Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 115–122

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Elucidation of potential bortezomib response markers in mutliple myeloma patients

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ARTICLE INFO

Article history:
Received 30 May 2008
Received in revised form
26 September 2008
Accepted 29 September 2008
Available online 17 October 2008

Keywords:
Biomarker
Drug response
Efficacy
Bortezomib
Velcade
Pharmacodynamics

ABSTRACT

Liquid chromatography coupled to mass spectrometry (LC/MS) was used to elucidate early biomarkers of bortezomib response in multiple myeloma patients. The change in serum myeloma M-protein level, maintained for a minimum of 6 weeks, is used as one of the main criteria to evaluate patient clinical response to therapy. The objective of this study was to identify biomarkers using LC/MS in order to predict patient response to bortezomib sooner and more accurately compared to serum M-protein levels. The plasma LC/MS biomolecular/biochemical profiles, comprised of thousands of endogenous small molecules, peptides and proteins, were determined for 10 multiple myeloma patients at predose and 24 h after initial dosing with bortezomib. The comparative analysis of the metabolic profiles of non-responders and partial responders provided an opportunity to investigate mechanisms related to disease progression and identify biomarkers related to drug response. The plasma levels of two potential efficacy response markers were significantly more abundant in the non-responsive patients compared to the responders at 24-h postdose. The potential response biomarkers, apolipoprotein C-I and apolipoprotein C-I′, were identified by mass spectral analyses and confirmed by authentic protein standards based on MALDI-TOF MS/MS sequencing of proteolytic peptides.

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1. Introduction

Multiple myeloma is a cancer of the plasma cells characterized by skeletal destruction, renal failure, anemia, and hypercalcemia. Plasma cells typically represent <5% of all bone marrow cells. In multiple myeloma, the cancerous plasma cells (myeloma cells) increase in number (representing >10% of marrow cells) and activity. The myeloma cells produce intact monoclonal immunoglobulins (IgA, IgG, IgD, and IgE, called M-proteins or paraproteins) or incomplete immunoglogulins (Bence-Jones protein light chains, LC). IgG myeloma accounts for the majority (about 60–70%) of all cases of myeloma. IgA myeloma accounts for about 20% of all cases. 15–20% of patients with myeloma produce Bence-Jones proteins. Paraproteins and Bence-Jones protein are present in the serum and urine, respectively, of all myeloma patients except the 1–2% of patients with non-secretory (NS) myeloma [1–3].

Changes in the levels of serum M-proteins and urinary Bence-Jones protein light chains are used to monitor disease progression and evaluate patient response to therapy. Patient response is typically evaluated according to the criteria set by European Group for Blood and Marrow Transplantation (EBMT) [1]. One of the main criteria is the change in the level of serum M-protein and/or urinary light chains, maintained for a minimum of 6 weeks; other criteria include the number of plasma cells in bone marrow, the size of soft tissue plasmacytomas (malignant monoclonal plasma cell tumors), and the size and number of bone lesions. A complete response to treatment requires a negative immunofixation of paraproteins in serum and urine. A partial response requires a ≥50% decrease in serum M-protein and/or a ≥90% reduction in urinary light chain excretion. Disease progression is usually defined as an increase of >25% in serum M-protein or urinary light chain excretion, and/or plasma cells in bone marrow, an increase in the size/number of bone lesions or soft tissue plasmacytomas and the development of hypercalcaemia.

Bortezomib (N-(2,3-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid, is a dipeptidyl boronic acid reversible inhibitor of the chymotrypsin like activity of the 26S proteasome. The proteasome is an enzyme found in all cells of the body. It plays an important role in protein degradation, cell-cycle regulation, and gene expression. Cancer cells are more susceptible than normal cells to the pro-apoptotic effects of proteasome inhibition because of deficiencies in protective mechanisms to manage cell-cycle dysregulation [4].

Early response biomarkers would help better predict clinical response to anticancer drugs, such as bortezomib, sooner and more accurately compared to serum M-protein levels. Patients

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doi:10.1016/j.jpba.2008.09.053

Table 1
Patient characteristics and clinical responses to bortezomib monotherapy as defined by the European Group for Blood and Marrow Transplantation (EBMT) criteria.

Clinical response	Myeloma type	Age (years)	Gender	Dose group (mg/m^2)	Peripheral neuropathy	Serum M-protein concentration	
						Predose (g/l)	24 weeks after postdose (g/l)
Partial response							
Patient 9-1	IGG	53	F	1.0	No	26.8	6.6 (−75%)
9-11	IGG	57	M	1.3	Yes	45.6	2.3 (−95%)
9-13	LC	52	M	1.3	Yes	N/A	N/A
No change							
9-3	IGG	62	F	1.0	No	20.2	13.1 (−35%)
9-4	IGG	56	F	1.3	Yes	27.3	19.1 (−30%)
9-9	IGA	69	M	1.3	Yes	16.5	19.2 (+16%)
9-14	IGG	48	M	1.3	Yes	68.2	26.4 (−61%)
9-15	LC	73	M	1.3	Yes	N/A	N/A
Progressive disease							
9-8	IGG	58	F	1.3	Yes	44.3	47.3 (+6%)
9-10	IGG	70	M	1.3	Yes	41.1	62 (+51%)

IGG: immunoglobulin G; NS: non-secretor; IGA: immunoglobulin A; LC: light chain; N/A: not applicable; F: female; M: male.

typically receive bortezomib twice weekly for 2 weeks followed by 1 week without treatment for up to eight 3-week cycles. A minimum of 6 weeks of treatment with bortezomib is required before a response can be assessed. It is also recognized that some patients develop increasing bone marrow palmacytosis despite falling serum M-protein levels (hyposecretory or non-secretory progression). Monitoring M-protein levels may not be applicable for hyposecretory or non-secretory patients [1].

The biomolecular/biochemical profiles of cells and tissues provide an integrated fingerprint of both physiological and pathophysiological processes. The application of analytical chemistry and novel bioinformatics for metabolic profiling of biofluids provides an opportunity to quantitatively evaluate thousands of endogenous metabolites (small molecules, peptides, and proteins). The comparative analysis of metabolic profiles can identify biomarkers related to drug response and/or toxic events. Metabolic profiles can also assist in examining mechanisms of drug action and disease development.

The focus of this study was to elucidate early markers of response to bortezomib in relapsed, refractory myeloma. LC/MS was used to determine levels of endogenous biochemical profiles in plasma samples taken from 10 multiple myeloma patients enrolled in a phase 2 study of bortezomib monotherapy. As described in Table 1, three of the patients were partial responders (PR), five showed no change (NC), and two were considered to have progressive disease (PD) as determined by the EBMT criteria. Predictive biomarkers would be useful to determine patient response to bortezomib sooner and more accurately compared to serum M-protein levels.

2. Materials and methods

2.1. Chemicals and reagents

The internal standards for LC/MS biomarker analysis (terbutaline, ketoprofen, quinidine, and doxorubicin) were purchased from Sigma–Aldrich (St. Louis, MA, USA). Formic acid (90%) and acetonitrile were purchased from JT Baker (Phillipsburg, NJ, USA). Dithiothreitol (DTT) was purchased from Amersham BioSciences (Piscataway, NJ). Sequencing grade modified porcine trypsin was obtained from Promega (Madison, WI, USA). Alpha-cyano-4-hydroxycinnamic acid MALDI matrix was purchased from Applied BioSystems (Foster City, CA). The apoC-I peptide standard was acquired from Sigma (St. Louis, MO).

The SDS-gel running buffer contained 50 mM Tris–HCl, pH 8.3, 192 mM glycine and 0.1% SDS. The gel de-staining solution was 50/50 aqueous ammonium bicarbonate; 50 mM/ACN (v/v). The trypsin rehydration solution contained 1 ng/μl trypsin in 50 mM ammonium bicarbonate with 0.1 mM calcium chloride (aq; pH 7.8). The MALDI matrix solution contained 10 mg/ml α-cyano-4-hydroxycinnamic acid in 50/50 water/ACN (v/v).

2.2. Plasma samples

Plasma samples were collected from 10 patients randomly selected from a phase 2 study of bortezomib in relapsed, refractory myeloma. The patients received bortezomib (1.0 or 1.3 mg per square meter of body-surface area) twice weekly (on days 1, 4, 8, and 11) for 2 weeks followed by a 10-day rest period in a 21-day cycle. The plasma samples used in this study were collected at predose and 24 h after the initial dosing with bortezomib. Of the 10 patients, seven had IgG type myeloma, one had IgA type myeloma, and two had light chain (LC) myeloma. The demographics and baseline characteristics of the 10 study patients are described in Table 1. Patient response to treatment was evaluated according to criteria set forth by EBMT [1]. Negative control plasma samples were obtained from 4 healthy individuals and 10 prostate cancer patients.

2.3. LC/MS biomarker analysis

2.3.1. Sample preparation

Plasma samples (250 μl) were mixed with internal standard (10 μl, 100 μM each of terbutaline, ketoprofen, quinidine, and doxorubicin) and prepared for analysis by adding cold acetonitrile (ACN) containing 0.1% formic acid (930 μl). The mixture was vortexed, allowed to stand for 5 min, and then centrifuged at 13,000 × *g* for 15 min in an Eppendorf microfuge. The supernatant was removed and concentrated under nitrogen to a volume of approximately 50 μl. The samples were reconstituted in 0.1% formic acid to a total volume of 200 μl and transferred to a 96-well microtiter plate.

2.4. LC–MS analysis

Liquid chromatography with mass spectrometry (LC/MS) was used to elucidate plasma biomarkers of response to bortezomib. The LC/MS system was comprised of a Gilson 235 auto-injector (Gilson, Inc., Middleton, WI), and an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA), and an MDS SCIEX API QStar Pulsar quadrupole time-of-flight (Q-TOF) mass spectrometer (Applied Biosystems, Foster City, CA). The LC/MS system performance was deemed acceptable based on pre-determined performance

criteria for linearity, precision, and carryover. Injections (200 μl were made onto a 150 mm × 2.00 mm Synergi Hydro-RP column (Phenomenex, Torrance, CA). A linear gradient from 100% mobile phase A to 80% mobile phase B over 58 min was used to elute the sample, where mobile phase A was 100/0.1 water/formic acid (v/v) and mobile phase B was 90/10/0.1 ACN/water/formic acid (v/v/v). The flow rate was 0.250 ml/min. Total run time, including column re-equilibration, was 90 min. The mass spectrometer was operated in TOF MS mode with positive electrospray ionization. The mass-to-charge ratio (m/z) 100 to 1500 full scan data were acquired; the charge state distribution of the whole peptide was apparent. On-line tandem mass spectrometry (TOF MS/MS) was used to determine the fragmentation pattern of the potential biomarkers. Product ion scans were acquired for intact peptides at multiple charge states.

The reproducibility of the sample preparation and LC/MS system performance was evaluated prior to data analysis using D_2-phenylalanine, ketoprofen, methotrexate, and doxorubicin as internal standards (LC/MS peak area, ±15% R.S.D.). The reproducibility of the apoC-I and apoC-I′ measurements were evaluated based on repeat injections ($n = 5$) of plasma from a single healthy individual (HS2). The average apoC-I and apoC-I′ protein intensity for the single healthy individual was 484 ± 93 ($n = 5$).

2.5. Biomarker identification

2.5.1. Separation by SDS–PAGE

The plasma protein sample (20 μl was denatured with dithiothreitol (disulfide bond reducing agent) and loaded onto a NuPAGE Novex 12% Bis-Tris Gel, 1.0 mm × 12 wells (Invitrogen Corp., Carlsbad, CA). The upper chamber of the electrophoresis cell was filled with 200 ml of SDS running buffer, and the lower chamber was filled with 600 ml of SDS running buffer. SeeBlue® pre-stained protein standard (Invitrogen Corp., Carlsbad, CA), including Insulin B Chain, Aprotinin, Lysozyme, Myoglobin, Carbonic Dehydrogenase, Glutamic Dehydrogenase, BSA, Myosin, was used for the visualization of protein molecular weight ranges during electrophoresis. Insulin (MW = 5.7 kDa) and ubiquitin (MW = 8.6 kDa) were used as standards to bracket the MW range of interest. The gel was run at 120 mÅ for 35 min, removed from the plate, and fixed in 50/40/10 water/methanol/acetic acid (v/v/v). The gel was then washed three times in water and stained in Coomassie Blue for 1 h, followed by destaining in water until the bands were visualized and the background was clean.

2.5.2. Peptide sequence analyses

A standard trypsin in-gel digestion protocol was used to generate proteolytic peptides from four protein gel bands (#1–4). The bands were carefully excised from the gel with a scalpel. An excised piece of blank gel from an unused lane served as a control. The gel bands were treated with de-staining solution (100 μl) three times with shaking, 30 min each time, discarding washes. After discarding the final wash, the bands were dried in a vacuum and then rehydrated in trypsin solution (15 μl). The bands were incubated in a sealed tube overnight at 37 °C in an Eppendorf Thermomixer R (Eppendorf North America, Westerbury, NY). An MDS SCIEX 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) was used for tryptic peptide mapping (MALDI TOF MS) and sequencing (MALDI TOF MS/MS). An aliquot (1 μl) of sample was removed from each tryptic digest. The reactions were quenched with a low pH MALDI matrix solution (1 μl). From the resulting solution, 1 μl as applied to the surface of a MALDI plate. The plate was air-dried and inserted into the sample introduction port of the 4700 Proteomics Analyzer. The samples were ionized with a 337 nm laser, and 1000 laser shots (scans) were used to produce each spectrum. The instrument was operated in reflector mode with delayed extraction and an accelerating voltage of 20 kV from the ion source. Mascot (Matrix Science, Inc., Boston, MA) was used to identify proteins from primary sequence databases (e.g., MSDB, NCBInr, SwissProt) based on mass spectrometry tryptic peptide m/z base peaks [5].

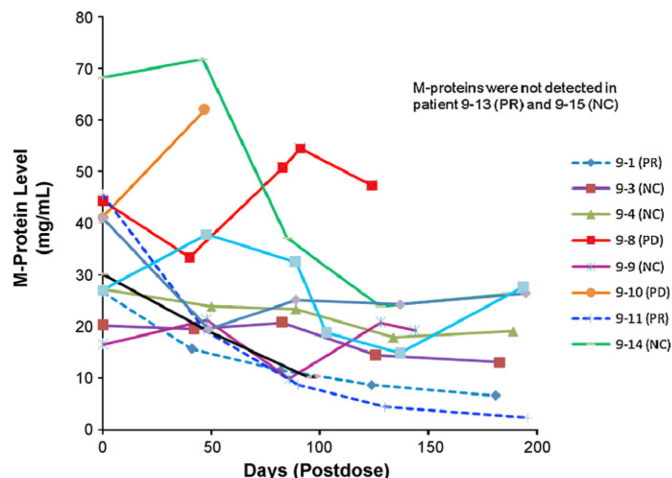

Fig. 1. The serum M-protein profiles of the individual IgG and IgA multiple myeloma patients over the 24-week treatment period with bortezomib. Patient clinical response was defined by the European Group for Blood and Marrow Transplantation (EBMT) criteria (PR: partial response; NC: no change in disease status; PD: progressive disease).

3. Results

3.1. Relationship between patient response and M-protein levels

Based on the EBMT criteria, three patients (9-1, 9-11, and 9-13) showed a partial response to treatment, five patients (9-3, 9-4, 9-9, 9-14, and 9-15) showed no change in disease status, and two patients (9-8 and 9-10) had progressive disease. As shown in Table 1, the change in serum M-protein levels correlated with clinical response for six of the eight IgG and IgA patients in this study. Fig. 1 shows the serum M-protein profile of the IgG and IgA patients over the 24-week treatment period. Two patients (9-1 and 9-11) with a partial response to treatment had significantly decreased M-protein levels at 24 weeks compared to pre-dose levels (−75% and −95%, respectively). Of the patients with no change in disease status, three patients (9-3, 9-4, and 9-9) showed serum M-protein levels that were similar to pre-dose levels, whereas one patient (9-14) showed levels that were significantly decreased (−61%) after 24-week postdose. One of the patients (9-10) with progressive disease showed significantly increased levels of M-protein (+51%), whereas the other (9-8) showed levels that were similar to predose levels. Serum M-proteins were not detected in patients 9-13 and 9-15.

3.2. LC/MS biomarker analysis

The levels of two unidentified biomolecules were significantly increased in the non-responder patients (i.e. no change and progressive disease) compared to the partial responders at 24-h postdose (Fig. 2A and B). Based on the m/z peak distribution, the potential response markers were expected to be multiply charged proteins with molecular weights (MW) of 6630 and 6432 Da (Fig. 3). Three of the multiple myeloma patients (9-11, 9-4, and 9-9) had increased marker levels at predose compared to the healthy subjects and the prostate cancer patients (Fig. 2B). The levels of the

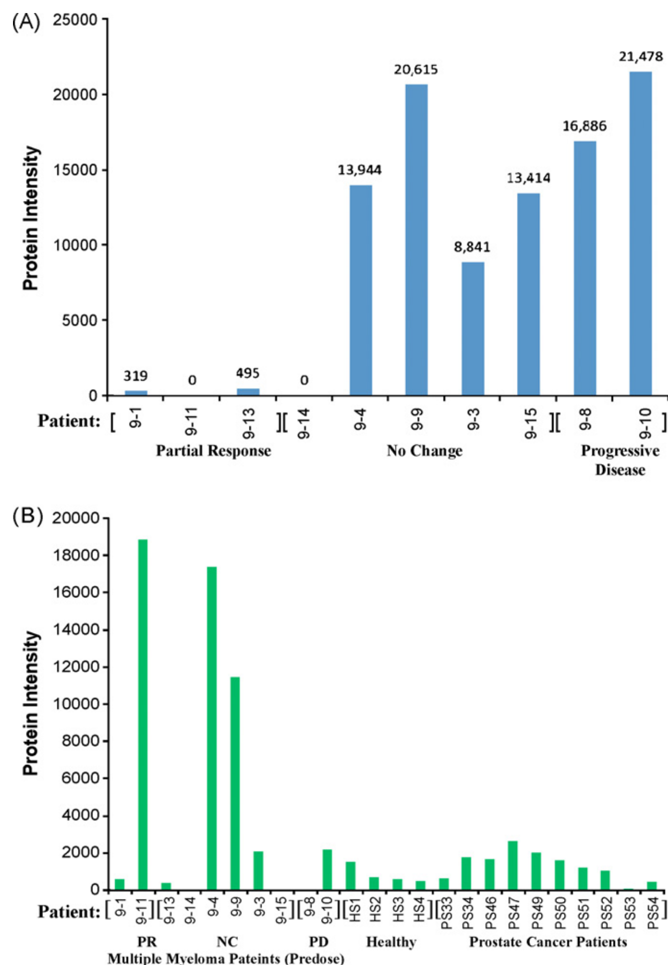

Fig. 2. (A) The plasma levels of apoC-I and apoC-I′ in the myeloma patients 24 h after the initial dosing with bortezomib. Patient clinical response was defined by the European Group for Blood and Marrow Transplantation (EBMT) criteria (PR: partial response; NC: no change in disease status; PD: progressive disease). (B) The plasma levels of apoC-I and apoC-I′ in the myeloma patients at predose, compared to the levels in healthy subjects and prostate cancer patients.

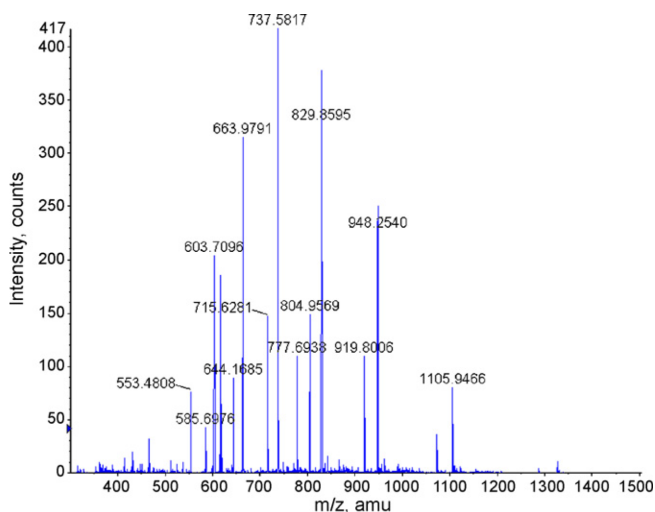

Fig. 3. The m/z peak distribution of apoC-I and apoC-I′ in the plasma of multiple myeloma patients.

markers in patient 9-11 (showing partial response to treatment) were significantly decreased at 24 h compared to predose. The levels of the markers in patients 9-4 and 9-9 (showing no change in disease status) were similar at 24 h and predose.

3.3. Biomarker identification

The potential biomarkers were isolated from plasma of normal volunteers and separated by SDS–PAGE as shown in Fig. 4. Four bands in the molecular weight range of interest were excised from the gel and digested with trypsin. Fig. 5 shows the MALDI-TOF/TOF MS/MS tandem mass spectra of the tyrptic peptides from gel band #4. The tryptic peptides matched the sequences for apolipoprotein C-I (TPDVSSALDKLKEFGNTLEDKARELISRIKQSELSAK MREWFSETFQKVKEKLKIDS, av. MW 6630.5 Da) and a truncated apoC-I isoform (apoC-I′) that lacks the N-terminal threonine and proline residues (DVSSALDKLKEFGNTLE DKARELISRIKQSELSAKMREWFSETFQKVKEKLKIDS, av. MW 6432.3 Da). The tryptic peptides in gel bands #1 and #2 matched the sequences of apoC-III and apoA-II, respectively (data not shown). Gel band #3 contained a mixture of peptides matching the truncated isoforms of apoC-III and apoA-II (Fig. 5).

The identities of the protein biomarkers were confirmed based on sequence analysis by MALDI-TOF/TOF mass spectrometry. The resulting sequence information for the tryptic peptides (m/z 1293.7, 1279.7, 1003.6, and 617.4) matched the theoretical sequence information for apoC-I and apoC-I′ in the databases. For example, Fig. 6 shows the peptide sequencing experiment for the tryptic peptide at m/z 1293.7. Under low energy MS/MS conditions (upper spectrum), the fragmentation is not extensive. Only high mass fragments are observed. Under high-energy MS/MS conditions (lower spectrum), the fragmentation is more extensive and low mass fragments are observed. The mass spectra were combined to confirm the amino acid sequence of the peptide as LKEFGNTLEDK, which corresponds to residues 11–21 of apoC-I. Similarly, m/z 1279.7, 1003.6 and 617.4 were identified as residues 13–23, 29–37, and 24–28, respectively.

4. Discussion

Apolipoprotein C-I (apoC-I) and its truncated isoform apolipoprotein C-I′ (apoC-I′) were identified in this study as potential early predictors of bortezomib response in relapsed, refractory myeloma patients. In the responders, plasma levels of apoC-I and apoC-I′ remained at (9-1 and 9-13) or returned to (9-11) normal levels after treatment with bortezomib (Fig. 2A and B). In the non-responders, however, plasma levels of apoC-I and apoC-I′ remained significantly high (9-4) or increased significantly (9-9, 9-3, 9-4, 9-15, 9-8, and 9-10) compared to normal levels. Plasma levels of apoC-I and apoC-I′ were significantly more abundant in the non-responsive patients (i.e. no change and progressive disease) compared to the partial responders at 24-h postdose bortezomib (Fig. 2A). The levels of apoC-I and apoC-I′ in patient 9-14 were much lower compared to the other non-responsive patients. Although serum M-protein levels are classically used for disease diagnostics and to monitor/predict drug efficacy in multiple myeloma patients, they are not presented in patients with light chain or non-secretory disease. The results of this study suggest that changes in the plasma levels of apoC-I and apoC-I′ after 24-h postdose may be used as potential markers of bortezomib efficacy response in different types of multiple myeloma patients. ApoC-I and apoC-I′ are not appropriate, however, for disease diagnostics in multiple myeloma because patients may have baseline plasma levels that are similar to healthy subjects

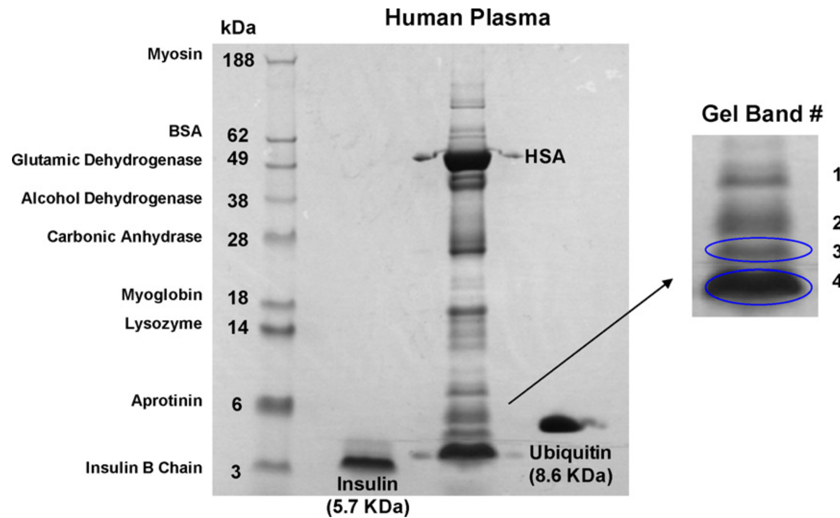

Fig. 4. Photograph of polyacrylamide gel electrophoresis of human plasma.

(Fig. 2B). ApoC-I and apoC-I′ were not drug–response predictive markers if these patients were not treated with bortezomib (i.e., predose).

The presence of both apoC-I and apoC-I′ in human serum and plasma is well established [6,7]. The mechanism for processing apoC-I to apoC-I′ may involve the action of didpeptidyl peptidase IV (EC 3.4.14.5), a widely distributed proline cleaving enzyme [8,9]. Functionally, the C apolipoproteins are low molecular weight exchangeable plasma proteins that are important regulators of lipid metabolism. C apolipoproteins account for about 30–40% of the total protein content of very low-density lipoprotein (VLDL) and are minor components of high-density lipoprotein (HDL). apoC-I is an activator of lecithin:cholesterol acyltransferase, a glycoprotein that catalyzes the conversion of plasma cholesterol to cholestryl ester [10]. It also inhibits the binding of apolipoprotein to the low-density lipoprotein (LDL) receptor, the LDL receptor-related protein, and the VLDL receptor [11–13].

VLDL, LDL and their major protein, apoB-100, play an essential role in lipid transport and metabolism. Intracellular apoB-100 is degraded through the ubiquitin-proteasome pathway [14,15]. When bortezomib is first dosed, the level of apoB-100 in multiple myeloma patients (either responders or non-responders) is presumably elevated due to proteasome inhibition. ApoC-I is normally regulated to inhibit LDL/VLDL receptors [13] or impede LDL/VLDL metabolism. The significantly higher concentrations of apoC-I and apoC-I′ in the non-responders may interrupt the regulation of lipid metabolism and be linked to the lack bortezomib efficacy.

Patients suffering from multiple myeloma show disturbances in serum cholesterol and lipoprotein levels [5]. Musolino et al.

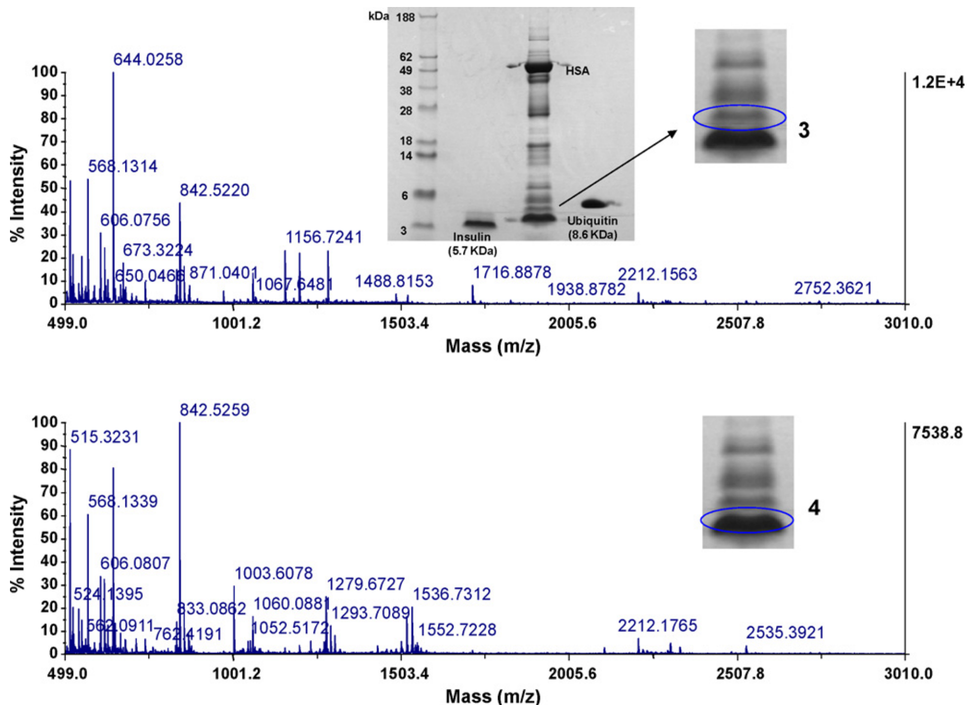

Fig. 5. MALDI-TOF spectra of tryptic peptide mapping by digesting selected gel band #3 and gel band #4.

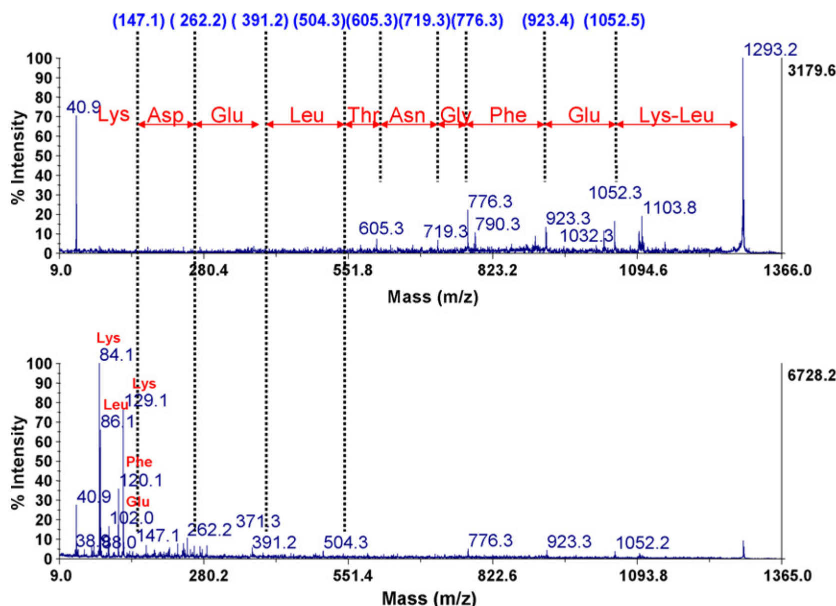

Fig. 6. MALDI-TOF/TOF sequencing of a tryptic peptide (m/z 1293) using low energy (top) and high energy (bottom) conditions.

[16] reported that the serum concentrations of apoA-I, apoA-II, and cholesterol in 43 multiple myeloma patients were significantly lower compared to normal subjects. Similar results have been reported by Musolino et al. [16], Levy et al. [17], and Hachem et al. [18]. ApoA-I and apoA-II are the primary apolipoproteins in normal HDL. Additional apolipoproteins, such as apoC-I and serum amyloid A protein (SAA), are present in normal HDL at relatively low levels. SAA displaces the majority of the apoA-I and apoA-II in the HDL of multiple myeloma patients [18–20]. The results of this study suggest that the apoA-I and apo-A-II in the HDL particles of the non-responders are also partially or considerably substituted for by apoC-I and apoC-I′.

As described in Table 1, of the 10 patients included in this study, 3 were partial responders, 5 showed no change, and 2 were considered to have progressive disease as determined by EBMT criteria. The change in serum paraprotein (M-protein) level is one of the major criteria used to define clinical response in IgG and IgA type myeloma patients. Patients with a ≥50% reduction in the level of serum M-protein over a minimum of 6 weeks are usually predicted to have at least a partial response to treatment. Patients that show a ≥25% increase in M-protein levels are predicted to have progressive disease. The change in serum M-protein levels correlated with clinical response for most of the heavy chain (IgG and IgA) patients in this study. Patient (9-14; IgG), however, showed M-protein levels that were significantly decreased (−61%) although no change in disease status was observed. Patient (9-8; IgG) showed M-protein levels that were similar to pre-dose levels (+6%) although progressive disease was experienced.

Dose-related peripheral neuropathy is a clinically significant adverse event associated with bortezomib treatment [21]. Symptoms can include a loss of reflexes, continuous burning pain, and abnormal sensory sensations (tingling, burning, and prickling). Richardson et al. [21] reported that 37% (84 of 228 patients) receiving bortezomib 1.3 mg/m^2 and 21% (6 of 28 patients) receiving bortezomib 1.0 mg/m^2 during phase II clinical studies had evidence of treatment-emergent peripheral neuropathy. Patients with and without neuropathy at baseline had similar overall frequencies of treatment-emergent neuropathy (36% for each patient group). The neuropathy was reversible in the majority of patients after dose reduction or discontinuation.

Richardson et al. [21] demonstrated that the peripheral neuropathy associated with bortezomib is a cumulative, dose-related adverse effect that increases in prevalence through the first five treatment cycles. Proteasome inhibition may alter specific aspects of neural mitochondrial homeostasis and lysosomal-mediated degradation of mitochondria [22]. Mitochondrial impairment is associated with increased production of reactive oxygen species (ROS) and decreased energy production. ROS generation is thought to play an important role in the initiation of the bortezomib-induced apoptotic cascade through disruption of the mitochondrial membrane potential and the release of cytochrome *c* [23].

Patients receiving bortezomib 1.3 mg/m^2 in this study had evidence of peripheral neuropathy (Table 1). There was no apparent relationship between the plasma levels of apoC-I and apoC-I′ (at predose or 24-h postdose bortezomib) with the incidence of peripheral neuropathy under the conditions of this study. The pharmacokinetic profile of each patient was evaluated over the initial 2 h following bortezomib administration, on Day 1 of the first cycle

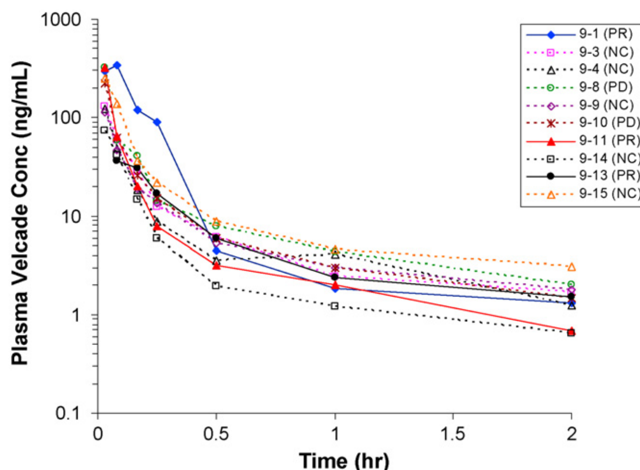

Fig. 7. The pharmacokinetic profile of individual patients evaluated over the initial 2 h following bortezomib administration, on Day 1 of the first cycle.

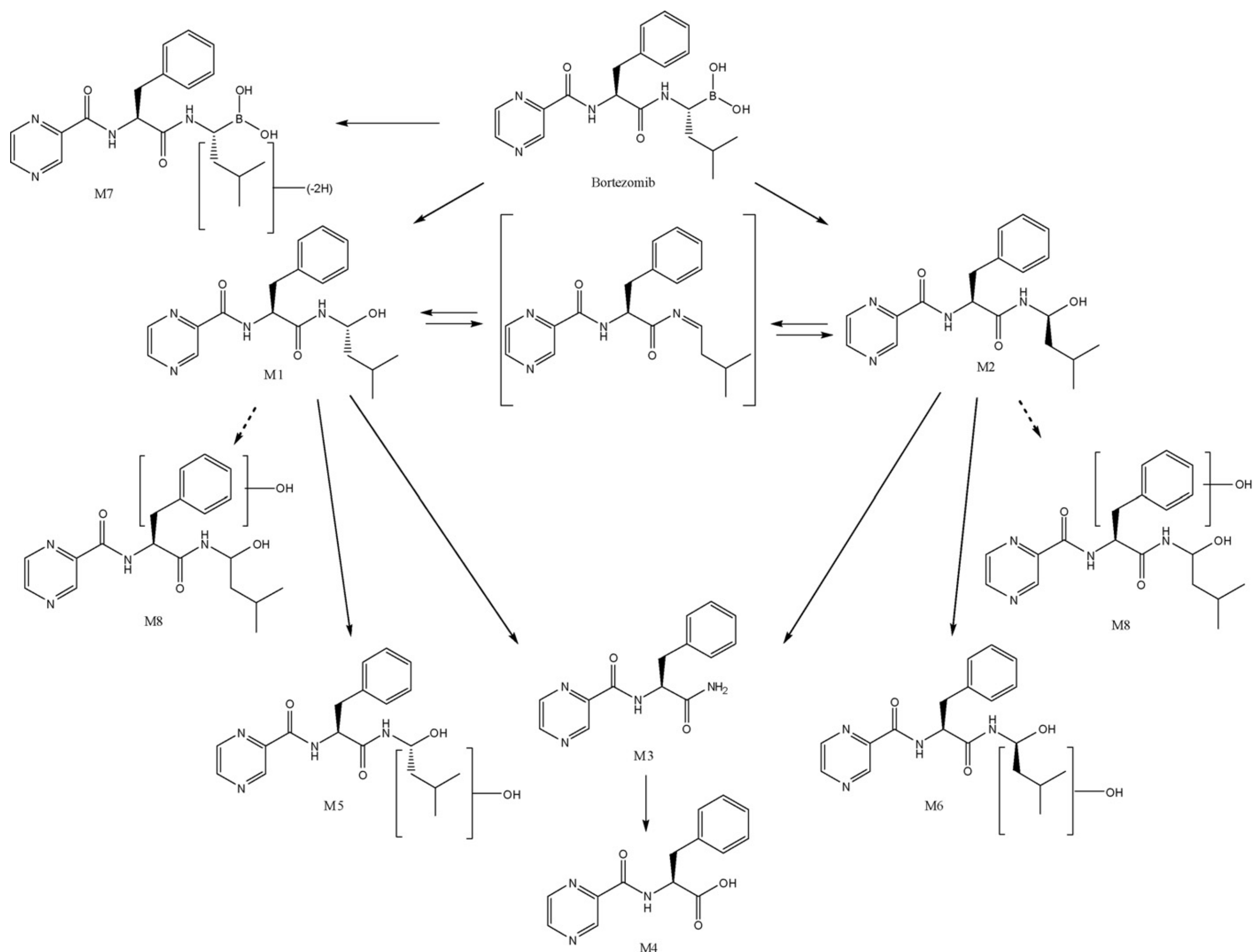

Fig. 8. Metabolites of bortezomib in human plasma.

(Fig. 7). Plasma samples were collected at predose, and at 2, 5, 10, 15, 30, 60, and 120 min after dosing [21]. No significant difference in the pharmacokinetic profiles of responders and non-responders (with or without neuropathy) was observed. The plasma samples were also analyzed for circulating bortezomib metabolites. Biotransformation of bortezomib includes CYP-mediated oxidation to deboronated and hydroxylated metabolites as shown in Fig. 8. Major metabolites of bortezomib, carbinolamide diastereomers M1 and M2, were observed in both the responders and non-responders. The results showed that the drug and drug metabolites of bortezomib in responders and non-responders were similar.

Additional plasma samples were collected from six patients in order to determine 20S proteasome activity at predose and at 1 and 24 h after bortezomib administration, on Day 1 of the first cycle. The maximum inhibition of 20S proteasome activity ranged from 35% (patient 9-14) to 70% (patient 9-15) at 1-h postdose. Five of the six patients had maximum levels of 20S proteasome inhibition in the range of 50–70%. Among these five patients, patients 9-11 (60% inhibition) and 9-13 (50%) experienced a partial clinical response, whereas 9-15 (70%) and 9-9 (60%) showed no change in overall status, and 9-10 (60%) had progressive disease. Peripheral neuropathy was observed in all of the patients, even though the efficacy response among them was different. In other words, the drug and drug metabolite exposure of bortezomib and the maximum inhibition of the 20S proteasome were similar in the patients, but the efficacy response to drug treatment was different.

5. Conclusions

Overall, the results of this study suggest that clinical response to bortezomib in multiple myeloma patients may be predicted through protein biomarker (apoC-I and apoC-I′) analysis at 24-h postdose. It is necessary however, to conduct a time course experiment in a larger patient population in order to investigate the utility of C appolipoproteins as predictors of bortezomib efficacy response. The integration of biomarker analysis with more classical criteria for evaluating disease progression (i.e. serum/urinary paraproteins, plasma cells in bone marrow, size of soft tissue plasmacytomas and size/number of bone lesions) would be useful during treatment of patients whose drug efficacy response is difficult to evaluate (i.e. hyposecretory and non-secretory patients). Impairments in apoC-I and apoC-I′ functioning and/or sorting may be related to the disturbances in the serum cholesterol and lipoprotein levels observed in patients suffering from myeloma. An understanding of the relationship between C apolipoproteins and lipid dysregulation in myeloma patients must be established, however, before the full potential of apoC-I and apoC-I′ as indicators of bortezomib response can be realized for clinical applications.

References

[1] J. Blade, D. Samson, D. Reece, J. Apperley, B. Bjorkstrand, G. Gahrton, Br. J. Haematol. 102 (1998) 1115–1123.
[2] P.G. Richardson, B. Barlogie, J. Berenson, S. Singhal, S. Jagannath, D.N. Irwin, Engl. J. Med. 348 (2003) 2609–2617.
[3] R.A. Kyle, S.V. Rajkumar, Blood 111 (2008) 2962–2972.
[4] T. Pekol, J.S. Daniels, J. Labuttie, I. Parsons, D. Nix, E. Baronas, F. Hsieh, L.S. Gan, G. Miwa, Drug Metab. Dispos. 33 (2005) 771–777.
[5] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Electrophoresis 20 (1999) 3551–3567.
[6] P.V. Bondarenko, S.L. Cockrill, L.K. Watkins, I.D. Cruzado, R.D. MacFarlane, J. Lipid Res. 40 (1999) 543–555.
[7] R.W. Mahley, T.L. Innerarity, S.C. Rall, K.H. Weisgraber, J. Lipid Res. 25 (1984) 1277–1294.
[8] H. Gotoh, M. Hagihara, T. Nagatsu, H. Iwata, T. Miura, Clin. Chem. 34 (1988) 2499–2501.
[9] G. Vanhoof, F. Goossens, I. De Meester, D. Hendriks, S. Scharpe, FASEB J. 9 (1995) 736–744.
[10] A. Rozek, T.J. Sparrow, K.H. Weisgraber, R.J. Cushley, Biochem. Cell Biol. 76 (1998) 267–275.
[11] T. Gautier, D. Masson, J.P. Pais de Barros, A. Athias, P. Gambert, D. Aunis, M.D. Metz-Boutigue, L. Lagrost, J. Biol. Chem. 275 (2000) 37504–37509.
[12] J. Poensgen, Biochim. Biophys. Acta 1042 (1990) 188–192.
[13] N.S. Shachter, Curr. Opin. Lipidol. 12 (2001) 297–304.
[14] P.J. Roberts-Thomson, G.S. Venables, A.C. Onitiri, B. Lewis, Postgrad. Med. J. 51 (1975) 44–51.
[15] H. Hachem, G. Favre, N. Ghalim, P. Puchois, J.C. Fruchart, G. Soula, J. Clin. Chem. Clin. Biochem. 25 (1987) 675–679.
[16] C. Musolino, L. Calabro, G. Bellomo, M. Cincotta, V. Di Giacomo, C. Pezzano, B. Loteta, V. Rizzo, S. Guglielmo, A. Alonci, Rec. Prog. Med. 93 (2002) 298–301.
[17] Y. Levy, M. Aviram, G. Spira, I. Tatarsky, G.J. Brook, A. Carter, Postgrad. Med. J. 60 (1984) 449–453.
[18] H. Hachem, G. Favre, G. Raynal, G. Soula, Ann. Biol. Clin. 41 (1983) 181–185.
[19] H. Hachem, G. Favre, G. Soula, Biochim. Biophys. Acta 963 (1988) 271–277.
[20] A.F. Strachan, W.F. Brandt, P. Woo, D.R. van der Westhuyzen, G.A. Coetzee, M.C. de Beer, E.G. Shephard, F.C. de Beer, J. Biol. Chem. 264 (1989) 18368–18373.
[21] P.G. Richardson, H. Briemberg, S. Jagannath, P.Y. Wen, B. Barlogie, J. Berenson, S. Singhal, D.S. Siegel, D. Irwin, M. Schuster, G. Srkalovic, R. Alexanian, S.V. Rajkumar, S. Limentani, M. Alsina, R.Z. Orlowski, K. Najarian, D. Esseltine, K.C. Anderson, A.A. Amato, J. Clin. Oncol. 24 (2006) 3113–3120.
[22] P.G. Sullivan, N.B. Dragicevic, J.H. Deng, Y. Bai, E. Dimayuga, Q. Ding, Q. Chen, A. Bruce-Keller, J.N. Keller, J. Biol. Chem. 279 (2004) 20699–20707.
[23] H.E. Ling, L. Liebes, Y. Zou, R. Perez-Soler, J. Biol. Chem. 278 (2003) 33714–33723.