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ORIGINAL ARTICLE

# Reactivation of hepatitis B virus in HBsAg-negative patients with multiple myeloma: two case reports

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**Abstract** It was recently reported that hepatitis B virus (HBV) reactivation had occurred in HBsAg-negative lymphoma patients who received rituximab plus steroid combination chemotherapy. HBV reactivation in myeloma patients have not been reported extensively. We describe here two cases of HBV reactivation in HBsAg-negative myeloma patients receiving systemic chemotherapy: one from the medical records of 40 patients and another from 61 patients with prospective HBV-DNA monitoring. In the first case positive for anti-HBs, HBV reactivation was diagnosed when hepatitis developed during conventional chemotherapy such as MP and MCP regimen in a relapsed patient after autologous stem cell transplantation (APBSCT); in the second case positive for anti-HBc and anti-HBs, elevation of HBV-DNA was recognized by serial HBV-DNA monitoring performed prospectively following APBSCT. Interestingly, these two cases had the reduction of the titer of anti-HBs during the treatment, followed by HBV reactivation. These clinical data suggest that the HBV-DNA monitoring is necessary for not only HBsAg-positive but also HBsAg-negative myeloma patients with anti-HBc-positive and/or anti-HBs-positive following transplantation and after conventional chemotherapy in the salvage setting. Establishment of a standard strategy to prevent HBV reactivation is important for myeloma patients receiving systemic chemotherapy.

**Keywords** Reactivation · HBV · Myeloma · Transplantation

**Abbreviations**

| | |
|---|---|
| HBV | Hepatitis B virus |
| HBsAg | Hepatitis B surface antigen |
| Anti-HBc | Hepatitis B core antibody |
| Anti-HBs | Hepatitis B surface antibody |
| AST | Aspartate transaminase |
| ALT | Alanine aminotransferase |
| RTD-PCR | Real-time detection polymerase chain reaction |
| APBSCT | Autologous peripheral blood stem cell transplantation |
| VAD | Vincristine, doxorubicin, dexamethasone |
| MP | Melphalan, prednisolone |
| MCP | Ranimustine, cyclophosphamide, prednisolone |
| MMCP | Melphalan, ranimustine, cyclophosphamide, prednisolone |
| BD | Bortezomib, dexamethasone |
| TD | Thalidomide, dexamethasone |
| CHOP | Cyclophosphamide, doxorubicin, vincristine, prednisolone |
| R-CHOP | Rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone |

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## 1 Introduction

Most cases of hepatitis B virus (HBV) reactivation have been reported in hepatitis B surface antigen (HBsAg)-positive cancer patients receiving systemic chemotherapy [1]. It was recently reported, however, that HBV reactivation also occurred in HBsAg-negative lymphoma patients who received rituximab plus steroid combination chemotherapy [2–5].

The clinical data on HBV reactivation in myeloma patients have not been reported extensively; therefore, we have performed retrospective and prospective analyses of HBV reactivation in 101 myeloma patients who received systemic chemotherapy at Nagoya City University Hospital. Based on these analyses, we report here two cases of HBV reactivation in HBsAg-negative myeloma patients.

## 2 Patients and methods

Between January 2001 and July 2009, 101 patients were diagnosed as multiple myeloma at Nagoya City University Hospital. We retrospectively analyzed the medical records of 40 patients for the development of hepatitis B who were diagnosed as multiple myeloma between January 2001 and December 2005. In 2006, we instituted the strategy described below to prevent HBV reactivation, and carried it out prospectively in 61 patients between January 2006 and July 2009. The serological markers for HBsAg, hepatitis B core antibody (anti-HBc) and hepatitis B surface antibody (anti-HBs) were tested to establish HBV infection status before the initial chemotherapy. HBsAg and anti-HBs were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBc of IgG classes was determined by radioimmunoassay (Abbot Japan) or CLEIA (Fujirebio). If the patient was positive for any of the serological markers, plasma HBV-DNA was measured by real-time detection polymerase chain reaction (RTD-PCR). If the patient was HBsAg-positive and/or had HBV-DNA before chemotherapy, prophylactic therapy with an antiviral drug was administered during and for at least 6 months after the chemotherapy. On the other hand, if the patient was HBsAg-negative, but seropositive for anti-HBc and/or anti-HBs (defined as resolved HBV infection), a serial monitoring of HBV-DNA was performed monthly by RTD-PCR during and for at least 1 year after the chemotherapy. If plasma HBV-DNA levels became detectable, antiviral therapy was started as soon as possible.

In this prospective HBV-DNA monitoring, each case of plasma HBV-DNA was measured at SRL Inc, using methods with the highest sensitivity available at the time in clinical practice; the assays included the following: TaqMan PCR assay (Roche Molecular Systems Inc, between April 2008 and July 2009), or Amplicor-PCR assay (Roche Molecular Systems Inc, between January 2006 and March 2008). The cutoff values of the TaqMan PCR assay and Amplicor-PCR assay were set at 1.8 log copies and 2.6 log copies/mL, respectively. In this retrospective analysis, serum HBV-DNA was measured at our laboratory of Nagoya City University using preserved specimen, and HBV-DNA sequences spanning the S gene were amplified by RTD-PCR in accordance with the previously described protocol with a slight modification; it has a detection limit of 2.0 log copies/mL [6].

The two patients with HBV reactivation provided written informed consent to the publication of this report.

## 3 Treatment for multiple myeloma

In patients younger than 65 years, autologous peripheral blood stem cell transplantation (APBSCT) was performed using high-dose melphalan (200 mg/m$^2$) following three courses of a VAD (vincristine, doxorubicin, and dexamethasone) regimen as induction therapy and a high-dose cyclophosphamide regimen as stem cell harvest therapy.

In patients who did not choose the transplantation treatment option, or from whom we could not collect enough hematopoietic stem cells, the initial treatment for symptomatic multiple myeloma was MP (melphalan plus prednisolone) or MMCP (melphalan, ranimustine, cyclophosphamide, prednisolone combination chemotherapy).

Patients over 65 years of age were not candidates for transplantation. The initial treatment regimens for these patients were MP, VAD, MMCP or VAD following MP. In relapsed and refractory patients, BD (bortezomib, dexamethasone: after December 2006), or TD (thalidomide, dexamethasone: after December 2008) or other regimens (MP, VAD, etc.) were administered as salvage treatments for all patients.

## 4 Results

### 4.1 A case with HBV reactivation on the basis of the medical records

Based on the retrospective analyses, only one patient developed HBV reactivation among 40 multiple myeloma patients diagnosed from January 2001 to December 2005. The clinical course is shown in Fig. 1. A 59-year-old woman diagnosed as symptomatic multiple myeloma (BJP-$\kappa$ type) received APBSCT as initial treatment. Before APBSCT, she was seronegative for HBsAg, but no

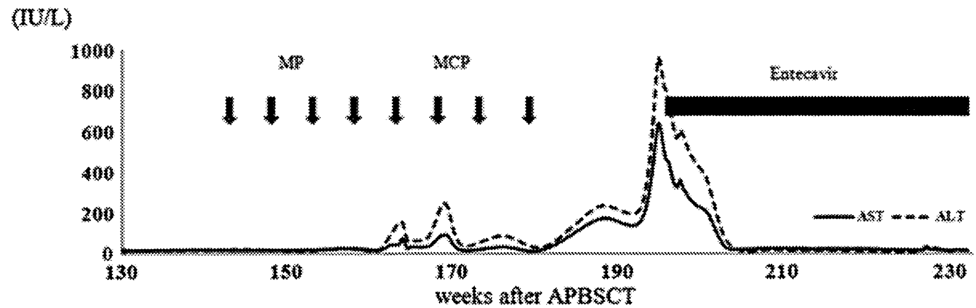


**Fig. 1** Clinical course of Case 1. *AST* aspartate transaminase, *ALT* alanine aminotransferase

screening tests for anti-HBc or anti-HBs were performed. Multiple myeloma recurred about 3 years after APBSCT, and MP was administered as salvage treatment. When MP therapy was started, she was seronegative for both HBsAg and anti-HBc, but seropositive for anti-HBs. Because MP could not control the disease, MCP (ranimustine, cyclophosphamide, prednisolone) therapy was administered as the next salvage regimen. Liver damage occurred 32 weeks after the initial salvage chemotherapy was started, and at that time HBsAg changed from negative to positive, and serum HBV-DNA was detectable at 6.2 log copies/mL, so we concluded that the liver damage was caused by hepatitis B virus.

Analyses of specimens preserved during and after salvage therapy showed that serum HBV-DNA was detectable at 2.2 log copies/mL at base line when MP therapy was started, as shown in Fig. 1. In other words, the patient had an occult HBV infection (defined as HBsAg-negative, but HBV-DNA detectable) before salvage chemotherapy.

Furthermore, the HBV gene sequences before and after salvage chemotherapy were confirmed identical in Case 1, so we judged that the liver damage was caused by HBV reactivation. HBV reactivation was reduced after entecavir (0.5 mg, once daily) was administered as an anti-HBV nucleotide analog, and HBV-DNA levels decreased to below the limit of detection.

### 4.2 HBV-DNA monitoring to prevent HBV reactivation (Fig. 2)

Among 61 patients with symptomatic multiple myeloma diagnosed between January 2006 and July 2009, 1 patient was seropositive for HBsAg, 15 patients were seropositive for anti-HBc and/or anti-HBs (indicating resolved HBV infection), and the remaining 45 patients were seronegative for either anti-HBc or anti-HBs according to the screening tests shown in Fig. 2. One HBsAg-positive patient was given an antiviral drug for prophylaxis before initial treatment. On the other hand, we prospectively performed serial HBV-DNA monitoring during and after myeloma treatment in the 15 patients with resolved HBV infection who had no occult infection.

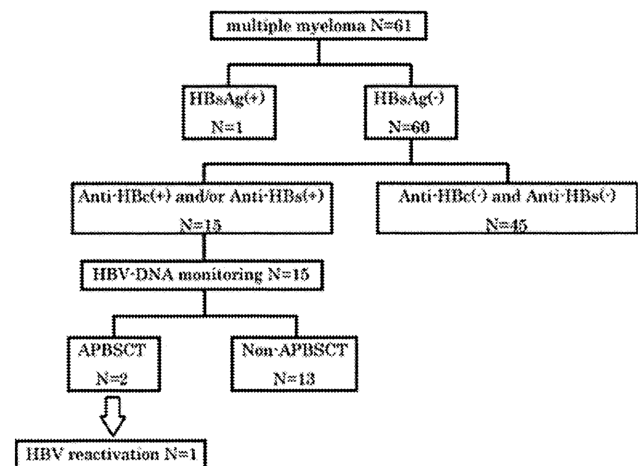


**Fig. 2** Screening tests for serological markers of HBV infection and the strategy to prevent HBV reactivation in 61 myeloma patients diagnosed between January 2006 and July 2009 at Nagoya City University Hospital. One patient was seropositive for HBsAg, 15 patients were seropositive for anti-HBc and/or anti-HBs (defined as resolved HBV infection), the remaining 45 patients were seronegative for either anti-HBc or anti-HBs. We prospectively performed serial HBV-DNA monitoring during and after myeloma treatment in 15 patients with resolved HBV infection: following APBSCT, 1 of 15 patients developed HBV reactivation without hepatitis and prior to liver damage because elevation of HBV-DNA was confirmed at an early stage

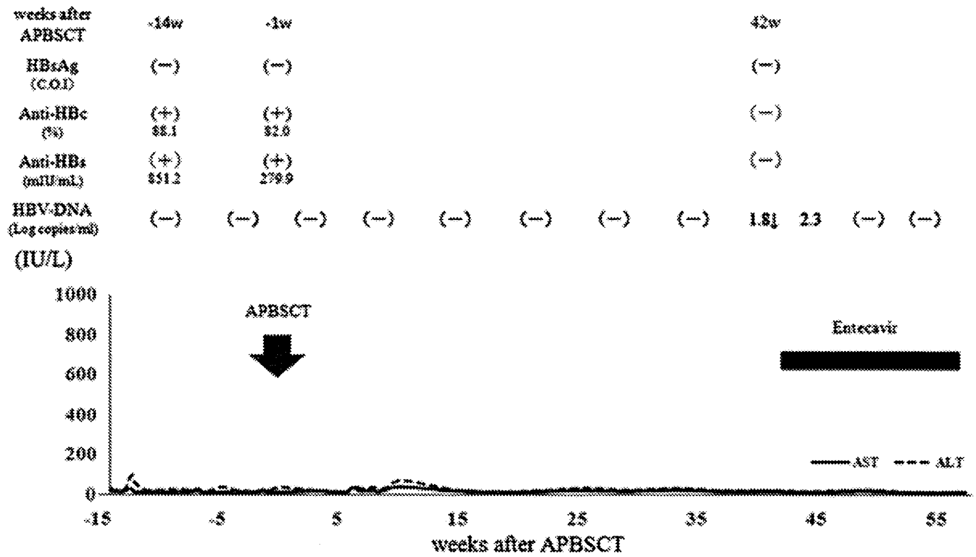


**Fig. 3** Clinical course of Case 2

In 8 of these 15 patients, the initial treatment was MP. Following VAD therapy, three patients received MP. Two patients underwent APBSCT. Each of the remaining two patients received MMCP therapy but no further treatment. For salvage treatment, 4 of the 15 patients received BD and/or TD.

One of the 15 patients developed HBV reactivation without hepatitis following APBSCT, after elevation of HBV-DNA was confirmed at an early stage prior to liver damage (Fig. 3). A 61-year-old woman was diagnosed as symptomatic multiple myeloma (BJP-$\lambda$ type). In screening tests before treatment, HBsAg was negative, both anti-HBc and anti-HBs were positive, and plasma HBV-DNA was below the limit of detection. Therefore, the patient's HBV status was confirmed as a resolved infection. Prospective serial HBV-DNA monitoring was performed monthly, but the testing was sometimes postponed up to 3 months on account of the patient.

Forty-two weeks (about 10 months) after APBSCT, the plasma HBV-DNA level was less than 1.8 log copies/mL but an amplification signal was detectable by the TaqMan PCR assay, and during the following month the HBV-DNA level became detectable with up to 2.3 log copies/mL, as shown in Fig. 2. HBV reactivation was diagnosed at that time, and entecavir (0.5 mg, once daily) was administered immediately as an anti-HBV nucleotide analogue. The plasma HBV-DNA decreased to an undetectable level without liver damage. At the time of HBV reactivation, all HBV serological markers (HBsAg, anti-HBc and anti-HBs) were negative, which suggested that the antibody titers may be reduced by the myeloma treatment. We performed a retrospective search of blood transfusions (red cells and platelets) received by this patient during the previous chemotherapy using the stored specimens from all the blood donors. As a result, it was concluded that the possibility of HBV infection through blood transfusion was extremely low.

## 5 Discussion

We reported two cases of HBV reactivation in myeloma patients who were seronegative for HBsAg before treatment. HBV was reactivated in a patient with occult infection and definitely diagnosed by a retrospective analysis of preserved specimens when the onset of liver damage occurred after salvage treatment 3 years after APBSCT. In another patient with resolved HBV infection, HBV reactivation at an early stage was detected by the serial HBV-DNA monitoring performed prospectively, and an antiviral drug was administered before liver damage had occurred.

Some HBsAg-negative patients have recently been reported to develop fatal hepatitis by HBV reactivation in the rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone (R-CHOP) or R-CHOP-like regimens, which combine rituximab and steroid for treatment of CD20-positive malignant lymphoma [2–5]. In 2006, Hui et al. [3] reported that 8 of 244 HBsAg-negative lymphoma patients receiving systemic chemotherapy developed hepatitis by HBV reactivation, and these eight patients were seropositive for either anti-HBc or anti-HBs. It was shown that rituximab plus steroid combination chemotherapy was a risk factor by multivariate analysis. Most recently, Yeo et al. [4] reported that 5 of 80 HBsAg-negative patients diagnosed as diffuse large B cell lymphoma and receiving R-CHOP or CHOP-like regimens had reactivated HBV. All five had received R-CHOP and all were positive for anti-HBc and negative for anti-HBs.

The HBV reactivation following APBSCT in patients with multiple myeloma has been reported sporadically.

Endo et al. [7] reported that 3 of 24 HBsAg-negative patients with resolved HBV infection developed new-onset hepatitis B following APBSCT, and all three patients were multiple myeloma patients. Uhm et al. [8] performed a retrospective analysis of the change of HBV serologic markers following APBSCT. Seven of 129 HBsAg-negative patients became HBsAg-positive after transplantation. All seven patients were seropositive for both anti-HBs and anti-HBc before treatment, and six of the seven patients had multiple myeloma. Furthermore, at reactivation after transplantation, it was shown that the titers of anti-HBs decreased in six of the seven patients. This phenomenon was also shown in our both cases. These data suggested that the decreased titer of anti-HBs may be associated with HBV reactivation, and that the pathophysiology of reactivation may be affected by the dysfunction of humoral immunity in multiple myeloma.

It is necessary to pay attention to the onset of HBV reactivation during salvage treatment; thus, if immunologic inhibition is strong over a longer period, the risk of HBV reactivation may be increased more in patients on second-line or third-line chemotherapy than in those undergoing chemotherapy for the first time [9]. As mentioned above, APBSCT may be one of the important risk factors for HBV reactivation in myeloma patients; however, the reactivation may occur even if the salvage treatment was performed with a mild myelosuppressive regimen such as MP and MCP after the autologous transplantation shown in Case 1. New molecular target drugs such as bortezomib, thalidomide, and lenalidomide improve the survival of myeloma patients remarkably [10–12], so the number of patients who will receive the immunosuppressive therapy for longer periods may increase in the future. Therefore, a standard strategy to prevent HBV reactivation may also become more important in myeloma treatment.

HBV reactivation may lead to fatal fulminant hepatitis, so we hematologists and oncologists should identify high-risk groups in advance before chemotherapy. The latest CDC and Japanese guidelines recommend that patients receiving cytotoxic or immunosuppressive therapy should be tested for serologic markers of HBV infection (i.e., HBsAg, anti-HBc, anti-HBs) [13, 14]. HBV infection status should be established before any chemotherapy or immunosuppressive therapy is initiated (when there is no immunologic inhibition), because antibody titers may be reduced by the treatment, as shown in Case 2. For patients positive for any of the HBV serological markers, the presence of HBV-DNA should be confirmed by RTD-PCR [5, 14].

Prophylaxis with antiviral drugs is essential for HBsAg-positive patients undergoing systemic chemotherapy as recommended by the latest American and Japanese guidelines [14, 15]. Because patients with serum HBV-DNA have more potential risk factors for HBV reactivation, they should be given antiviral drugs as well [5, 14].

If a patient is seropositive for anti-HBc and/or anti-HBs, no standard strategy to prevent HBV reactivation has been established, but making an early diagnosis of HBV reactivation is critical to enable early initiation of active antiviral therapy. Preemptive therapy by serial HBV-DNA monitoring is a reasonable strategy recommended by the latest Japanese guidelines [14]. If HBV-DNA levels become detectable, antiviral therapy should be started as soon as possible.

Only a few studies have reported on the optimal frequency and duration of HBV-DNA monitoring. Hui et al. [3] reported on malignant lymphoma patients that the median time from the elevation of serum HBV-DNA to hepatitis onset was 18.5 weeks (range 12–28 weeks). Most recently, Fukushima et al. [16] conducted a prospective study to monitor HBsAg monthly and HBV-DNA every 3 months during and after systemic chemotherapy in HBsAg-negative but anti-HBc-positive patients with malignant lymphoma; they found that 1 of 24 patients developed HBV reactivation, which was diagnosed by elevation of HBV-DNA level, while their HBsAg was still negative. In fact, as shown in Case 2, we were able to diagnose HBV reactivation at an early stage by the monthly HBV-DNA monitoring and avoid liver damage and decrease plasma HBV-DNA to below the limit of detection by starting the antiviral drug administration.

It is also necessary to make a differential diagnosis in order to distinguish transmission of HBV by blood transfusion from HBV reactivation, because blood transfusion may be received during systemic chemotherapy. In Case 2, we performed a retrospective search of blood transfusion (red cells and platelets) received during previous chemotherapy, using the stored specimens of all blood donors. It was concluded that the possibility of HBV infection through blood transfusion was extremely low.

In conclusion, these clinical data suggest that the HBV-DNA monitoring is necessary for not only HBsAg-positive but also HBsAg-negative myeloma patients with anti-HBc-positive and/or anti-HBs-positive following transplantation and after conventional chemotherapy in the salvage setting. Preemptive therapy by serial HBV-DNA monitoring may be a useful and cost-effective option for preventing HBV reactivation in patients with resolved HBV infection. Establishment of a standard strategy to prevent HBV reactivation is important for myeloma patients receiving systemic chemotherapy.

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ORIGINAL ARTICLE

# Lenalidomide plus dexamethasone treatment in Japanese patients with relapsed/refractory multiple myeloma

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**Abstract** We conducted a multicenter, open-label study to investigate the safety, efficacy, and pharmacokinetics of lenalidomide in Japanese patients with relapsed or refractory multiple myeloma The study was composed of the "monotherapy phase", a dose-escalation phase, to determine the tolerability to single agent lenalidomide and the "combination phase" to determine the safety and obtain preliminary data on the efficacy of lenalidomide plus dexamethasone. The primary end points were the tolerability to 25 mg lenalidomide and safety. Nine and six patients were enrolled in the monotherapy phase and the combination phase, respectively. Since 25 mg of monotherapy treatment did not satisfy the DLT criteria, this dose was employed in the combination phase. The major adverse event was myelosuppression. At the planned interim analysis (median study duration, 26.3 weeks), grade 3 or grade 4 neutropenia was observed with high frequency (66.7%). However, all adverse events observed were clinically manageable. In the combination cohort, the overall response rate (≥PR) was 100%. The pharmacokinetics of lenalidomide showed rapid absorption and elimination after both single and multiple doses. In conclusion, 25 mg of lenalidomide was given safely as a single agent or in combination with dexamethasone in Japanese patients. The good efficacy of the combination therapy was also demonstrated in this study.

**Keywords** Multiple myeloma · Lenalidomide · Dexamethasone · Pharmacokinetics

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## 1 Introduction

Lenalidomide is one of the immunomodulatory drugs (IMiD® brand drugs) developed by Celgene Corporation. Two phase III studies, MM-009 and MM-010, designed to compare lenalidomide plus high-dose dexamethasone combination therapy (LD therapy) with dexamethasone monotherapy (D therapy) in previously treated patients with multiple myeloma (MM) were conducted in US/Canada and Europe/Israel/Australia, respectively [1, 2]. In these studies, 25 mg of lenalidomide was administrated at days 1–21 of a 28-day cycle; 40 mg of dexamethasone was co-administrated with lenalidomide on days 1–4, 9–12, and 17–20 for the first 4 cycles, and on days 1–4 after the 4th cycle. Superiority of the LD regimen was demonstrated based on the following significant differences from D therapy [3]: overall response rate (≥PR) of 60.6% (vs. 21.9% for D therapy), time to progression (TTP) of 13.4 months (vs. 4.6 months), progression-free survival (PFS) of 11.1 months (vs. 4.6 months) and overall survival period of 38 months (vs. 31.6 months). With regard to the safety, the adverse events (AEs) of LD therapy were mainly related to bone marrow suppression, e.g., neutropenia, and all AEs were manageable by supportive care, dose reduction, or interruption of lenalidomide. These data led to approval of lenalidomide as a treatment in combination with dexamethasone for patients with MM who had been treated previously with at least one therapeutic regimen by the US Food and Drug Administration (FDA) in 2006, the European Medicines Evaluation Agency (EMEA) in 2007, and by regulatory agencies on many other countries. The combination therapy is recommended by the clinical practice guidelines of the National Comprehensive Cancer Network (NCCN) as a salvage therapy (category 1) for relapsed or refractory MM [4]. The combination therapy is also category 1 primary therapy for newly diagnosed MM.

Lenalidomide has not yet been approved in Japan. No data are available regarding the safety, efficacy, or pharmacokinetics of LD therapy in Japanese patients. This study was a multicenter, non-randomized, and open-label study to examine the safety, efficacy, and pharmacokinetics of lenalidomide as a single agent or in combination with dexamethasone in Japanese patients with relapsed/refractory MM. The study also examined the pharmacokinetics of higher dose dexamethasone (40 mg), which has not been previously reported.

## 2 Patients and methods

### 2.1 Patients

Patients who fulfilled the following inclusion criteria were enrolled in the study: Japanese MM patients aged 20 years or older who were previously treated with at least one prior therapy for myeloma and evaluated to have progressive disease (PD)/disease progression during or after the prior treatment; serum M protein ≥0.5 g/dL or urinary M protein (as measured in a 24-h urine sample) ≥0.2 g; Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2. Patients were excluded from the study if they met any of the following exclusion criteria: patients with acute myocardial infarction within the past 6 months, or patients with a history of deep venous thrombosis (DVT) or pulmonary embolism within the past 3 years; pregnant or lactating females; absolute neutrophil count of less than 1,000/μL ($1.0 \times 10^9$/L); platelet count of less than 75,000/μL ($75 \times 10^9$/L); serum creatinine level of over 2.5 mg/dL.

### 2.2 Study design

The study design is based on the previously conducted MM-009/010 studies. Figure 1 illustrates the outline of the study design. The primary end points were the tolerability of 25 mg of single agent lenalidomide and the safety of lenalidomide given alone or in combination with dexamethasone in Japanese patients. The secondary end points

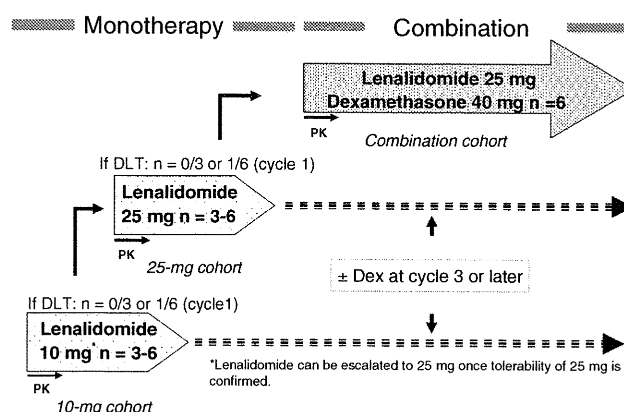


**Fig. 1** Study design. This study was composed of the monotherapy phase and the combination phase. In the monotherapy phase, the standard "3 + 3" design of phase I study was employed to determine the safety of single agent lenalidomide. After confirming the tolerability of 25 mg dose, a combination cohort was initiated. In the combination cohort, 6 patients were enrolled. In the 10- or 25-mg monotherapy cohort, patients received 10 or 25 mg lenalidomide once daily, respectively, for 1–21 days a 28-day cycle. In the combination cohort, in addition to the lenalidomide administration, 40 mg of dexamethasone was co-administered with lenalidomide on days 1–4, 9–12, and 17–20 for the first 4 cycles and only on days 1–4 after the 4th cycle. Interim analysis was performed after all patients completed 24 weeks at least after the initiation of the study. Patients who participated in the 10- or 25-mg cohort were allowed to receive 40 mg of dexamethasone and 25 mg of lenalidomide from cycle 3 or later. Pharmacokinetic analysis was performed in cycle 1 in each cohort. Dexamethasone or lenalidomide was not administered on day 1 or 2, respectively, in cycle 1 in the combination cohort to evaluate plasma concentration of each drug when administered alone

were pharmacokinetics and efficacy (response rate, duration of response, and PFS). AEs were reported according to MedDRA Ver. 10. The grade of the AEs was evaluated according to the Common Terminology Criteria for Adverse Events, Ver. 3.0 (NCI-CTCAE). The relationship of AEs to drug was based on the investigators' assessment. Efficacy was evaluated according to the modified EBMT/IBMTR/ABMTR criteria [1, 2, 5].

The study consisted of two phases: the "monotherapy phase" during which patients received lenalidomide alone, followed by the "combination phase" during which lenalidomide was administered in combination with dexamethasone (Fig. 1). A treatment cycle with both phases consisted of 28 days. During each cycle, lenalidomide was administered orally once daily from days 1 to 21, followed by 7 days off therapy. In the monotherapy phase, patients were treated in the "3 + 3" design of phase I study at two different dose levels. DLT (dose limiting toxicity) evaluation was conducted at the end of cycle 1. In this study, DLT was defined as ≥grade 4 neutropenia or thrombocytopenia, or ≥grade 3 febrile neutropenia or non-hematological AEs. The initial dose level tested was 10 mg (10-mg cohort). If no DLTs occurred in 3 patients, a next cohort at 25 mg of lenalidomide was tested. If a DLT occurred in 1 of 3 patients, an additional 3 patients were enrolled; if incidence of DLT resulted in 1 of 6 patients, a 25-mg cohort was enrolled. As with the 10-mg cohort, if DLT did not occur in 3 patients or occurred in 1 of 6 patients in the 25-mg cohort, the 25 mg dose was used for future studies. After the safety of 25 mg of single agent lenalidomide was confirmed, a combination phase with 6 new patients was investigated for the safety and efficacy of LD treatment. In the combination phase, lenalidomide was administered at 25 mg from days 1 to 21 of each cycle. Dexamethasone at a strength of 40 mg (ten 4-mg tablets) was administered orally in combination with lenalidomide once daily from days 1 to 4, 9 to 12, and 17 to 20 for the first 4 cycles, and from days 1 to 4 after cycle 4. Treatment could be continued for up to 156 weeks (3 years) at the investigators' discretion. Patients who participated in the monotherapy phase were allowed to receive 40 mg of dexamethasone and a higher dose (25 mg) of lenalidomide from cycle 3 or later to evaluate the safety of long-term treatment, once the safety of 25 mg dose of lenalidomide was determined.

Interim analysis was performed after all patients completed at least 24 weeks of treatment. Administration of DVT prophylaxis was recommended for the patients who received combination treatment of lenalidomide and dexamethasone. Administration of G-CSF was permitted for treatment of neutropenia.

Dose reduction or dose interruption (temporary suspension of the treatment) of the study drugs due to the study drug-related adverse events was permitted. In accordance with the principles of Good Clinical Practice (GCP), the study protocol was approved by IRB of each institution, and written informed consent was obtained from each patient enrolled in the study.

### 2.3 Pharmacokinetics

Pharmacokinetic analysis was performed in cycle 1 in each cohort. In the 10- and 25-mg cohorts in the monotherapy phase, plasma concentration of lenalidomide following a single administration or multiple administrations was determined on day 1 or 12, respectively. In the combination phase, to determine the PK of lenalidomide and dexamethasone given alone and in combination, dexamethasone was held on day 1 to determine the plasma concentration of lenalidomide administered alone (day 1). On day 2, dexamethasone was administered but lenalidomide held, so as to determine the plasma concentration of dexamethasone administered alone. On day 12, plasma concentrations of both lenalidomide and dexamethasone were determined with the two drugs given concurrently. Drugs were administrated under fasting conditions.

Blood samples for lenalidomide were collected before and 0.5, 1, 1.5, 2, 4, 6, 9, 12, and 24 h after the drug administration. Blood samples for dexamethasone were collected before and 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, and 24 h after the drug administration. The concentrations of *R* and *S*-lenalidomide in plasma were determined by chiral liquid chromatography–tandem mass spectrometry (LC–MS/MS). The concentration of dexamethasone in plasma was determined by LC–MS/MS.

## 3 Results

### 3.1 Patient characteristics

A total of 15 patients were enrolled from July 2007 to August 2008. During the monotherapy phase, 3 patients were enrolled in the 10-mg cohort and 6 patients in the 25-mg cohort. After completion of the monotherapy phase, an additional 6 patients were enrolled in the combination cohort. The characteristics of the 15 patients are shown in Table 1. The median age of the patients was 64.0 years (range 43.0–81.0 years), and the median time from initial diagnosis to the screening was 2.0 years (range 0.8–7.6 years). Thirteen of the 15 patients had received more than or equal to 2 prior regimens for MM and 11 patients had previous autologous stem cell transplant (ASCT). Types of myeloma included 6 patients of IgG, 5 of IgA, 2 of IgD, 1 of Bence-Jones protein (BJP)-kappa, and 1 of BJP-lambda type. In patients whose cytogenetic data by fluorescence in

**Table 1** Patient characteristics

| Characteristic | 10 mg ($n$ = 3) | 25 mg ($n$ = 6) | Combo ($n$ = 6) | Total ($n$ = 15) |
|---|---|---|---|---|
| Age (years) | | | | |
| Median | 64.0 | 64.5 | 64.0 | 64.0 |
| Range | 54.0–68.0 | 43.0–76.0 | 47.0–81.0 | 43.0–81.0 |
| Female:male ($n$) | 1:2 | 2:4 | 2:4 | 5:10 |
| Time since MM diagnosis (years) | | | | |
| Median | 4.4 | 2.1 | 1.9 | 2.0 |
| Range | 1.4–7.6 | 0.8–4.8 | 1.4–4.1 | 0.8–7.6 |
| No. of previous therapies, $n$ (%) | | | | |
| 1 | 0 | 2 (33.3) | 0 | 2 (13.3) |
| ≥2 | 3 (100) | 4 (66.7) | 6 (100) | 13 (86.7) |
| Type of therapy, $n$ (%) | | | | |
| Thalidomide | 1 (33.3) | 1 (16.7) | 1 (16.7) | 3 (20.0) |
| Bortezomib | 0 | 0 | 1 (16.7) | 1 (6.7) |
| ASCT | 2 (66.7) | 4 (66.7) | 5 (83.3) | 11 (73.3) |
| Cytogenetic abnormality by FISH | | | | |
| t(4;14) | 1 | 2 | 1 | 4 |
| t(11;14) | 0 | 1 | 2 | 3 |
| del(13q) | 1 | 4 | 3 | 8 |

*ASCT* autologous stem cell transplant

situ hybridization (FISH) were available, t(4:14) (p16;q32) was detected in 4 of 9 patients and del(13q) (13S319) in 8 of 11 patients. Del(17p) was detected in none of the 7 patients.

The median study duration at the data cutoff was 48.3 (range 41.1–51.1), 24.0 (12.0–36.1), and 25.3 (22.7–28.0) weeks in the 10-mg, 25-mg, and the combination cohorts, respectively. It was 26.3 (12.0–51.1) weeks for all cohorts. Two patients had discontinued the study due to AE ($n$ = 1) or PD ($n$ = 1) in the 25-mg cohort, while none of the patients in the 10-mg or the combination cohort had discontinued the study at the time of data cutoff.

### 3.2 Safety in the monotherapy phase

Grade 3 or higher lenalidomide-related AEs reported during cycle 1 in the 10-mg cohort were anemia (grade 4, $n$ = 1), leukopenia (grade 3, $n$ = 1), lymphopenia (grade 3, $n$ = 1), and neutropenia (grade 3, $n$ = 1). Since none of the AEs corresponded to DLT, 6 patients were additionally enrolled to receive a higher dose of 25 mg.

≥Grade 3 AEs related to lenalidomide during cycle 1 in the 25-mg cohort were leukopenia (grade 3, $n$ = 1), neutropenia (grade 3, $n$ = 1), lymphopenia (grade 3, $n$ = 1), and hypoxia (grade 3, $n$ = 1). The grade 3 hypoxia corresponded to DLT. Since only one of the 6 patients in the 25-mg cohort developed DLT, the dose of lenalidomide to be used in Japanese patients with MM was set at 25 mg, as specified in the study protocol. The safety data of the 25 mg dose was reviewed and recommended for the combination cohort by the Independent Data Monitoring Committee. Patients who experienced DLT discontinued the study at cycle 1.

### 3.3 Safety

All patients who received at least one dose of lenalidomide ($n$ = 15) were included in the safety evaluation. Grade 3 or 4 lenalidomide-related AEs were reported in 11 of 15 patients at the data cutoff (Table 2). The major AE was myelosuppression. Neutropenia ($n$ = 10) was reported with the highest frequency. None of the patients in the combination cohort had any grade 4 AEs. Regarding neutropenia, median duration of grade 3 or 4 neutropenia was 15 days (range 3–29 days). Median frequency of grade 3 or 4 neutropenia per patient was 1.5 (range 1–5). To manage neutropenia, lenalidomide were interrupted in 2 patients. None of the patients experienced drug reduction due to neutropenia. As dexamethasone-related AEs, hyperglycemia (grade 3, $n$ = 2), and osteomyelitis (grade 3, $n$ = 1), which was considered equivalent to a recurrence of osteonecrosis of the jaw (ONJ), were reported. The patient who experienced ONJ had a history of bisphosphonate treatment. Peripheral neuropathy, DVT, pulmonary embolism, or thrombosis was not reported in any cohort. Seven of the 12 patients in whom lenalidomide and dexamethasone were administered received low-dose aspirin to prevent DVT.

None of the patients in the 25-mg or combination cohort experienced dose reduction of lenalidomide. Eight of the 15 patients, including the 3 in the 10-mg cohort, 3 in the

**Table 2** Lenalidomide-related adverse events with NCI-CTCAE grade 3 or 4 ($n = 15$)

| Events | Grade 3 | Grade 4 | Grade 3 + 4, $n$ (%) |
|---|---|---|---|
| Patients with at least one ≤grade 3 adverse event | – | 2 (13.3) | 11 (73.3) |
| Anemia | 3 (20.0) | | 2 (13.3) |
| Leukopenia | 3 (20.0)[a] | – | 3 (20.0) |
| Lymphopenia | 9 (60.0) | 1 (6.7) | 3 (20.0) |
| Neutropenia | 1 (6.7) | – | 10 (66.7) |
| Hypoxia | 1 (6.7) | – | 1 (6.7) |
| Malaise[b] | 1 (6.7) | – | 1 (6.7) |
| Hepatic function abnormality[b] | 1 (6.7) | – | 1 (6.7) |
| Decreased blood phosphorus | 1 (6.7) | – | 1 (6.7) |
| Increased alanine aminotransferase[b] | | | 1 (6.7) |

A subject with multiple occurrence of an adverse event is counted once

[a] In 3 cases of lymphopenia, one was reported as a lenalidomide and/or dexamethasone-related AE

[b] Lenalidomide and/or dexamethasone-related AEs

25-mg cohort and 2 in the combination cohort experienced dose interruption of lenalidomide due to lenalidomide-related AEs including, anemia, neutropenia, malaise, pyrexia, and rash that developed in 2 patients each.

One patient in each cohort experienced lenalidomide-related serious AEs (SAEs) including malaise (grade 2) and pyrexia (grade 1) in the 10-mg cohort, hypoxia (grade 3) and interstitial pneumonia (grade 2) in the 25-mg cohort, and hepatic function abnormality (grade 3) in the combination cohort. None of the SAEs induced study discontinuation except interstitial pneumonia, which might have triggered the hypoxia, a DLT. All lenalidomide-related AEs observed in this study had been previously reported. Moreover, all the AEs were manageable by supportive care or dose interruption/reduction of study drugs.

### 3.4 Efficacy

All 6 patients in the combination cohort achieved PR at the data cutoff. The overall response rate (ORR), defined as partial or complete response, was 100%. Within 2 months of the interim analysis, CR was achieved in 2 of the 6 patients (33%) in the combination cohort. Median time to response, the time from study start to PR entry, was 4.1 weeks (range 4.0–4.3 weeks) in the combination cohort. Median response duration and progression-free survival (PFS) as determined by the Kaplan–Meier method were not estimable at the time of the interim analysis. The response was continued in 5 of the 6 patients in the combination cohort at the time of data cutoff. Patients in the combination cohort who had a history of prior bortezomib or thalidomide therapy achieved PR at the time of the interim analysis.

### 3.5 Pharmacokinetics

Mean plasma lenalidomide concentrations following single and multiple doses of 10 or 25 mg lenalidomide are shown in Fig. 2a. Mean plasma lenalidomide concentrations when administered alone (day 1) or in combination with dexamethasone (day 12) are shown in Fig. 2b. The plasma pharmacokinetic parameters of lenalidomide are listed in Table 3.

The profile of the plasma lenalidomide concentration over time was similar between days 1 and 12 following administration of the drug alone at 10 or 25 mg: The plasma concentration of lenalidomide reached a peak at approximately 1 h postdose and levels of lenalidomide declined rapidly in a monophasic manner at both dose levels and on both days. The $C_{max}$ and AUC increased in a dose-dependent manner. Mean accumulation ratios between days 1 and 12 for $C_{max}$ (AR [$C_{max}$]) or $AUC_{\tau}$ (AR [$AUC_{\tau}$]) were nearly 1.00, suggesting no drug accumulation following multiple doses of lenalidomide. There were also no meaningful differences in the $V_z/F$, CL/F, or $t_{1/2}$ between the two dose levels as well as between days 1 and 12.

After oral administration of 25 mg lenalidomide in combination with dexamethasone (day 12), plasma lenalidomide $C_{max}$ was observed at 1 h later than that observed after administration of lenalidomide alone (day 1). A higher variation in the $t_{max}$ value was observed after co-administration of lenalidomide with dexamethasone ($t_{max} = 0.53$–$4.02$ h) compared to administration of lenalidomide alone ($t_{max} = 1$–$1.97$ h). A higher intersubject variability in $C_{max}$ was also observed on day 12 (CV% = 46.1% on day 12 and 27.1% on day 1). There were no marked differences in $t_{1/2}$ when lenalidomide was administered with or without dexamethasone. The $AUC_{\tau}$ was slightly reduced from days 1 to 12. The accumulation ratio of day 12 to day 1 for the $C_{max}$ and $AUC_{\tau}$ (AR [$C_{max}$] and AR [$AUC_{\tau}$]) was 0.914 and 0.868, respectively. These observations indicate a modest change in lenalidomide oral absorption when co-administered with large quantities of dexamethasone tablets (4 mg × 10), which is not considered clinically relevant since other PK parameters were almost the same between days 1 and 12.

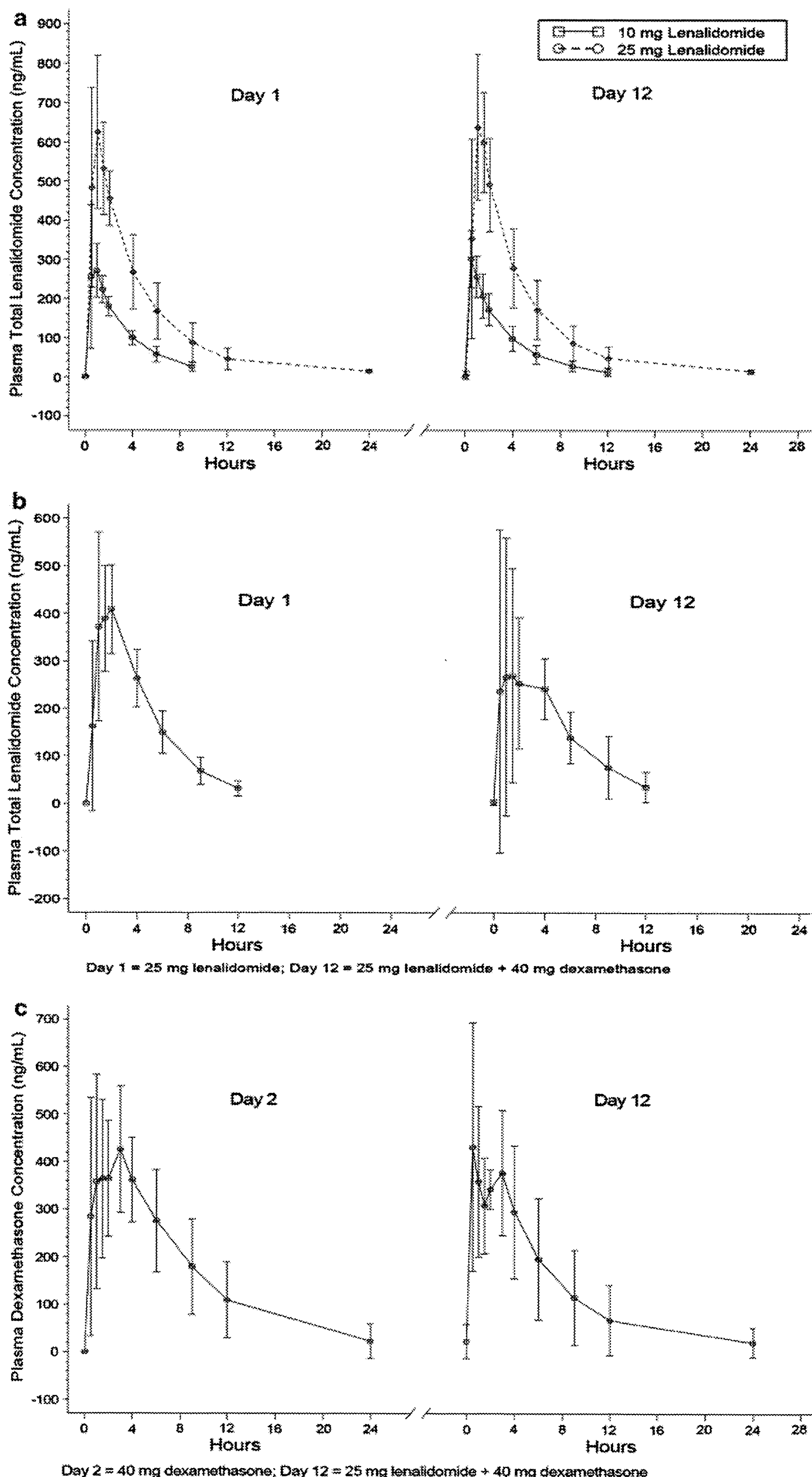


**Fig. 2** **a** Mean (±SD) plasma lenalidomide concentrations versus time on days 1 and 12 (monotherapy cohorts). Plasma lenalidomide concentrations following single (day 1) and multiple doses (day 12) of 10 mg ($n = 3$) or 25 mg ($n = 6$) lenalidomide. Data points at 24 h in 10 mg lenalidomide are missing because of BLQ (below the limit of quantitation; 5 ng/mL). **b** Mean (±SD) plasma lenalidomide concentrations versus time on days 1 and 12 (combination cohort). Plasma lenalidomide concentrations when administered alone (day 1, $n = 6$) and in combination with dexamethasone (day 12, $n = 6$). Data points at 24 h are missing because of BLQ. **c** Mean (±SD) plasma dexamethasone concentrations versus time on days 2 and 12 (combination cohort). Plasma dexamethasone concentration when administered alone (day 2, $n = 6$) and in combination with lenalidomide (day 12, $n = 6$)

**Table 3** Plasma lenalidomide pharmacokinetic parameters when administered alone or in combination with dexamethasone

| Parameters | 10-mg cohort | | 25-mg cohort | | Combination cohort | |
|---|---|---|---|---|---|---|
| | Day 1 (single dose) | Day 12 (multiple dose) | Day 1 (single dose) | Day 12 (multiple dose) | Day 1 (single dose) Len | Day 12 (multiple dose) Len + Dex |
| Number of patients | 3 | 3 | 3 | 6 | 6 | 6 |
| $t_{max}$ (h)[a] | 0.93 (0.50, 1.00) | 0.50 (0.45, 1.00) | 1.01 (0.43, 2.00) | 0.97 (0.45, 1.47) | 1.70 (1.00, 1.97) | 2.76 (0.53, 4.02) |
| $C_{max}$ (ng/mL) | 315 (39.5) | 311 (22.5) | 622 (29.3) | 714 (15.2) | 474 (27.1) | 433 (46.1) |
| $AUC_t$ (ng h/mL) | 962 (29.8) | 973 (26.5) | 2600 (39.0) | 2687 (34.6) | 2063 (10.9) | 1817 (20.1) |
| $AUC_\tau$ (ng h/mL) | 1034 (30.2) | 1024 (27.6) | 2686 (36.3) | 2773 (32.1) | 2177 (12.6) | 1890 (17.4) |
| $AUC_\infty$ (ng h/mL) | 1037 (30.5) | NA | 2710 (37.2) | NA | 2183 (12.8) | NA |
| $t_{1/2}$ (h) | 2.52 (24.3) | 2.43 (16.5) | 3.11 (25.8) | 3.14 (30.6) | 2.56 (14.0) | 2.55 (23.0) |
| CL/F (mL/min) | 161 (30.5) | 163 (27.6) | 154 (37.2) | 150 (32.1) | 191 (12.8) | 221 (17.4) |
| $V_z$/F (L) | 35.0 (17.4) | 34.2 (10.8) | 41.4 (18.8) | 40.8 (17.8) | 42.2 (11.9) | 48.7 (17.2) |
| AR ($C_{max}$) | NA | 0.988 (27.6) | NA | 1.15 (24.0) | NA | 0.914 (25.1) |
| AR ($AUC_\tau$) | NA | 0.991 (13.8) | NA | 1.03 (9.6) | NA | 0.868 (7.59) |

Geometric mean (CV%)

*AR* accumulation ratio, *Dex* dexamethasone, *Len* lenalidomide, *NA* not applicable

[a] Median (minimum–maximum)

**Table 4** Plasma dexamethasone pharmacokinetic parameters when administered alone or in combination with lenalidomide

| Parameters | Combination cohort | |
|---|---|---|
| | Day 2 (single dose) Dex | Day 12 (multiple dose) Dex + Len |
| Number of patients | 6 | 6 |
| $t_{max}$ (h)[a] | 2.49 (1.00, 4.00) | 1.75 (0.47, 3.07) |
| $C_{max}$ (ng/mL) | 499 (33.0) | 523 (33.9) |
| $AUC_t$ (ng h/mL) | 3528 (38.4) | 2633 (44.5) |
| $AUC_\tau$ (ng h/mL) | 3526 (38.3) | 2687 (43.0) |
| $AUC_\infty$ (ng h/mL) | 3661 (43.8) | NA |
| $t_{1/2}$ (h) | 4.24 (31.9) | 3.85 (32.3) |
| CL/F (mL/min) | 182 (43.8) | 248 (43.0) |
| $V_z$/F (L) | 66.9 (23.3) | 82.7 (21.3) |
| AR ($C_{max}$) | NA | 1.05 (18.7) |
| AR ($AUC_\tau$) | NA | 0.762 (16.2) |

Geometric mean (CV%)

*AR* accumulation ratio, *Dex* dexamethasone, *Len* lenalidomide, *NA* not applicable

[a] Median (minimum–maximum)

Mean plasma dexamethasone concentrations are demonstrated in Fig. 2c. Table 4 summarizes the plasma pharmacokinetic parameters for dexamethasone when administered alone (day 2) and in combination with lenalidomide (day 12). The mean $C_{max}$ and median $t_{max}$ values were comparable between days 2 and 12, indicating that the oral absorption rate of dexamethasone was not altered significantly after multiple doses. Mean $AUC_\tau$ was 24% lower (3526 ng h/mL on day 2 to 2687 ng h/mL on day 12), while mean CL/F was 36% higher on day 12 compared to day 2 (182 mL/min on day 2 to 248 mL/min on day 12). There were no considerable differences in $t_{1/2}$ when dexamethasone was administered with or without lenalidomide.

## 4 Discussion

In this study, the 25 mg dose of lenalidomide, which was the dose used in the pivotal phase III studies MM-009/010 of lenalidomide conducted outside of Japan, was found to be safe in Japanese patients with relapsed/refractory MM. Safety data in this study were also comparable with those reported in clinical studies conducted previously. The most common AE was neutropenia in this study. Ten of 15 patients (67%) experienced grade 3 or 4 neutropenia. Two patients experienced interruption of lenalidomide for 14 or 21 days, respectively, to manage neutropenia. In the patient in whom lenalidomide was interrupted for 21 days, neutropenia disappeared 10 days after treatment interruption. However, lenalidomide was not administered until the next cycle in accordance with the protocol. Although administration of G-CSF was permitted for neutropenia treatment, only one patient received G-CSF. Other treatment-related adverse events were also clinically manageable. Grade 2 interstitial pneumonia (IP) was reported in 1 patient in the 25-mg cohort on day 29. This patient had grade 3 hypoxia,

corresponding to DLT, and IP-like findings were seen on diagnostic imaging studies. Pulse steroid treatment was given for IP; after the steroid treatment, there was improvement clinically and based on imaging studies by day 40. Although the M protein was significantly reduced, this patient discontinued the study. Occurrences of IP-like syndrome associated with lenalidomide have previously been reported [6, 7]. Given the high incidence of thromboembolic events reported in several clinical trials of LD therapy, international groups of multiple myeloma experts including the International Myeloma Working Group (IMWG) have recommended prophylactic anticoagulation for patients treated with LD therapy according to the risk factors [8, 9]. In our study, dexamethasone and lenalidomide were co-administered to 12 patients and 7 of them received low-dose aspirin at the investigators' discretion. None of the patients developed DVT, pulmonary embolism, or thrombosis during the study. Patients with a prior history of DVT in the previous 3 years or ones with prior history of acute myocardial infraction in the previous 6 months were not enrolled in the study.

All 6 patients in the combination cohort achieved PR at the time of interim analysis. In addition, 2 of the 6 patients achieved CR after the interim analysis. The median time to response (TTR) in the combination cohort was 4.1 weeks. A recent compassionate use study of lenalidomide and dexamethasone reported a median TTR of about 4 weeks [10], which indicates rapid response of LD therapy in relapsed/refractory MM patients. In the monotherapy cohorts, all patients were on stable disease at the end of cycle 3. By cycle 3, the patients were not allowed to receive dexamethasone. At the interim analysis, one patient in the 10-mg cohort achieved minimal response. Two patients in the 25-mg cohort showed responses of PR. It should be noted that two patients in the 10-mg cohort and 4 patients in the 25-mg cohort started the combination therapy (lenalidomide + dexamethasone) by interim analysis. Duration of the combination therapy varied among the patients. Moreover, patients in the 10-mg cohort received 25 mg of lenalidomide after its tolerability was confirmed.

It has been reported that LD therapy was effective in patients with previous exposure to thalidomide or bortezomib [1, 2, 10, 11]. Patients who had a history of bortezomib or thalidomide therapy, respectively, achieved PR at the time of the interim analysis in the combination cohort. Responses were also seen in patients with high-risk cytogenetic features. In the combination cohort, a patient with t(4;14) achieved PR at the interim analysis and CR thereafter. This patient had del(13q) in addition to t(4;14). The other two patients with del(13q) achieved PR at the data cutoff in the combination cohort. Also, two patients with t(4;14) who were treated with lenalidomide in the 25-mg cohort showed responses of MR and PR, respectively, when combined with dexamethasone. In the MM-016 study, LD therapy induced durable responses among relapsed t(4;14) or del(13q) diseases [12].

When administered alone to Japanese MM patients under fasting conditions, the pharmacokinetics of lenalidomide show rapid absorption and elimination, with a $t_{max}$ and $t_{1/2}$ of about 1 and 3 h, respectively after both single and multiple doses. The lack of a significant difference in any of the pharmacokinetic parameters between days 1 and 12 indicated no plasma accumulation after multiple doses of lenalidomide. The $C_{max}$ and AUC increased in a dose-dependent manner. These pharmacokinetic characteristics are comparable to those reported for Japanese MDS patients and Caucasian healthy subjects [13, 14]. It is also reported that a majority of lenalidomide is eliminated unchanged through urinary excretion [15]. Co-administration with multiple doses of dexamethasone had no significant effect on the elimination of lenalidomide, as evidenced by the identical mean values of the $t_{1/2}$ for lenalidomide alone and lenalidomide plus dexamethasone. Judged by the wider range of $t_{max}$ and the higher variability in $C_{max}$, the oral absorption of lenalidomide appeared mildly affected when co-administered with large quantities of dexamethasone tablets. These changes are considered negligible and not clinically relevant since there was no marked influence on the other PK parameters.

After a single oral 40 mg dose, dexamethasone showed a median $t_{max}$ of 2.5 h and a mean $t_{1/2}$ of 4.2 h. These data are comparable to those reported at a lower oral dose (1 mg) in adult healthy subjects [16]. Dexamethasone is also known to be eliminated through urinary excretion [17]. Upon multiple dosing, dexamethasone displayed a 36% increase in CL/F and a 24% decrease in $AUC_t$ with little change in $t_{max}$ and $C_{max}$, indicating a slightly faster elimination from plasma after multiple doses. It has been known that higher doses of dexamethasone can induce CYP3A4 activity, the primary enzyme responsible for dexamethasone metabolism [18]. Thus, dexamethasone may accelerate its own metabolism via enzyme induction, thereby causing a lower exposure in plasma with multiple high doses. Lenalidomide is not likely to affect dexamethasone metabolism because it is not an inducer of the human cytochrome P450 enzymes.

**Conflict of interest statement** Conflicts of interest of all authors are as follows; H. Lau, K. Takeshita, and M. Takatoku are employees of Celgene Co., Ltd. S. Iida, T. Chou, S. Okamoto, H. Nagai, K. Hatake, H. Murakami, T. Takagi, K. Shimizu, and T. Hotta received acceptance research expenses for this trial from Celgene KK, Tokyo. S. Iida received research grants from Kyowa Hakko Kirin Co., Ltd. and Chugai Pharmaceutical Co., Ltd. H. Murakami received grants from Janssen Pharmaceutical K.K. and Novartis Pharma K.K. S. Iida declares honoraria from Janssen Pharmaceutical K.K.

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## ORIGINAL ARTICLE

# Bortezomib-resistant myeloma cell lines: a role for mutated *PSMB5* in preventing the accumulation of unfolded proteins and fatal ER stress

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**Bortezomib is an effective agent for treating multiple myeloma (MM). To investigate the underlying mechanisms associated with acquired resistance to this agent, we established two bortezomib-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ, the 50% inhibitory concentration values of which were respectively 24.7- and 16.6-fold higher than their parental cell lines. No activation of caspase and BH3-only proteins such as Noxa was noted in bortezomib-resistant cells after exposure to the drug. The accumulation of polyubiquitinated proteins was reduced in bortezomib-resistant cells compared with the parental cells, associated with avoidance of catastrophic ER stress as assessed by downregulation of CHOP expression. These resistant MM cells have a unique point mutation, G322A, in the gene encoding the proteasome β5 subunit (*PSMB5*), likely resulting in conformational changes to the bortezomib-binding pocket of this subunit. KMS-11 parental cells transfected to express mutated PSMB5 also showed reduced bortezomib-induced apoptosis compared with those expressing wild-type PSMB5 or the parental cells. Expression of mutated PSMB5 was associated with the prevention of the accumulation of unfolded proteins. Thus, a fraction of MM cells may acquire bortezomib resistance by suppressing apoptotic signals through the inhibition of unfolded protein accumulation and subsequent excessive ER stress by a mutation of the *PSMB5* gene.**
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**Keywords:** bortezomib; drug resistance; MM; PSMB5; cell line

## Introduction

Bortezomib, a proteasome inhibitor, is widely used in the treatment of multiple myeloma (MM), resulting in remarkable response rates in both relapsed/refractory MM and newly diagnosed MM.[1,2] However, bortezomib treatment often achieves only very short-duration responses and drug resistance rapidly develops.[3,4] Therefore, understanding the mechanisms underlying this drug resistance is necessary to develop novel approaches to overcome this problem. Bortezomib was originally developed as a proteasome inhibitor, which blocked the degradation of ubiquitinated IκBα, a negative regulator of the canonical nuclear factor (NF)-κB pathway, and prevented its translocation into the nucleus.[5] However, several investigators have proposed additional mechanisms for its antitumor effects, especially focusing on the expression of BH3-only proteins, including Noxa, Bid, puma and Bik,[6–9] and on misfolded protein accumulation followed by endoplasmic reticulum(ER) stress-associated apoptosis.[10,11] When proteasome function is inhibited, damaged proteins including unfolded or oxidatively modified proteins accumulate in the intracellular environment, which causes ER overload, well recognized as an ER stress.[12,13] This in turn induces cellular protective responses, so-called 'unfolded protein responses' (UPR) that promote refolding or elimination of unfolded proteins, but can ultimately trigger apoptosis by activating CHOP, caspase-4 and caspase-12 if the accumulation of damaged protein is excessive.[14] Administration of low doses of proteasome inhibitors can disrupt this mechanism, protecting cells from the effects of damaged protein accumulation, particularly effectively in cells such as MM and pancreatic tumors, which actively secrete proteins.[15] For this reason, modifications to the mechanism for disposal of misfolded proteins and avoidance of catastrophic ER stress caused by their accumulation may be one of the means by which MM cells acquire bortezomib resistance.

Several investigators have reported on the mechanisms of bortezomib tolerance in different tumor cell lines induced by continuous exposure to stepwise-increasing doses of bortezomib. Lu *et al.*[16] and Oerlemans *et al.*[17] have proposed either mutation of the gene for the proteasome β5 subunit (*PSMB5*) (a single G to A nucleotide shift at the position 322) or overexpression of this protein as possible mechanisms associated with bortezomib resistance in the T-lymphoblastic/leukemia cell line JURKATB, and the monocytic/macrophage cell line, THP1/BTZ, respectively. In another study, Rückrich *et al.*[18] proposed that the suppression of protein biosynthesis contributes to the adaptation to impaired proteasome activity in myeloid leukemia HL60a cells, which have acquired resistance to bortezomib. These investigators also established a bortezomib-resistant MM cell line, designated AMO-1a, but did not report any details with regard to resistance mechanisms. To the best of our knowledge, there have been no published studies on the mechanisms responsible for bortezomib resistance in MM cells. Here, we established two bortezomib-resistant MM cell lines, which tolerated the drug even at doses 10-fold higher than the 50% inhibitory concentration ($IC_{50}$) values for parental cells. Using these new resistant lines, we investigated the alteration of *PSMB5*, misfolded protein accumulation, ER stress and apoptosis signals including BH3-only proteins at clinically achievable drug concentrations. Our study demonstrates that preventing the accumulation of misfolded proteins and avoidance of catastrophic ER stress has a crucial role in bortezomib resistance by suppressing apoptosis-inducing signals in MM cells. We also document that the mechanism for this effect involves a unique point mutation of *PSMB5* in bortezomib-resistant MM cells, which contributes to reducing the accumulation of misfolded proteins and alleviates catastrophic ER stress in MM cells.

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## Materials and methods

### *Establishment of bortezomib-resistant MM cell lines*

KMS-11 was kindly provided by Professor T Otsuki, Kawasaki Medical University (Okayama, Japan). OPM-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Two bortezomib-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ, were established from their parental lines, KMS-11 and OPM-2, under continuous exposure to bortezomib in RPMI-1640 medium supplemented with 10% fetal bovine serum over a half year. During this time, the concentration of bortezomib was increased stepwise weekly after confirmation of the maintained viability of the cells at the previous dose. After their establishment, the bortezomib-resistant cell lines were incubated in bortezomib-free medium for 2 weeks to confirm the stability of resistance trait, and then subjected to all assays used in our study.

### *Antibodies, reagents and western blot analysis*

Bortezomib was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Antisera against caspase-12 and CHOP were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). Antisera against Bcl-Xl/s, Mcl-1, Noxa, ubiquitin and actin were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Antisera against Bid, Bcl-2, β-galactosidase, V5-tag and caspase-4 were purchased from Medical & Biological Laboratories (Nagoya, Japan). Antisera against β5, β1 and β2 subunits of the 26S proteasome were purchased from BIOMOL International, L.P. (Plymouth Meeting, PA, USA).

Western blot analysis was performed as previously described.[8] In brief, protein samples were electrophoresed by SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk, membranes were incubated with primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. The protein band was detected using chemiluminescent substrate.

### *Cell proliferation and apoptosis assays*

The cell proliferation assay was previously described.[8] Calculation of the $IC_{50}$ value used XLfit 4.2 curve-fitting software for Excel. Apoptotic cells after exposure to bortezomib were evaluated using Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ, USA).

### *Chymotrypsin-like activity assay*

A total of $5 \times 10^5$ cells were incubated with or without 10 nM bortezomib for the indicated times. After washing twice with cold phosphate-buffered saline, cells were resuspended in 50 mM Tris (pH 7.4) buffer containing 5 mM $MgCl_2$ and 0.2 μg/ml digitonin, which permeabilizes the cell membrane without disrupting it. Cells were transferred into black 96-well flat-bottom plates at a final concentration of $4 \times 10^4$ cells in 160 μl of buffer in each well. Thereafter, 40 μl of fluorogenic substrate, Suc-LLVY-amc (BIOMOL International, L.P.), was added to each well. After incubation for 3 h at 37 °C, fluorescence was measured at 380 nm excitation wavelength and 460 nm emission wavelength.

### *DNA sequencing*

Total RNA extraction from MM cell lines, followed by reverse transcription into cDNA, was performed as previously reported.[8] Exon II of the *PSMB5* gene was amplified by means of PCR using the following primer set: forward, 5′-TTCCGCCATGGAGTCATA-3′; and reverse, 5′-GTTGGCAAGCAGTTTGGA-3′. After purification, the PCR product was directly sequenced by the dye terminator method with the aid of an ABI377 (Applied Biosystems, Foster City, CA, USA).

### *Transfection by lentiviral infection*

The cDNA encoding wild-type or mutated *PSMB5* was obtained from KMS-11 or KMS-11/BTZ cells, respectively, by PCR amplification using 5′-attB-added PCR primers (Gateway Technology, Invitrogen, Carlsbad, CA, USA) followed by sequencing. The lentivirus-based expression vector was constructed from the combination of each cDNA-containing entry vector, cytomegalovirus (CMV) promoter-containing vector, and plenti6.4/R4R2/V5-DEST multisite gateway vector by attB-attP and attL-attR reaction. The 293FT packaging cell line was transfected with plenti/CMV/wPSMB5/V5 or plenti/CMV/mPSMB5/V5 plasmids for 24 h and each viral supernatant was collected. After equalization of viral titer, KMS-11 cells were infected by each viral supernatant for 24 h and then selected by incubation with 5 μg/ml blasticidin. After selection, KMS-11 cells stably expressing wild-type *PSMB5* (KMS-11/wPSMB5-V5) or mutated *PSMB5* (KMS-11/mPSMB5-V5) were assayed for apoptosis after exposure to bortezomib, and were also used for immunoblot analysis. As a control for lentiviral expression, plenti/CMV/lacZ/V5 plasmid was similarly constructed and transfected into KMS-11 cells (KMS-11/lacZ-V5).

## Results

### *Two MM cell lines, KMS-11/BTZ and OPM-2/BTZ, demonstrate acquired resistance to bortezomib treatment*

Two bortezomib-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ, showed a 24.7-fold ($IC_{50}$ 148.3 nM) and 16.6-fold ($IC_{50}$ 51.6 nM) higher resistance to bortezomib, respectively, compared with their parental cells, KMS-11 ($IC_{50}$ 6 nM) and OPM-2 ($IC_{50}$ 3.1 nM) after a 72-h exposure (Figure 1a). In addition, these cells showed cross-resistance against another proteasome inhibitor, MG132, but not against doxorubicin (Figure1a and Table 1). In experiments measuring apoptosis induced by dose-escalated bortezomib treatment (Figure 1b), KMS-11/BTZ showed remarkable tolerance to between 3.3 and 100 nM of the drug, and OPM-2/BTZ between 3.3 and 33.3 nM, whereas the parental cells underwent apoptosis even at lower concentrations, 10 nM in KMS-11 and 3.3 nM in OPM-2. We next investigated the biological differences between resistant and parental cells at 10 nM bortezomib in further analyses.

### *Bortezomib-resistant cells do not activate apoptosis-executing signals induced by bortezomib treatment*

To compare the apoptosis-regulating signals following bortezomib treatment, we investigated alterations in the expression of different caspases, Bcl-2 family members, BH3-only proteins, NF-κB activation, and ER stress signaling in the bortezomib-resistant cells and their parental cells in the presence of 10 nM bortezomib for 48 h. As shown in Supplementary Figure S1 (also refer Supplementary Materials and methods), immunoblot analysis indicated that KMS-11/BTZ and OPM-2/BTZ cells failed to activate caspases, that is, cleavage of caspase-3, -8 and -9, which did occur in the parental cells after bortezomib exposure. In Figure 2a, two bcl-2 family proteins, Bcl-2 and Bcl-xL, are

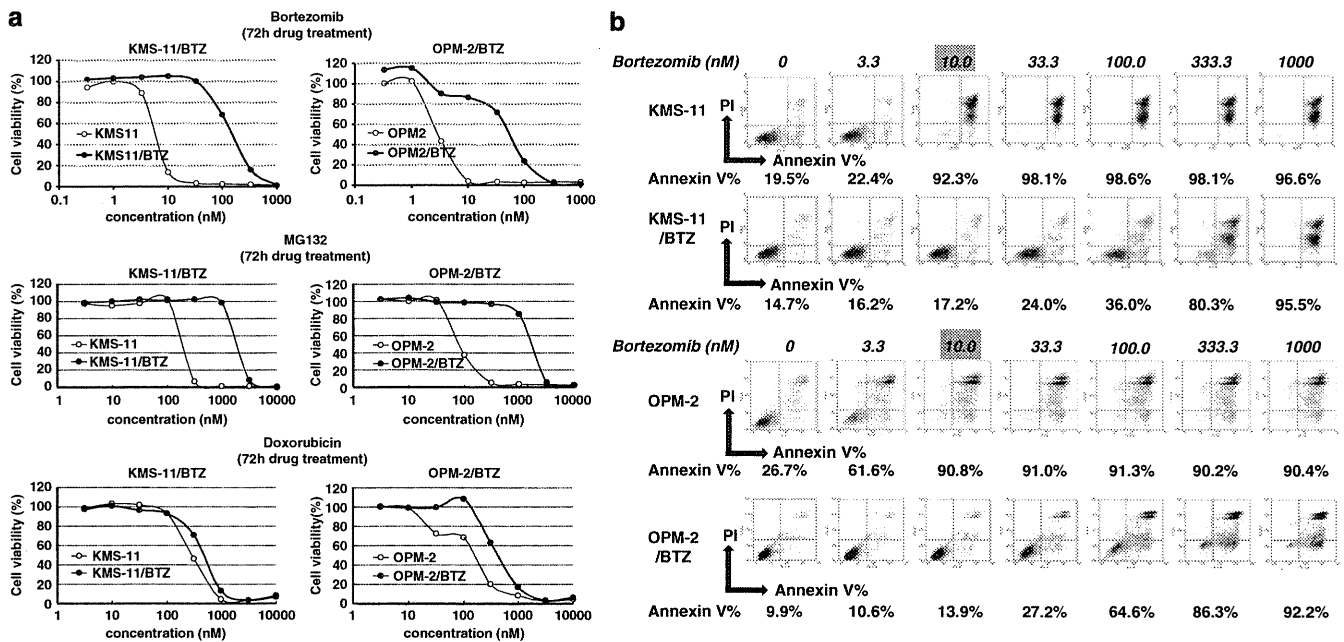


**Figure 1** Bortezomib-resistant myeloma cell lines, KMS-11/BTZ and OPM-2/BTZ. (**a**) Cell viability of bortezomib-resistant cell lines, KMS-11/BTZ and OPM-2/BTZ, and their parental cell lines after exposure to different concentrations of three drugs, bortezomib, MG132 and doxorubicin for 72 h. (**b**) Bortezomib-induced apoptosis at 72 h after exposure to different concentrations of the drug assessed by flow cytometric analysis of Annexin V and PI double staining.

**Table 1** The $IC_{50}$ of KMS11/BTZ and OPM-2/BTZ and their parental cell lines in each drug

| | $IC_{50}$ (nM) | | $IC_{50}$ ratio | $IC_{50}$ (nM) | | $IC_{50}$ ratio |
|---|---|---|---|---|---|---|
| | KMS-11 | KMS-11/BTZ | | OPM-2 | OPM-2/BTZ | |
| Bortezomib | 6 | 148.3 | 24.7 | 3.1 | 51.6 | 16.6 |
| MG132 | 260 | 2000 | 7.7 | 110 | 1900 | 17.27 |
| Doxorubicin | 300 | 450 | 1.5 | 190 | 370 | 1.94 |

Abbreviation: $IC_{50}$, 50% inhibitory concentration.
The $IC_{50}$ of KMS-11/BTZ and OPM-2/BTZ and their parental cell lines when exposed to each of the drugs for 72 h. The ratio of $IC_{50}$ value of resistant to parental cell line is shown on the right.

shown to be overexpressed in OPM-2/BTZ cells compared with their parental cells, and this was maintained during exposure to bortezomib. However, this was not the case for KMS-11/BTZ cells. We also noted that another Bcl-2 family protein, Mcl-1, was not altered in the bortezomib-resistant cells after exposure to bortezomib, whereas parental cells showed accumulation of long, short and cleaved forms of Mcl-1 after treatment. A BH3-only protein, Noxa, which was rapidly upregulated by bortezomib treatment in parental cells, was significantly suppressed in bortezomib-resistant cells. Similarly, the basal level of Bid, a caspase-8-dependent BH3-only protein, which declined in parental cells after bortezomib exposure, was not changed in bortezomib-resistant cells. Most recently, Wang *et al.*[19] proposed that Noxa was transcriptionally activated by the complex consisting of ATF3 and ATF4, which were upregulated by an inhibitor of ER-associated protein degradation or by bortezomib treatment in tumor cells. As shown in Supplementary Figure S2 (also refer Supplementary Materials and methods), there were no obvious differences in the induced levels of ATF3 and ATF4 between KMS-11/BTZ and the parental KMS-11 line. Moreover, both OPM-2/BTZ and OPM-2 showed low levels of ATF3 expression before and after bortezomib treatment.

Gel shift assays demonstrated that even in the presence of bortezomib, resistant cells maintained NF-κB activation as represented by three (KMS-11/BTZ) or four (OPM-2/BTZ) different DNA–protein complexes, and one of them became abundant at 48 h after bortezomib exposure (depicted by an asterisk in Supplementary Figure S3 and refer Supplementary Materials and methods). On the other hand, in the parental cells, NF-κB activity was suppressed after bortezomib treatment, whereas only one of the complexes (indicated by three asterisks) showed a transient increase at 6 h after bortezomib exposure, followed by a decrease at 12 h and later. Most recently, Hideshima *et al.*[20] have proposed that bortezomib activates the canonical NF-κB pathway through activation of IKKβ resulting in IκBα phosphorylation and degradation, a process mediated by the proteasome. Following this report, the over-expressed band (depicted by triple asterisks in Supplementary Figure S3a) after bortezomib exposure may correspond to the one resulting from activation of the canonical NF-κB pathway. In fact, we have also demonstrated that bortezomib treatment resulted in phosphorylation of p65 and IκBα followed by degradation of IκBα in bortezomib-sensitive MM cells even when they are committed to cell death (Supplementary Figure S3b). On the other hand, two bortezomib-resistant MM cells

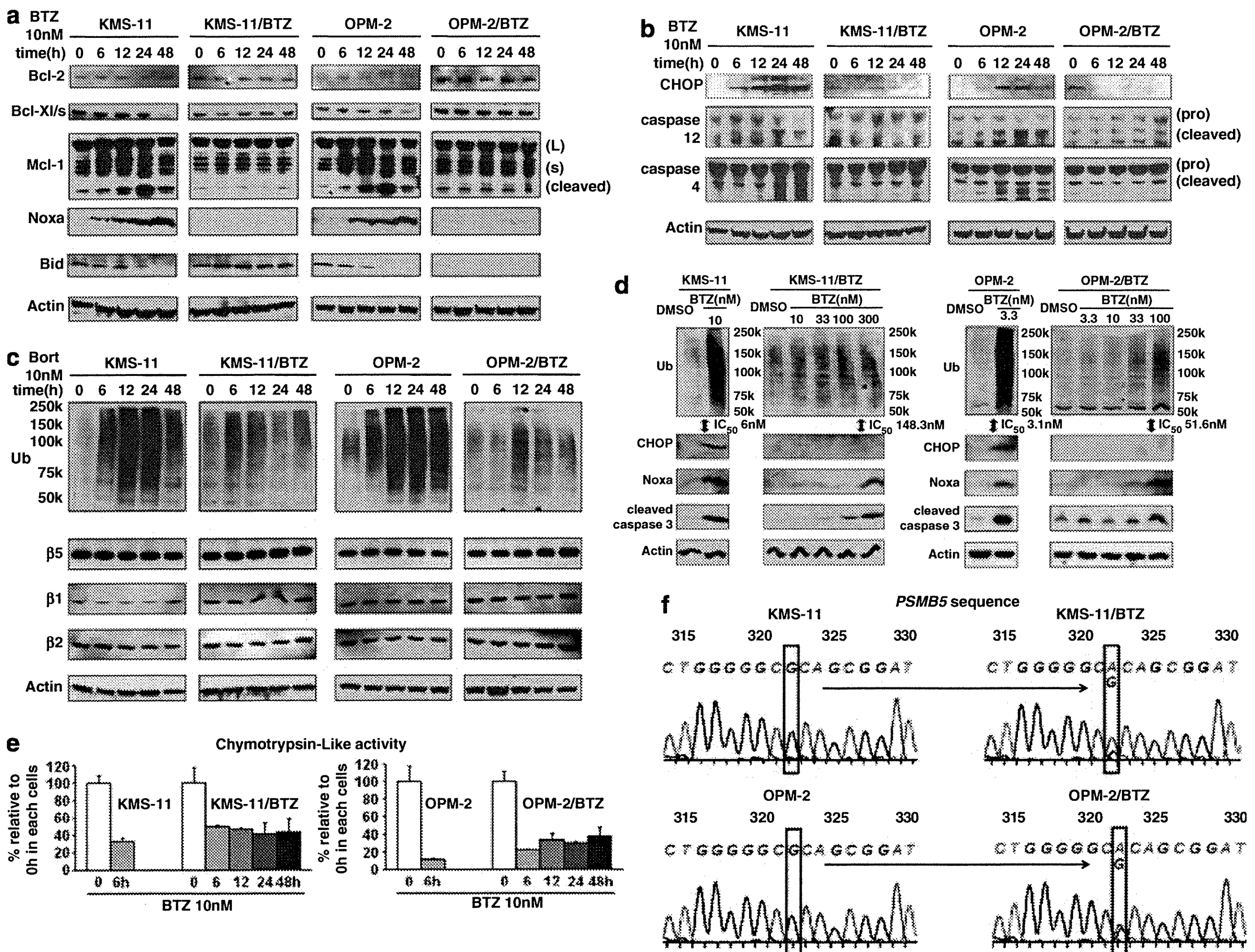

**Figure 2** Kinetic changes of apoptosis-related protein expression, chymotrypsin-like activity, and sequencing of the proteasome β5 subunit in parental KMS-11, OPM-2, and the bortezomib-resistant KMS-11/BTZ and OPM-2/BTZ lines. (**a**) Altered expression of antiapoptotic Bcl-2 family proteins such as Bcl-2, Bcl-XL and Mcl-1 and proapoptotic proteins, that is, BH3-only proteins, such as Noxa and bid. (**b**) Kinetic changes of CHOP expression and cleavage of caspase-12 and -4 evaluated by western blot analysis in KMS-11/BTZ, OPM-2/BTZ and their parental cell lines. (**c**) Accumulation of polyubiquitinated proteins and altered expression of 20S proteasome subunits, including β1, β2 and β5 before and after exposure to 10 nM bortezomib for the indicated times, evaluated by western blot analysis. (**d**) Dose-dependent alterations of accumulated polyubiquitinated proteins, expression levels of CHOP and Noxa, and activation of cleaved caspase-3 in bortezomib-resistant cells and in their parental cells. (**e**) Chymotrypsin-like activity measured using specific fluorogenic peptides after exposure to 10 nM bortezomib for the indicated times. The values represent the means of three independent experiments. (**f**) At nucleotide position 322, wild-type *PSMB5* in KMS-11 and OPM-2 indicates only G, whereas a double peak (G/A) is present at the same site in KMS-11/BTZ and OPM-2/BTZ cells.

showed neither phosphorylation of IκBα and p65 nor degradation of IκBα, indicating that the canonical NF-κB pathway in these two resistant lines was not altered by bortezomib exposure. Our study demonstrated that even in the presence of bortezomib, resistant cells maintained constitutively active NF-κB.

We next examined the expression of ER stress-related markers including cytosolic and ER chaperones, which might prevent the aggregation of misfolded proteins and promote their refolding.[14] We also assessed the activation of CHOP and caspase. As shown in Supplementary Figure S4 (also refer Supplementary Materials and methods), compared with the parental cells, bortezomib-resistant cells had neither upregulated cytosolic chaperones nor ER chaperones such as Bip and Grp94, thioredoxin family (PDI) or lectin family proteins (calreticulin) with the exception of calnexin. On the other hand, CHOP expression was upregulated immediately after bortezomib treatment in the two parental MM cells. The already-low levels of CHOP completely disappeared in bortezomib-resistant cells (Figure 2b). In addition, the resistant cells showed no activation of caspase-4 and -12, which were induced in susceptible cells.

### *The ubiquitin–proteasome pathway is altered in bortezomib-resistant cells*

To determine whether unfolded proteins accumulated after exposure to bortezomib, intracellular misfolded proteins recognized as polyubiquitinated were assessed by western blot analysis in bortezomib-resistant MM and parental cells before and after bortezomib treatment. As shown in Figure 2c, both KMS-11 and OPM-2 cells gradually accumulated polyubiquitinated proteins after bortezomib treatment. However, only transient accumulation of polyubiquitinated proteins was observed both in KMS-11/BTZ and OPM-2/BTZ cells, which