipal Hospital and 252 German patients treated at Saarland University Medical School (Homburg/Saar, Germany). Serum protein electrophoresis identified a monoclonal spike in these samples, which was shown to contain a monoclonal IgA, IgD, or IgG paraprotein by immunofixation. The German control group consisted of 200 healthy employees of Saarland University Medical School and the Japanese control group consisted of 278 Japanese healthy blood donors. "Healthy" was defined as being healthy in the pre-donation medical check-up and having no monoclonal immunoglobulin by serum electrophoresis and immunofixation. In addition, the healthy donors who were identified by isoelectric focusing (IEF) as pP-7 carriers were contacted and they confirmed that they were without any major health problems and explicitly without malignant disease, in particular.

The same criteria were applied in our previous study to the German population of 252 MGUS/MM patients. The German control group was the same as in the previous study. There was no difference with respect to age, gender (all patients), progression to MM, or stage of the disease between patients with a P-7-specific paraprotein. Patients were listed under MGUS if the first blood sample was obtained while they had still MGUS, even if they later progressed into MM. Whenever possible, human materials were obtained during routine diagnostic or therapeutic procedures and stored at  $-80^{\circ}\text{C}$  until use.

**Western blot sample preparation.** For Western blot analyses, blood samples were centrifuged and washed with PBS. The pellet was resuspended in 8 M urea, 0.1 M phosphate, 10 mM Tris-HCl (pH 8.0), and 0.1% NP-40 and incubated at  $20^{\circ}\text{C}$  for 15 min.

**Isoelectric focusing for determination of pP-7 carrier state.** Blood samples were centrifuged and washed with PBS followed by lysis in lysis buffer containing 8 M urea, 0.1 M  $\text{NaH}_2\text{PO}_4$ , 0.01 M Tris-HCl, and 0.1% NP40 (15 min,  $20^{\circ}\text{C}$ ) and stored at  $-20^{\circ}\text{C}$  until use. Equal volumes of sample and loading buffer were mixed. Samples were analysed by IEF on a gel with a fixed pH gradient (pH 3.0–10.0) according to the manufacturer's instructions (Novex pH 3.0–10.0; Invitrogen, Karlsruhe, Germany) followed by an immunoblot screening.

**Immunoblot staining.** After lysates from whole peripheral blood were separated by IEF or SDS-PAGE under reducing conditions, the proteins were transferred to an Immobilon-P PVDF membrane (Immobilon; Millipore, Eschborn, Germany) by semi-dry blotting. The membrane was blocked overnight at  $4^{\circ}\text{C}$  in TBST/milk buffer (10% [v/v] milk in 10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% [v/v] Tween 20), washed and incubated for 1 h at room temperature with serum in TBST (paraprotein-containing serum from patients at a dilution of  $1:10^8$  and from controls at a dilution of  $1:10^3$ ). After three washings in TBST, the membranes were incubated for 1 h at room temperature with mouse anti-human IgG-POX antibody (BioRad, Dreieich, Germany) diluted 1:3000 in TBST. The

membranes were then washed in TBST, followed by detection using Pharmacia's ECL (enhanced chemiluminescence) system (General Electric Healthcare, Fairfield, CT, USA).

**Paratarg-7 ELISA for detection of paraproteins with specificity to P-7.** To determine the antigen specificity of the paraproteins, the P-7 ELISA using full-length recombinant paratarg-7 was carried out as described previously.<sup>19)</sup>

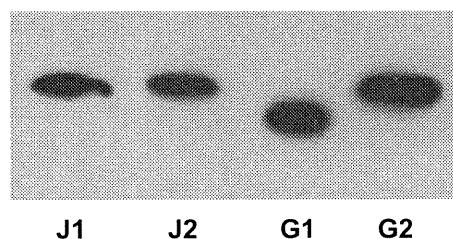
## Results

Thirty-five of 252 (13.9%) paraproteins from the German MGUS/MM patients were shown by ELISA to react with paratarg-7. In contrast, only 5/111 Japanese patients (4.5%) had a paraprotein specific for paratarg-7 (Table 1) and were carriers of pP-7 (Fig. 1). This difference is significant with a *P*-value of 0.009 (chi-square test). The anti-paratarg-7 reactivity of these sera had titres ranging from  $1:10^8$  to  $1:10^{10}$ . None of the sera from healthy controls reacted at a dilution of  $\leq 1:10^2$ . Lower serum dilutions were not tested because they cause too much background in the P-7 ELISA. As was the case with the German IgG paraproteins with specificity for P-7,<sup>19)</sup> all Japanese P-7 reactive IgG paraproteins (4/78) belonged to the IgG<sub>3</sub> subtype, with 4/38 IgG<sub>3</sub> (10.5%) paraproteins displaying this specificity. The prevalence of healthy pP-7 carriers in the healthy Japanese population, as determined by IEF (Fig. 1) followed by immunoblot staining, was lower (1/278 or 0.36%) than in the German (4/200 or 2.0%) population. This is a strong trend but, due to the low prevalence of a pP-7 carrier state in healthy controls, did not reach significance (*P* = 0.166; Fisher's exact test). Nevertheless, the relative risk for pP-7 carriers to develop MGUS/MM is significant in both ethnic groups with an odds ratio of 13.1 (95% confidence interval, 1.5–113.1; *P* = 0.020) in

**Table 1. Detection of paratarg-7-specific paraproteins in 252 German and 111 Japanese consecutive patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM)**

	MGUS (%)	MM (%)	Total (%)
Japanese patients			
IgA	0/3 (0)	1/22 (4.5)	1/25 (4.0)
IgD	0/0 (0)	0/8 (0)	0/8 (0)
IgG	0/8 (0)	4/70 (5.3)+	4/78 (5.1)+
Total	0/11 (0)	5/100 (5.0)	5/111 (4.5)
German patients			
IgA	2/24 (8.3)	4/21 (19.0)	6/45 (13.3)
IgD	0/0 (0)	0/0 (0)	0/0 (0)
IgG	15/117 (12.8)+	14/90 (15.5)+	29/207 (14.0)+
Total	17/141 (12.1)	18/111 (16.2)	35/252 (13.9)

+All pP-7 reactive IgG paraproteins were of the IgG<sub>3</sub> subclass.



**Fig. 1.** Western blot analysis after isoelectric focusing of paratarg-7 (P-7) derived from Japanese and German patients with P-7-reactive paraproteins. Bands of whole peripheral blood lysates from two Japanese patients with multiple myeloma (J1, J2) with anti-P-7 paraproteins, one German patient with a P-7-specific paraprotein (G2) and one German patient with a non-P-7-specific paraprotein (G1) migrated differently.

the Japanese and 7.9 (95% confidence interval, 2.8–22.6;  $P = 0.0001$ ) in the German population.

## Discussion

This is the first study to show that pP-7, which was identified as the first dominantly inherited risk factor for any hematological neoplasm in White people, is also found in Japanese patients with MGUS/MM and is a strong and highly significant risk factor for developing MGUS/MM in both Japanese and German pP-7 carriers. A causal relationship between MGUS/MM and chronic antigenic stimulation has been suggested by the results of several studies; however, results have generally been inconsistent.<sup>(4,5,10,11,28–37)</sup> The specificity of the P-7 binding paraproteins was extensively discussed in our previous published study<sup>(19)</sup> and has recently been confirmed by cloning the B-cell receptors of two patients with a P-7-specific paraprotein.<sup>(38)</sup>

The MGUS/MM patients in this study carrying pP-7, and those with paraproteins that did not bind to P-7, showed no significant difference with respect to age, sex, or course of disease (data not shown). Even though the frequency of P-7 as a paraprotein target is only 4.5% in Japanese patients, it is still much higher than expected by chance and suggests a direct or indirect role of pP-7 in the pathogenesis of these diseases in both the Japanese and German populations. All IgG paraproteins with specificity for P-7 belonged to the IgG<sub>3</sub> subclass, both in the Japanese patients (4/38 or 10.5%) and in the German patients (24/57 or 42.1%).<sup>(19)</sup> The reason for this is unknown, but indicates that additional factors might be necessary for the recognition of pP-7 as an auto-antigen by the autologous immune system. Recent results from our laboratory show that the difference between “wild-type” and pP-7 is due to a phosphorylation at a single site (serine 17 of the molecule; unpublished data). Because of the dominant inheritance of P-7 hyperphosphorylation, a polymorphism in one of the plethora of kinases is more likely to be responsible for the phosphorylation differences than a deficiency or decreased activity of a phosphatase, which should be compensated by the second allele.

Several reports suggest that gene mutations or genetic polymorphisms might be associated with the risk of MM.<sup>(39–41)</sup> However, results have been inconsistent and significant findings have not been replicated convincingly.<sup>(42)</sup> Hyperphosphorylated P-7 is the first molecularly characterized structure that provides a plausible explanation for the familial clustering of cases of MGUS/MM, at least in cases with a P-7-specific paraprotein. Indeed, we observed two pedigrees with familial MGUS/MM, and all affected members in these two families were carriers of pP-7.<sup>(43)</sup> It is now possible to investigate whether previously reported cases of familial MGUS/MM<sup>(2,3,5,35)</sup> can also be explained by the carrier state of pP-7.

The frequency of the carrier state of pP-7 among patients with MGUS/MM and in healthy controls reveals a 13.1-fold increased risk to develop MGUS/MM for Japanese carriers and a 7.9-fold increased risk for German carriers. These are, to the best of our knowledge, the highest odds ratios for an MGUS/MM risk factor reported to date in either ethnic

group.<sup>(2,4,5,44)</sup> The number of families with MGUS/MM patients carrying pP-7 is still too small to estimate the risk of a family member carrying pP-7; it is at least 13.1 and 7.9 in the Japanese and German population, respectively, but is probably much higher, because other, yet unidentified, genetic factors shared between family members might further increase the risk for MGUS/MM among family members.

The odds ratios for carriers of pP-7 to develop MGUS/MM was significant for the German study group (4/200 vs 35/252;  $P = 0.0001$ ; chi-square test) and Japanese study group (1/278 vs 5/111;  $P = 0.008$ ; Fisher's exact test). Testing of family members for the pP-7 carrier state by simple IEF enables the identification of family members who are at increased risk of developing MGUS/MM.

In contrast to the carrier state of pP-7, which is under exclusive genetic control, the nature of the immune response against pP-7 is complex and might involve both genetic and environmental factors. The fact that genetic factors are relevant is suggested by the previous findings that all IgG paraproteins with specificity for P-7, analyzed to date, are of the IgG<sub>3</sub> subclass and 42.1% of all IgG<sub>3</sub> paraproteins react with pP-7.<sup>(19)</sup> The frequency of pP-7 as an antigenic target and/or stimulus for paraprotein-producing clones and the availability of many families with MGUS/MM patients with the pP-7 carrier state now allow for the analysis of tumor-host interactions in the presence and absence of the antigen in the respective patients and family members, and to study more specifically the role of environmental factors and immunoregulatory deficiencies, such as the recently reported dysfunction of regulatory T cells<sup>(45)</sup> in patients with MGUS and MM.

The fact that pP-7 functions as the antigenic target of the paraproteins of all MGUS/MM patients with pP-7, suggests that the hyperphosphorylated protein plays a role in the development of sporadic and familial MGUS/MM. The hyperphosphorylation of P-7 appears to be the most obvious likely reason for its auto-immunogenicity. Whether pP-7 induces the development of MGUS/MM by chronic antigenic stimulation or whether it is only a marker or an epiphenomenon of another dominantly inherited susceptibility to develop MGUS/MM can now be investigated in the respective patients and their (not yet) affected relatives.

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## Disclosure Statement

KDP and MP have applied for a relevant patent. None of the other authors have a conflict of interest.

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**Short Communication**

## Melphalan–Prednisolone and Vincristine–Doxorubicin–Dexamethasone Chemotherapy followed by Prednisolone/Interferon Maintenance Therapy for Multiple Myeloma: Japan Clinical Oncology Group Study, JCOG0112

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A multicenter phase III study for untreated multiple myeloma was conducted to investigate a switch-induction chemotherapy with melphalan–prednisolone and vincristine–doxorubicin–dexamethasone followed by randomization on maintenance therapy for patients achieving plateau. Between November 2002 and November 2005, 34 patients were registered. The study was closed early because of poor accrual. Thirty-three eligible patients, with a median age of 65 years (range: 47–77 years) were analyzed for the secondary purpose. For induction therapy, 16 patients were treated with vincristine–doxorubicin–dexamethasone and 17 with melphalan–prednisolone initially. In eight cases, induction therapy was switched because of a poor response. Both regimens were well tolerated, but neutropenia, anorexia, constipation and infection with neutropenia were more frequent for vincristine–doxorubicin–dexamethasone. Best response rates were 44% (95% confidence interval, 20–70) and 47% (95% confidence interval, 23–72), respectively, for vincristine–doxorubicin–dexamethasone and melphalan–prednisolone. Vincristine–doxorubicin–dexamethasone/melphalan–prednisolone switch-induction therapy might be feasible and effective for Japanese patients with multiple myeloma.

*Key words:* myeloma – Chemo-Phase III – MP/VAD – IFN/PSL

### INTRODUCTION

The administration of melphalan and prednisone (MP) has been the standard treatment for multiple myeloma (MM) since the 1960s. A review of clinical trials found that MP results in a 60% overall response rate (ORR) with the response lasting 18 months and an overall survival (OS) period of 24–36 months (1). To develop more effective treatments that both produce a higher response rate and lengthen OS, combination chemotherapy using various

regimens has been compared with MP or MP-like regimens in numerous randomized studies including ours (2). Among the many alternatives to MP, vincristine, doxorubicin and dexamethasone (VAD), first reported as an effective salvage therapy for MM refractory to melphalan or other alkylators, is frequently chosen especially for patients with significant symptoms related to MM partly because the achievement of a response with relief of symptoms occurs sooner than with MP (3). Also, VAD has been chosen for patients undergoing

high-dose chemotherapy followed by autologous stem cell transplantation (HDC-aSCT), because of less damage to hematopoietic stem cells after this regimen when compared with an alkylator-containing regimen such as MP (4). Therefore, VAD has been considered a standard treatment for untreated MM. However, VAD is more toxic than MP and requires hospitalization for continuous drip infusion. It was reported that MP was also effective as a salvage treatment for relapse after VAD and HDC-aSCT (5).

Despite the effective induction chemotherapies, most patients with MM will ultimately relapse. Therefore, attempts to prolong the remission with maintenance treatment have been made. Meta-analyses suggested that MM patients receiving  $\alpha 2$  interferon (IFN) as maintenance therapy have a slightly prolonged OS (6,7). It is well documented that glucocorticoids have antitumor activity in MM patients (8,9). In a prior Southwest Oncology Group (SWOG) study, 89 patients responding to induction of VAD chemotherapy receive maintenance therapy with either prednisone at 50 mg three times per week with IFN, or IFN alone (10). Progression-free survival (PFS) was increased from 9 to 19 months for patients given the combination compared with those given IFN alone. A subsequent study by SWOG comparing alternate-day, oral treatment with prednisone at pharmacologic doses (50 mg) vs. physiologic doses (10 mg) for maintaining remission in MM patients who responded to chemotherapy revealed the benefit of the former regimen (OS: 37 vs. 26 months;  $P = 0.05$ ) (11). However, the role of maintenance therapy in MM patients remains controversial, especially the usefulness of IFN when compared with prednisone. We considered that both the MP and VAD were standard as the induction therapy for untreated MM and each was substitutable for the other as salvage therapy. Therefore, we conducted a multicenter prospective randomized controlled trial to investigate switch-induction chemotherapy with MP and VAD followed by maintenance therapy to compare less toxic prednisolone (PSL) alone with PSL + IFN- $\alpha$  for the treatment of overt MM.

## PATIENTS AND METHODS

### PATIENTS

Patients were eligible for the first registration if they were <80 years and had overt MM diagnosed according to the SWOG criteria and not previously treated with chemotherapy (12). Patients were required to have a performance status (PS) of 0–3 according to the criteria of the Eastern Cooperative Oncology Group (13), to have no severe organ dysfunction (Hgb  $\geq 6.0$  g/dl, neutrophils  $\geq 1.0 \times 10^9/l$ , platelets  $\geq 20 \times 10^9/l$ , alanine aminotransferase/aspartate aminotransferase  $\leq 2.5$  times the upper normal limit, total bilirubin  $\leq 2.0$  mg/dl, serum creatinine  $\leq 5.0$  mg/dl, PaO<sub>2</sub>  $\geq 60$  torr or SaO<sub>2</sub>  $\geq 90\%$ , electrocardiogram without arrhythmia and/or ischemia requiring treatment) and to be negative for hepatitis B virus surface antigen and/or hepatitis C virus antibody. Patients were not eligible if they had indolent myeloma or

non-secretory MM, active primary cancer in other organs or any serious concomitant disease, including diabetes mellitus requiring insulin therapy, uncontrolled hypertension, psychiatric disease, a history of myocardial infarction, unstable angina, renal diseases with abnormal function, interstitial pneumonitis or autoimmune hepatitis, or if they were pregnant and/or breast feeding. The study protocol and the informed consent document were approved by both the Japan Clinical Oncology Group (JCOG) Protocol Review Committee and the institutional review board of each participating institution.

Eligibility for the second registration was identical to that of the first except for the achievement of a plateau phase of MM, a PS of two or less, a platelet count of  $\geq 50 \times 10^9/l$  and a serum creatinine concentration of  $\leq 2.0$  mg/dl.

### TREATMENT

As for induction therapy, either VAD or MP was chosen. After the achievement of a plateau phase, maintenance therapy with either IFN + PSL or PSL alone was applied by randomization with a minimization method for balancing institution, induction chemotherapy and risk grouping by  $\beta 2$  microglobulin and C-reactive protein.

### SWITCH-INDUCTION CHEMOTHERAPY

As for induction chemotherapy, patients received either MP or VAD following the primary physician's decision. If the response was less than partial response just before the fifth course of VAD or the seventh course of MP, a switch to the other induction therapy was allowed. The maximum number of courses for VAD and MP was 10 and 15, respectively. The maximum number in cases with switching was 20.

VAD consists of 0.4 mg vincristine per day and 10 mg/m<sup>2</sup> of doxorubicin per day, both administered by continuous infusion on Days 1–4; 40 mg of dexamethasone per day by drip infusion on Days 1–4. Treatment was repeated at 21 day intervals. MP consists of oral melphalan given at 8 mg/m<sup>2</sup> on Days 1–4 and oral PSL at 60 mg/m<sup>2</sup> on Days 1–4, every 4 weeks.

### MAINTENANCE THERAPY

For maintenance therapy, after the achievement of a plateau phase and an evaluation of eligibility, patients were assigned to receive either PSL alone or PSL + IFN- $\alpha$ . IFN- $\alpha$  and PSL consist of natural IFN- $\alpha$  (NAMALWA, Sumiferon<sup>®</sup>) 3 MU s.c.  $\times 3$ /week and PSL 50 mg p.o.  $\times 3$ /week. PSL alone consists of PSL 50 mg p.o.  $\times 3$ /week. The therapy was continued unless progressive disease or severe toxicity occurred.

### EVALUATION OF RESPONSE AND ADVERSE EVENTS

Response criteria followed those of the European Group for Blood and Marrow Transplantation/International Blood and Marrow Transplant Research/Autologous Blood and Marrow



Transplant Registry (14). An objective response was evaluated after every two courses of induction and maintenance therapy by the following five factors: serum M-protein concentration, 24 h urine Bence-Jones protein, plasma cells in bone marrow, extramedullary plasmacytoma detected by computed tomography or magnetic resonance imaging and radiological changes in bone lesion.

Adverse events were graded according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC, version 2.0, 30 April 1999) (15).

STATISTICAL ANALYSIS

The primary endpoint was PFS, defined as the time from randomization at the second registration until death from any cause, relapse or progressive disease and censored at the last follow-up. The secondary endpoints were toxicity and OS after the second registration, and toxicity, ORR and OS after the first registration for induction therapy. The planned duration of patient accrual was 4 years, and the planned follow-up time was 4 years.

The study was designed as a non-inferiority trial of maintenance therapy comparing less toxic PSL alone with PSL + IFN-α. The required sample size for the second registration was 160 with 75% power, non-inferiority margin with a hazard ratio of 1.32 (corresponding to a 10% difference in the 4 year PFS rate when that in the PSL + IFN-α arm is 35%), and a one-sided alpha value of 0.05. The planned sample size for the first registration was 230 assuming that 70% of the registered patients would achieve a plateau phase of MM after induction therapy and randomization.

The JCOG Data Center collected and managed case report forms. In-house interim monitoring for quality control was performed at the Center, and the monitoring reports were semi-annually submitted to the JCOG Data and Safety Monitoring Committee.

RESULTS AND DISCUSSION

Between November 2002 and November 2005, 34 patients were enrolled from 15 participating institutions. The study was then closed early because of poor accrual. One patient was deemed ineligible after randomization because he was judged to have smoldering myeloma. Thirty-three eligible patients were analyzed for the secondary purpose. For the first induction therapy, VAD and MP were chosen for 16 and 17 patients, respectively (Table 1). Median ages were 63 and 70 years in patients initially treated with VAD and MP, respectively. In eight cases (five for VAD and three for MP), the induction therapy was switched because of a poor response. The median numbers of courses of first induction therapy were six (range: 2–10) and nine (range: 1–15), respectively, for VAD and MP.

Both regimens were well tolerated, but Grade 4 neutropenia was more frequent for VAD than for MP (63 vs. 11%)

and also, Grade 3/4 anorexia, constipation and infection with Grade 3/4 neutropenia were more frequent for VAD (25 vs. 17%, 31 vs. 6% and 25 vs. 6%, respectively). The ORR including that after the switch was 44% [95% confidence interval (CI), 20–70%] and 47% (95% CI, 23–72%), respectively, for VAD and MP (Table 2). In all of them the

Table 1. Patient characteristics

	Eligible patients (n = 33) <sup>a</sup>	Initial therapy	
		VAD (n = 16)	MP (n = 17)
Age, years [median (range)]	65 (47–77)	63 (47–70)	70 (56–77)
Gender			
Male	19 (58)	6 (38)	13 (77)
Female	14 (42)	10 (63)	4 (24)
Stage (Durie and Salmon)			
IIA	6 (18)	74 (25)	2 (12)
IIB	0	0	0
IIIA	24 (73%)	10 (63)	14 (82)
IIIB	3 (9)	2 (13)	1 (6)
PS (stratification: 0,1/2,3,4)			
0	5 (15)	1 (6)	4 (24)
1	18 (55)	11 (69)	7 (41)
2	2 (6)	1 (6)	1 (6)
3	8 (24)	3 (19)	5 (29)
Ig subtype			
G/A/D/BJP	19/7/2/5 (58/21/6/15)	11/2/1/2 (69/13/6/13)	8/5/1/3 (47/29/6/18)

Percentage values are represented in parenthesis. VAD, vincristine–doxorubicin–dexamethasone; MP, melphalan–prednisolone; PS, performance status; BJP, Bence-Jones protein.

<sup>a</sup>One patient was ineligible because of inaccurate staging.

Table 2. Response to treatment

	VAD as initial therapy (n = 16)	MP as initial therapy (n = 17)	P value
CR (%)	0	0	
PR (%)	44	47	
MR (%)	25	29	
NR (%)	31	12	
PD (%)	0	12	
Not evaluable	0	0	
CR + PR (%) (95% CI)	44 (20–70)	47 (23–72)	NS <sup>a</sup>

CR, cytogenetic response; PR, partial response; MR, minor response; NR, no response; PD, progressive disease; CI, confidence interval.

<sup>a</sup>Fisher’s exact test (two sided).

responses were partial. Only eight patients (three and five treated with PSL + IFN- $\alpha$  and PSL alone, respectively) were registered for the randomization on the maintenance therapy. The OS rate at 2 years was 63% (95% CI, 44–77%) for the 33 patients after the first registration.

This is the first prospective study evaluating MP and VAD as the induction therapy for Japanese patients with MM, though there was a recent report on VAD followed by HDC-aHST (16). As reported from Europe and the USA, the toxicity of the VAD, such as hematological effects, infections, constipation and anorexia, was greater than that of the MP (1–4). The ORRs in this study, 44 and 47%, respectively for VAD and MP, were also consistent with previous reports. Therefore, it is likely that both induction regimens, despite the small number of eligible patients and switch to the other regimen, were feasible and might be effective for Japanese patients with MM.

There were several reasons for the poor accrual of the patients in the present multicenter phase III study. The poor accrual might have been partly caused by the application of HDC-aHST for relatively young patients as consolidation therapy. Furthermore, over the past few years, treatment of MM has been evolving rapidly due to the introduction of more effective drugs, such as bortezomib, thalidomide and lenalidomide, though the new drugs were not available in Japan during the registration period of the present trial, which might have led to less interest among the participating physicians to evaluate the efficacy of MP or VAD, which had been already established as the standard chemotherapy in Western countries. The significance of maintenance therapy using a combination of IFN and steroids remains controversial. However, thalidomide and other new agents are now considered promising for maintenance as well as induction therapy (17).

In conclusion, MP/VAD switch-induction therapy is feasible and might be effective for Japanese patients with MM, although this study was not completed. Maintenance therapy as well as induction therapy for MM should be explored incorporating new effective agents.

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## Conflict of interest statement

None declared.

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# Delayed treatment with vitamin C and *N*-acetyl-L-cysteine protects Schwann cells without compromising the anti-myeloma activity of bortezomib

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**Abstract** Bortezomib-induced peripheral neuropathy (BIPN) emerges as a disabling adverse effect. As rat models for BIPN have demonstrated damage in nerve Schwann cells, we screened for cytoprotective agents to devise a method of rescuing Schwann cells from the cytotoxic effects of bortezomib without compromising its anti-myeloma effects. Schwann cells underwent macroautophagy along with cytoplasmic inclusion body and vacuole formation, and appeared much less susceptible to bortezomib-induced cytotoxicity than did myeloma cells. Vitamin C or *N*-acetyl-L-cysteine (NAC) achieved near-complete rescue of Schwann cells treated with bortezomib at 30 nM or less, and these agents in combination are able to cooperatively inhibit the morphological changes and the cytotoxicity in Schwann cells with higher doses of bortezomib. The delayed addition of vitamin C and/or NAC after the exposure to bortezomib alleviated the cytotoxicity

in Schwann cells but not myeloma cells. These results suggest that delayed treatment with these agents may be instrumental in prophylaxis of BIPN.

**Keywords** Myeloma · Bortezomib-induced peripheral neuropathy · Vitamin C · *N*-Acetyl-L-cysteine

## 1 Introduction

Bortezomib has become a mainstay in the treatment of multiple myeloma in both first-line and relapsed/refractory settings. However, bortezomib-induced peripheral neuropathy (BIPN) emerges as a disabling adverse effect which often limits the dose of bortezomib and duration of treatment [1–4]. As bortezomib has become more widely available, the need for neuroprotective strategies to prevent BIPN has become more urgent.

Although the pathogenic mechanism underlying BIPN remains largely unknown, a preclinical animal model for BIPN demonstrated pathological changes representing intracytoplasmic vacuolization due to mitochondrial and endoplasmic reticulum (ER) damage predominantly in peripheral nerve Schwann cells and satellite cells in dorsal root ganglia [5]. Various agents have been demonstrated to protect cells from cytotoxic stimuli including antioxidants and autophagy inducers. Vitamin C [6], vitamin E [7] and *N*-acetyl-L-cysteine (NAC) [8] exert antioxidant activity. Rapamycin has been demonstrated to act as an autophagy inducer to protect dopaminergic PC12 cells from lactacystin-induced injury [9]. Nerve growth factor (NGF) is a neuroprotective cytokine [10]. Carnitine [11] and valproic acid [12] alleviate painful diabetic neuropathy. In the present study, we therefore examined the effects of the above cytoprotective agents on the viability and morphological

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and biological changes of Schwann cells upon treatment with bortezomib. We demonstrate that Schwann cells are less susceptible to bortezomib than myeloma cells, and that delayed treatment with vitamin C and/or NAC protects Schwann cells from bortezomib's cytotoxicity without compromising its anti-myeloma activity.

## 2 Materials and methods

### 2.1 Reagents

The following reagents were purchased commercially as indicated: vitamin C from Wako (Osaka, Japan); NAC from Nakarai tesque (Kyoto, Japan); vitamin E, acetylcarnitine, valproic acid, and mouse monoclonal antibodies against  $\beta$ -actin from SIGMA (Saint Louis, MO); rapamycin and LY294002 from Calbiochem (Darmstadt, Germany); recombinant human (rh) IL-6 from PEPROTECH EC (London, UK); and rh NGF from R&D systems (Minneapolis, MN). The rabbit monoclonal antibodies against protein kinase-like ER kinase (PERK) and phosphoPERK, rabbit polyclonal antibodies against poly (ADP ribose) polymerase (PARP) and microtubule-associated protein 1 light chain 3B (LC3B), HRP-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA); rabbit monoclonal anti-ubiquitin antibody from Millipore (Temecula, CA); and FITC-goat anti-rabbit IgG from Zymed Laboratories (San Francisco, CA). Bortezomib was obtained from Millennium Pharmaceuticals, Inc. (Cambridge, MA).

### 2.2 Cells and cultures

Rat RT4-D6P2T Schwannoma cell line, rat RSC96 Schwann cell line and RPMI8226 myeloma cell line were obtained from American Type Culture Collection (ATCC) (Rockville, MD). The myeloma cell lines INA-6 and MM.1S were kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany) and Dr. Steven Rosen (Northwestern University, Chicago, IL), respectively. The RT4-D6P2T and RSC96 cells were cultured in  $\alpha$ MEM supplemented with 10% fetal bovine serum (SIGMA), 2 mM L-glutamine (SIGMA), 100 U/mL penicillin G (SIGMA) and 100  $\mu$ g/mL streptomycin (SIGMA). The myeloma cell lines were cultured in RPMI1640 medium with 5% fetal bovine serum, 100 U/mL penicillin G and 100  $\mu$ g/mL streptomycin. rh IL-6 was added at 1 ng/mL to the culture of INA-6 cells.

### 2.3 Assays of cell viability

Viable cells were counted using Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the instructions

provided. Briefly, cells were incubated in culture plates with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8). The absorbance of each well was measured at 450 nm with a microtiter plate reader (Model 450 micro-plate reader; Bio-Rad Laboratories, Hercules, CA).

### 2.4 Wright–Giemsa staining

Cells cultured in 24-well plates were stained with Wright solution (Merk KGaA, Darmstadt, Germany) for 5 min, followed by Giemsa solution (Merk KGaA) at a dilution of 1:10 for 20 min. Cells were visualized under an Olympus BX50 microscope equipped with a UMPlanFI 40X/0.75 objective lens (Olympus, Tokyo, Japan). Images were recorded with an Olympus SC35 CCD camera and Viewfinder Lite Software (Pixera, Los Gatos, CA).

### 2.5 Immunoblotting

Cells were collected and lysed in a cell lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail solution (Sigma). Equal amounts of protein sample were loaded, electrophoresed in SDS-PAGE gels, and blotted onto polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk, the membranes were and incubated with primary antibodies overnight at 4°C and washed. Horse-radish-conjugated secondary antibody was added for 1 h, and protein bands were visualized with an Enhanced Chemoluminescence Plus kit (GE Healthcare Bio-sciences AB, Uppsala, Sweden).

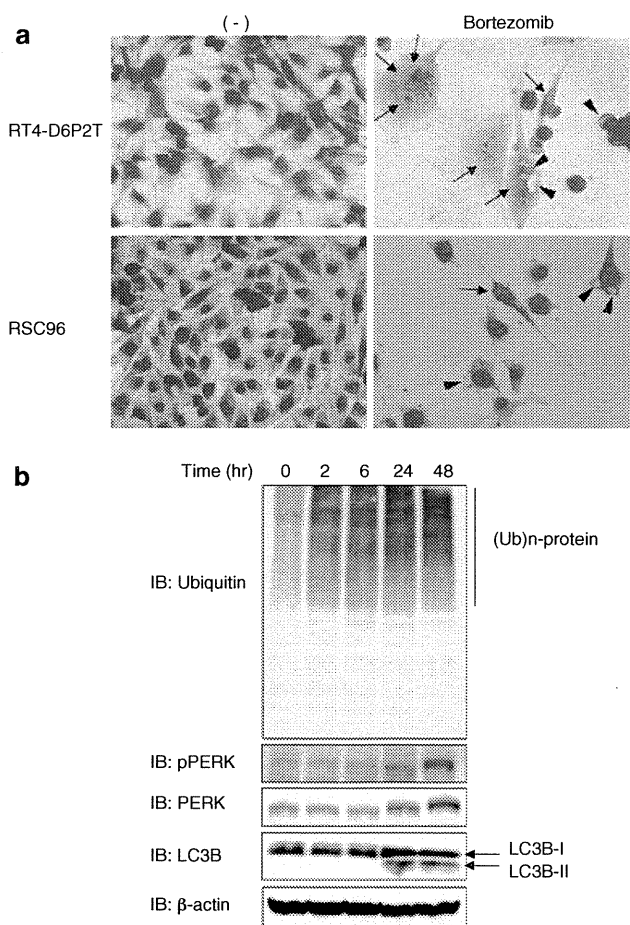
### 2.6 Statistical analysis

Statistical significance was determined using Student's *t* tests. The minimal level of significance was  $P = 0.05$ .

## 3 Results

### 3.1 Bortezomib induces ER stress and macroautophagy in Schwann cells

Because a preclinical rat model for BIPN demonstrated damage predominantly in peripheral nerve Schwann cells and satellite cells in dorsal root ganglia [5], we examined the cytotoxic and morphologic effects of bortezomib on rat RT4-D6P2T Schwannoma and RSC96 Schwann cell lines. These Schwann cells are spindle-shaped and adherent when cultured on plastic dishes. After exposure to bortezomib at 50 nM, however, they became detached over time; the remaining adherent cells showed distinct morphological



**Fig. 1** Bortezomib induces ER stress and macroautophagy in rat Schwann cell lines. **a** Rat Schwann cell lines, RT2-D6P2T and RSC96, were treated for 48 h with 50 nM of bortezomib. The cells were stained with Wright and Giemsa solutions. Intracytoplasmic inclusion bodies are indicated by *arrows* and vacuoles by *arrowheads*,  $\times 400$ . **b** RT4-D6P2T cells were treated with 50 nM bortezomib for the periods indicated, and cell lysate was harvested. The accumulation of ubiquitinated proteins, phosphorylation of PERK, and conversion of LC3B were determined by immunoblotting.  $\beta$ -Actin was used as a loading control

changes with perinuclear inclusion bodies and vacuoles in the cytoplasm on Wright–Giemsa staining (Fig. 1a). These morphological changes appear to be similar to those observed in Schwann cells in a preclinical rat model for BIPN [5], and suggested the induction of macroautophagy.

Because bortezomib inhibits the proteasomal degradation of ubiquitinated proteins, we next explored the induction of ER stress and macroautophagy in the Schwann cells. Bortezomib treatment time dependently accumulated ubiquitinated proteins in Schwann cells along with the phosphorylation of PERK, a marker of ER stress (Fig. 1b). ER stress induces macroautophagy to protect cells, although excessive ER stress results in cell death [13]. During macroautophagy, microtubule-associated protein 1 light chain 3B (LC3B)-I is cleaved to produce LC3B-II

which associates with autophagosomes [13]. Exposure to bortezomib over 24 h induced the cleavage of LC3B (Fig. 1b), suggesting the induction of macroautophagy. Although macroautophagy may contribute to survival of Schwann cells, majority of the cells appeared to be dead through excessive ER stress after the treatment with bortezomib.

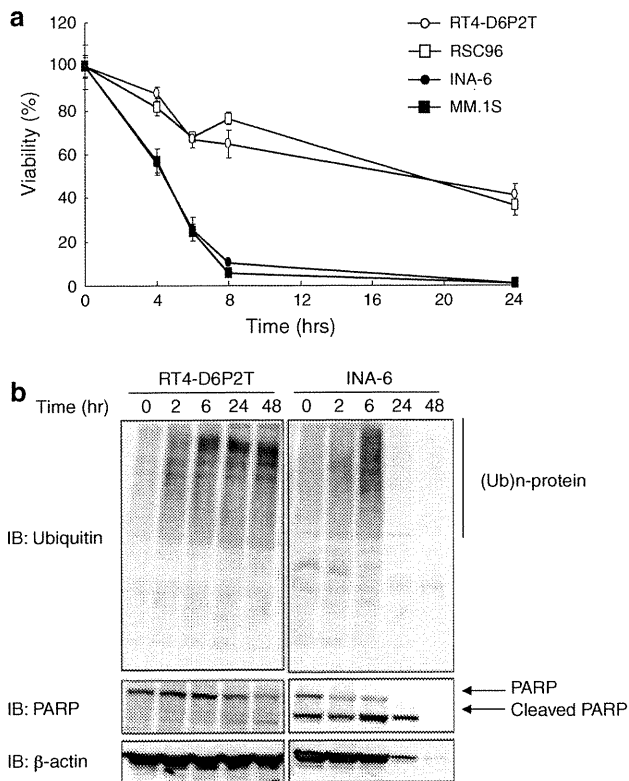
### 3.2 Schwann cells are less susceptible to bortezomib than myeloma cells

Myeloma cells over-produce a monoclonal immunoglobulin and have higher levels of ER stress, and are regarded as a cell type with high susceptibility to bortezomib [14]. Indeed, bortezomib induces cell death in myeloma cells but not in normal hematopoietic cells in patients with myeloma. We next examined the susceptibility of Schwann cells to bortezomib. After exposure to bortezomib at 50 nM for 8 h, most Schwann cells remained intact, while the INA-6 and MM.1S cells were mainly dead (Fig. 2a). After 24 h, about 40% of Schwann cells remained alive, whereas almost all the myeloma cells had died (Fig. 2a).

Bortezomib time dependently increased the levels of ubiquitinated proteins in RT4-D6P2T Schwann cells as well as INA-6 myeloma cells (Fig. 2b). However, viable INA-6 cells were substantially decreased in number and yielded little cell lysate when harvested at 24 h. To determine the involvement of apoptosis, we examined the cleavage of PARP. In RT4-D6P2T cells, PARP was not cleaved within 6 h of exposure to bortezomib, but started to be cleaved at 24 h (Fig. 2b). The bortezomib-induced cleavage was, however, only partial even after 48 h. In INA-6 cells by contrast, bortezomib markedly enhanced the cleavage of PARP at as early as 2 h, and further increased it over time (Fig. 2b). These results suggest that Schwann cells are less susceptible to bortezomib than myeloma cells.

### 3.3 Vitamin C and NAC abolished the cytotoxicity of bortezomib in Schwann cells

To identify possible neuroprotective effects against bortezomib, we screened various clinically available agents with cytoprotective activity. A variety of drugs including antioxidants, inducers of autophagy, and known neuroprotective agents were added to Schwann cells in the absence or presence of bortezomib. Among these drugs, vitamin C and NAC ameliorated bortezomib's cytotoxicity in both RT4-D6P2T and RSC96 Schwann cells (Fig. 3a). Vitamin C dose dependently reduced the cytotoxic effects of bortezomib on Schwann cells at concentrations ranging from 50 to 200  $\mu$ M (Fig. 3b), which have been demonstrated to be clinically achievable [15]. NAC was used at



**Fig. 2** Schwann cell lines are less sensitive to bortezomib-induced cytotoxicity than MM cells. **a** RT4-D6P2T (*open circles*) and RSC96 (*open squares*) Schwann cells, and INA-6 (*filled circles*) and MM.1S (*filled squares*) MM cells were cultured in triplicate in the absence or presence of 50 nM bortezomib. After culturing for the periods indicated, viable cells were counted. Data are expressed as a percentage of control (mean  $\pm$  SD). **b** RT4-D6P2T and INA-6 cells were treated with 30 nM bortezomib for the periods indicated, and cell lysate was harvested. The accumulation of ubiquitinated proteins and cleavage of PARP were analyzed by immunoblotting.  $\beta$ -Actin was used as a loading control

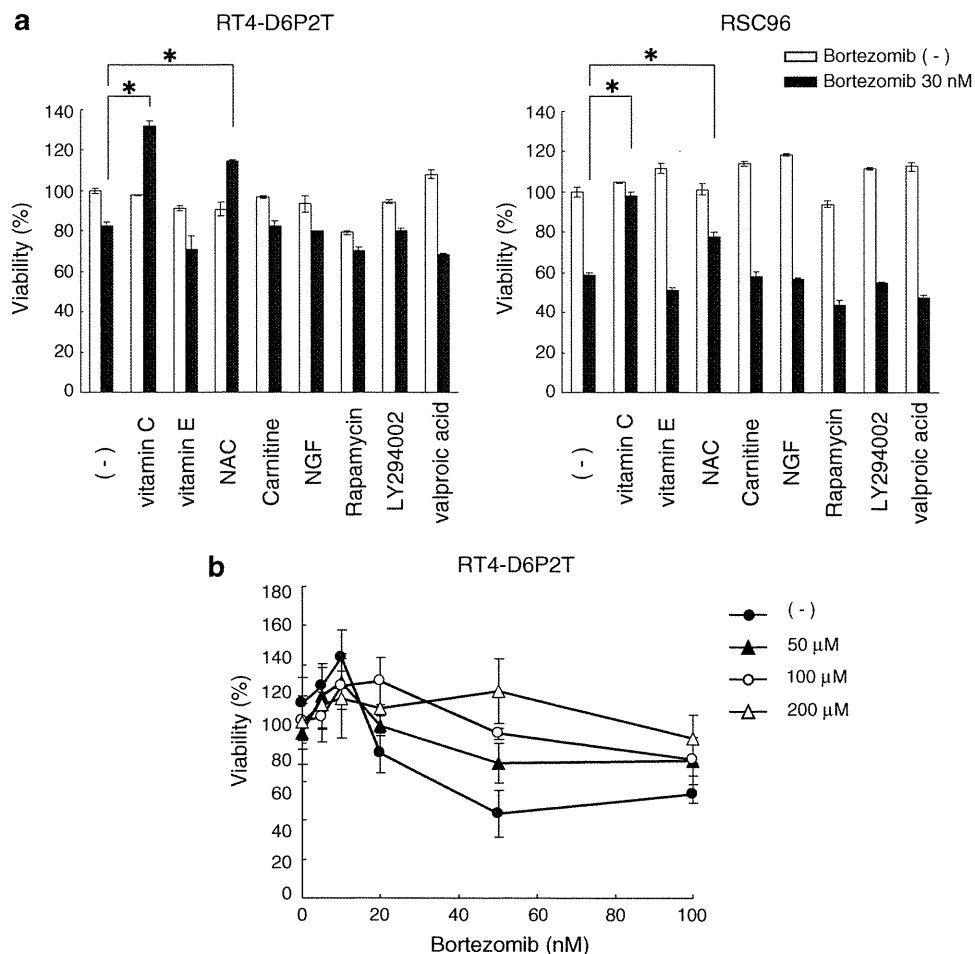
1 mM, a concentration demonstrated to be achieved after the intravenous administration of NAC [16]. Vitamin C at 200  $\mu$ M or NAC at 1 mM almost completely rescued Schwann cells exposed to bortezomib at 30 nM or less (Fig. 3c). Interestingly, these agents in combination cooperatively enhanced Schwann cell viability even with a highly toxic dose of bortezomib, 100 nM, whereas vitamin C or NAC alone only partially rescued the cells (Fig. 3c). In addition, the treatment with vitamin C or NAC alone reduced morphological changes by bortezomib with perinuclear inclusion bodies and vacuoles in the cytoplasm in RT4-D6P2T and RSC96 cells in parallel with the increase in viable cell numbers (Fig. 3d). Schwann cells appeared to be intact without such morphological changes when the two agents were used in combination (Fig. 3d). We further examined their effect on accumulation of ubiquitinated proteins induced by bortezomib. Vitamin C or NAC inhibited accumulation of ubiquitinated proteins upon

treatment with bortezomib (Fig. 3e). These results suggest vitamin C and NAC as a protective agent for Schwann cells and cooperative effects of these agents in combination against the bortezomib-induced toxicity at least in part through reduction of ubiquitinated protein accumulation.

### 3.4 Delayed addition of vitamin C and/or NAC rescues Schwann cells without compromising the anti-myeloma activity of bortezomib

Bortezomib rapidly induced cell death in myeloma cells, while Schwann cells appear to be much less susceptible to bortezomib-induced cytotoxicity and remain alive after treatment with bortezomib (Fig. 2a). We therefore hypothesized that delaying the addition of vitamin C may rescue Schwann cells from cell damage without compromising the anti-myeloma effects of bortezomib although vitamin C has been reported to reduce the anti-myeloma effects of bortezomib [17, 18]. To test our hypothesis, we examined the effects of vitamin C on the viability of myeloma cells and Schwann cells added simultaneously or at different time points within 8 h after the initiation of bortezomib treatment. Vitamin C added together with or 1 hour after bortezomib mostly reduced the cytotoxic effects of bortezomib on RPMI8226 cells (Fig. 4a). When vitamin C was added at 4 and 8 h after the bortezomib treatment, however, the suppression of the anti-myeloma cytotoxic effects of bortezomib was mostly abolished. On the other hand, the cytoprotective effects on RT4-D6P2T Schwann cells were observed when vitamin C was added at 8 h or earlier after the bortezomib treatment at 20 nM and at 4 h or earlier with bortezomib at 50 nM (Fig. 4a). Thus, delaying the addition of vitamin C by 4 or 8 h is suggested to allow bortezomib to exert its anti-myeloma effects while protecting Schwann cells.

We further investigated the effects of a 4-h delay in the administration of vitamin C and/or NAC following treatment with bortezomib on the viability of myeloma cells and Schwann cells. The simultaneous addition of vitamin C almost completely inhibited the cytotoxic effects of bortezomib on INA-6 and RPMI8226 myeloma cells, whereas NAC had somehow less potent cytoprotective effects on these myeloma cells with bortezomib at 20 and 30 nM (Fig. 4b). However, the addition of vitamin C or NAC 4 h after the treatment with bortezomib at 20 and 30 nM still resulted in cytotoxic activity, although vitamin C alone and the combination of vitamin C and NAC partially minimized the cytotoxic effects of bortezomib on RPMI8226 cells. In contrast, delaying the treatment with vitamin C and/or NAC mostly suppressed the cytotoxic effects on Schwann cells of bortezomib at 30 nM (Fig. 4c). The delayed treatment with vitamin C plus NAC compromised bortezomib's cytotoxicity against Schwann cells but not



**Fig. 3** Vitamin C and NAC abrogate bortezomib-induced cytotoxicity in Schwann cells. **a** RT4-D6P2T and RSC96 Schwann cells were cultured in triplicate in the absence (open bar) or presence (closed bar) of 30 nM bortezomib for 24 h. Various agents including 200 μM vitamin C, 200 μM vitamin E, 1 mM NAC, 1 mM acetylcarnitine, 10 ng/ml rh NGF, 60 μM rapamycin, 3 μM LY294002 and 100 μg/ml valproic acid were added as indicated. **b** RT4-D6P2T cells were cultured in triplicate in the absence or presence of bortezomib. Vitamin C was simultaneously added at 0 (filled circles), 50 (filled triangles), 100 (open circles) and 200 μM (open triangles). **c** RT4-D6P2T and RSC96 Schwann cells were cultured in triplicate in the absence or presence of bortezomib. Vitamin C (200 μM), NAC (1 mM), or both were simultaneously added. Filled circles, filled triangles, open circles and open triangles represent the addition of

neither agent, vitamin C alone, NAC alone, and the two agents in combination, respectively. After culturing for 24 h, viable cells were counted. Data are expressed as a percentage of control without any agents (mean ± SD). **d** RT4-D6P2T and RSC96 Schwann cells were cultured in the presence of 50 nM bortezomib. Vitamin C (200 μM), NAC (1 mM), or both were simultaneously added as indicated. After culturing for 48 h, cells were fixed and stained with Wright and Giemsa solutions. Intracytoplasmic inclusion bodies and vacuoles are indicated by arrows and arrowheads, respectively. **e** RT4-D6P2T cells were cultured for 20 h in the presence or absence of bortezomib (10 nM). Vitamin C (200 μM), NAC (1 mM), or both in combination were added as indicated. The cell lysates were harvested after the treatment. β-Actin was used as a loading control

myeloma cells even at 100 nM (Fig. 4c). These results suggest that the delayed treatment with vitamin C and/or NAC protects Schwann cells without compromising the anti-myeloma activity of bortezomib.

#### 4 Discussion

Dysfunction of ubiquitin–proteasome system has been implicated in the pathogenesis of various neurodegenerative diseases, including Charcot-Marie-Tooth disease type

1A, Alzheimer’s disease, Parkinson’s disease and polyglutamine diseases [19]. Autophagic inclusion bodies and vacuoles are observed in affected nerves and are suggested to degrade disease-related proteins. Bortezomib also induced the formation of inclusion bodies and vacuoles in Schwann cells (Fig. 1a). By analogy with these pathological conditions, the accumulation of neurotoxic ubiquitinated proteins by bortezomib treatment may contribute to the emergence of BIPN. Because vitamin C and NAC in combination markedly restored the viability and did not induce the formation of inclusion bodies and vacuoles in

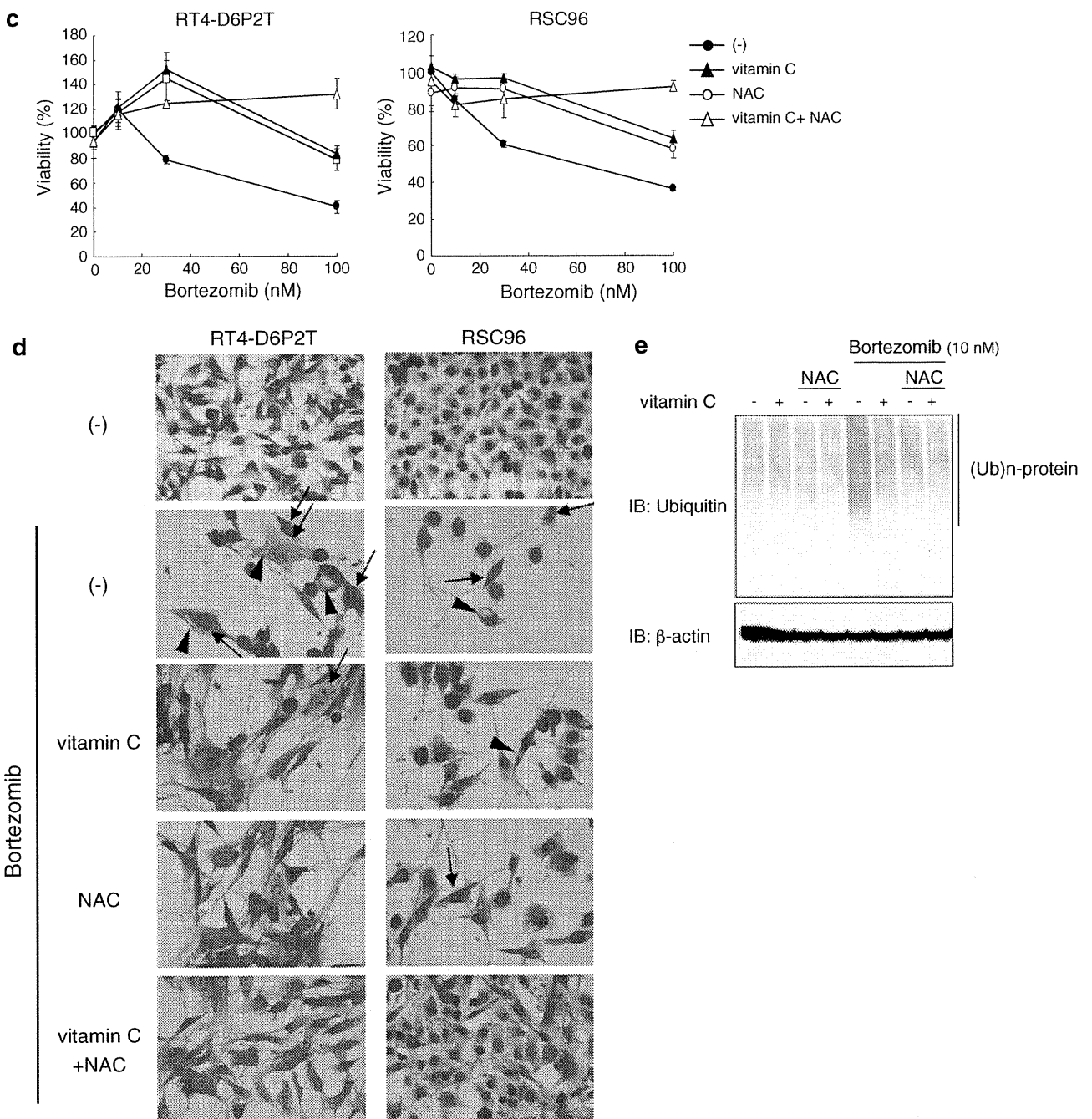
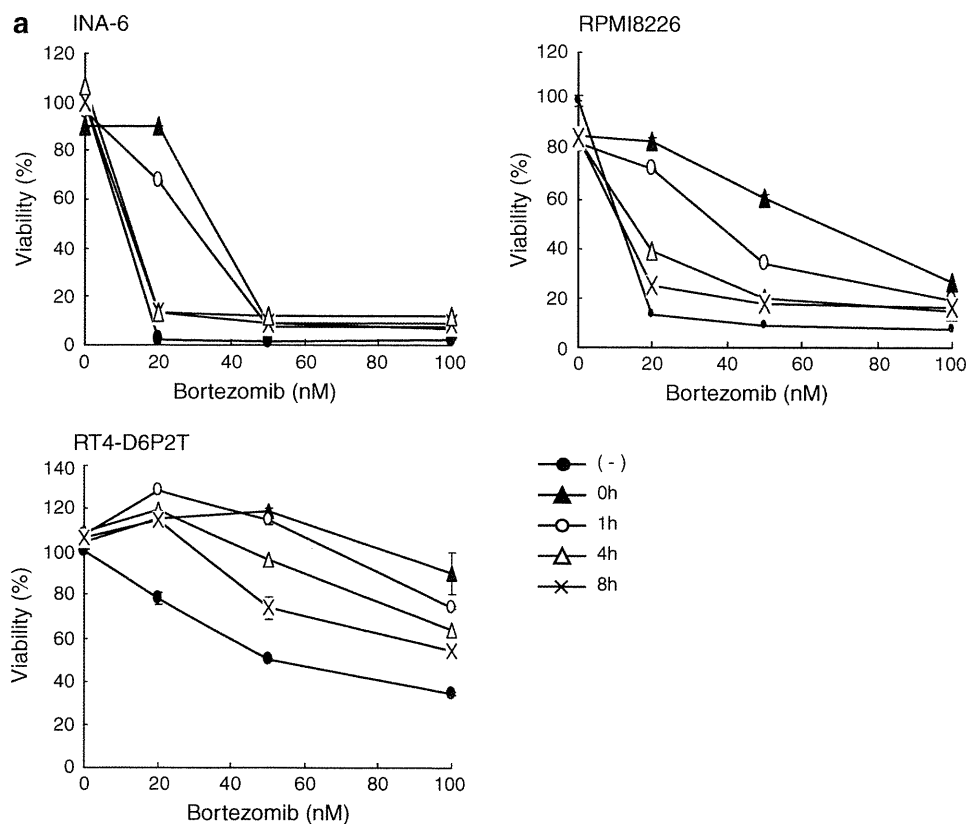


Fig. 3 continued

Schwann cells (Fig. 3c, d), vitamin C and NAC may affect the ubiquitin–proteasome metabolic process of neurotoxic ubiquitinated proteins. Watanabe et al. [20] recently demonstrated using a Schwannoma cell system that aggresome formation is a possible mechanism of BIPN, and that the pretreatment of suberoylanilide hydroxamic acid, 17-allylamino-17-demethoxy-geldanamycin and clonazepam enhances chaperon-mediated autophagy to alleviate

BIPN. In our study, we investigated ER stress and macroautophagy induced by bortezomib in similar cell systems, and found that vitamin C and NAC reduced ER stress induced by bortezomib. In addition, although bortezomib generates reactive oxygen species (ROS) to cause apoptosis, vitamin C and NAC, as potent antioxidants, may reduce ROS-mediated cytotoxic effects of bortezomib [21, 22]. Furthermore, NAC is converted to glutathione in cytoplasm





**Fig. 4** Delayed treatment with vitamin C and NAC rescues Schwann cells but not MM cells from bortezomib. **a** INA-6 and RPMI8226 MM cells, and RT4-D6P2T Schwann cells were cultured in triplicate in the presence of bortezomib at different concentrations. Vitamin C (200  $\mu$ M) was added together with (filled triangles) or 1 (open circles), 4 (open triangles) and 8 h (times) after bortezomib. **b** INA-6 and RPMI8226 MM cells were cultured in triplicate in the presence of bortezomib at different concentrations. Vitamin C (200  $\mu$ M), NAC (1 mM), or both were added together with (left) or 4 h after bortezomib (right). Filled circles, filled triangles, open circles and

open triangles represent the addition of neither agent, vitamin C alone, NAC alone, and the two in combination, respectively. **c** RT4-D6P2T Schwann cells and RPMI8226 cells were cultured in triplicate in the presence of bortezomib at different concentrations (left). Vitamin C (200  $\mu$ M), NAC (1 mM), or both were added 4 h after bortezomib. Filled circles, filled triangles, open circles and open triangles represent the addition of neither agent, vitamin C alone, NAC alone, and the two agents in combination, respectively. After culturing for 24 h, viable cells were counted. Data are expressed as a percentage of control without any agents (mean  $\pm$  SD)

to activate vitamin C [23], which may contribute to the cooperative effects of both agents for the protection of Schwann cells. However, the precise role of vitamin C and/or NAC in ubiquitin–proteasome metabolic process remains to be elucidated. Because there may be a difference in the drug sensitivity between species, these observations should be recapitulated in human Schwann cells.

Minimizing the adverse effects of anti-tumor agents without diminishing their clinical efficacy is critical to cancer treatment. The delayed administration of antidote/rescue agents is used to achieve anti-tumor activity and reduce unwanted side effects. One approach widely used in the treatment of cancers including lymphomas of the central nervous system and osteosarcoma is to administer leucovorin after delivering a high-dose methotrexate [24, 25]. The delayed administration of leucovorin prevents potentially life-threatening side effects of methotrexate

without compromising its potent anti-tumor effects. We demonstrated in the present study that delayed treatment with vitamin C and/or NAC rescued Schwann cells from bortezomib-induced toxicity but did not compromise the agent’s anti-MM activity. These observations suggest that vitamin C and/or NAC can rescue cells from BIPN, and that a neuroprotective effect is achieved with the delayed administration of these agents. We are expecting to extend our study in myeloma animal models after establishing the evaluation methods for BIPN-associated neurological signs and pathological features.

To make the best use of this important anti-myeloma agent for longer while eliminating the risk of BIPN, the delayed administration of vitamin C and/or NAC may be worth considering, especially for responders to bortezomib with a high risk of neuropathy or impending BIPN. Because vitamin C and NAC affect the anti-tumor effect of

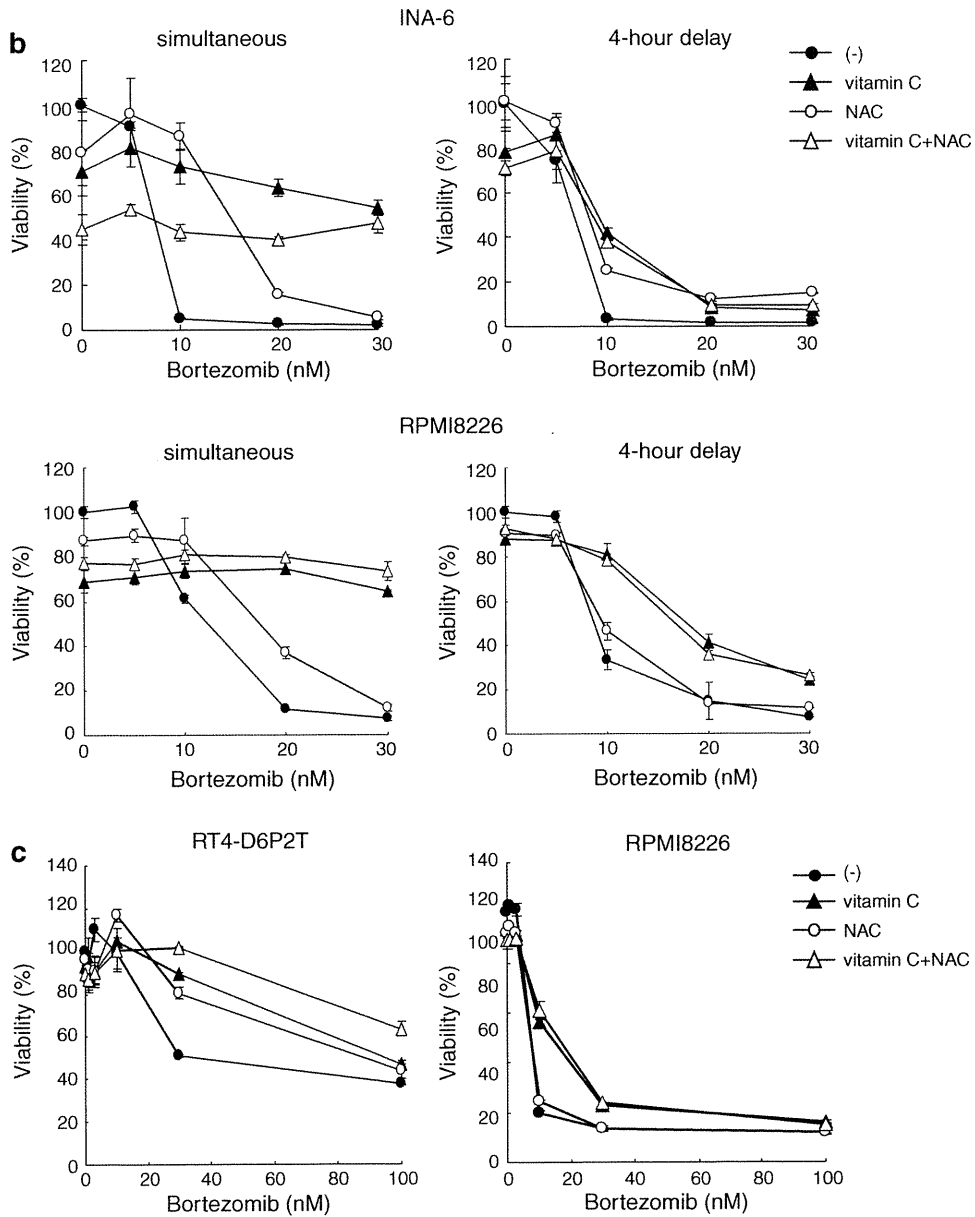


Fig. 4 continued

bortezomib, overall efficacy should be carefully evaluated in a well-designed clinical study.

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# Efficacy of Long-Term Treatment with Low-Dose Thalidomide for Patients with Relapsed/Refractory Multiple Myeloma

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## ABSTRACT

**Introduction:** We report the results of a prospective study of long-term treatment with single-agent thalidomide in patients who had responded in a preceding trial of the use of thalidomide for relapsed/refractory myeloma. **Patients and Methods:** Nineteen patients were enrolled: 11 patients (57.9%) treated at a dosage of 100 mg/day; 2 patients (10.5%) at a dosage of 200 mg/day; 2 patients (10.5%) at a dosage of 300 mg/day; and 4 patients (21.1%) at a dosage of 400 mg/day. The median follow-up from the start of the preceding study was 3.0 years. At the time of entry to this study, 5 patients (26.3%) had partial response (PR), another 5 patients (26.3%) had a minimal response (MR), and the remaining 9 patients (47.4%) had shown no change (NC). **Results:** The cumulative MR rate was 78.9% (at the 32<sup>nd</sup> week) and the cumulative PR rate was 47.4% (at the 112<sup>th</sup> week). The median progression-free survival was 104 weeks and the median time to next treatment was 144 weeks. No patients experienced grade 4 or greater hematologic toxicity or grade 3 or greater non-hematologic toxicity. **Conclusion:** Long-term thalidomide maintenance therapy induced an increase in response rate, suppressed the progression to active myeloma without severe adverse events, and contributed to long survival with good activities of daily living.

**Keywords:** Maintenance Therapy, Progression-Free Survival, Time to Next Treatment, Efficacy, Safety

## 1. Introduction

Whereas the positive role of thalidomide as a consolidation treatment after high-dose therapy with autologous stem cell transplantation (HDT-ASCT), in the context of newly diagnosed myeloma, has been clarified in the patients without high-risk cytogenetics who have obtained less than a very good partial response [1,2], its role as a maintenance therapy remains controversial because of its toxicity and the concern of potential induction of resistance to subsequent treatment [3-5]. In addition, we cur-

rently have much less information on the role of thalidomide as maintenance therapy, in the context of cases of relapsed/refractory multiple myeloma, after successful salvage treatment. Based on the results of phase II trials in which the cumulative dose of thalidomide did not have an impact on the efficacy of maintenance therapy and toxicity increased above a dosage of 200 mg/d [6,7], treatment with low-dose thalidomide is now the preferred option.

We have conducted a prospective study to evaluate the efficacy and safety of thalidomide given as a single-agent maintenance therapy to patients with relapsed and/or re-