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Stability of the proteasome inhibitor bortezomib in cell based assays determined by ultra-high performance liquid chromatography coupled to tandem mass spectrometry

Jannick Clemens[a], Magdalena Longo[a], Anja Seckinger[b], Dirk Hose[b], Walter Emil Haefeli[a], Johanna Weiss[a,1], Jürgen Burhenne[a,*,1]

[a] Department of Clinical Pharmacology and Pharmacoepidemiology, University Hospital Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany
[b] Department of Internal Medicine V, Oncology, Hematology, and Rheumatology, University Hospital Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

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ABSTRACT

Bortezomib represents the first clinically approved proteasome inhibitor for multiple myeloma. Research conducted on its intracellular kinetics in target cells and on possibly related mechanisms of resistance is sparse so far. We therefore developed and validated a highly sensitive ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS) method for bortezomib quantification within cultured myeloma cells and media. Fast gradient UPLC based on a BEH C18 column (1.7 μm particle size) with aqueous formic acid and acetonitrile as mobile phase. Selective extraction procedures using protein precipitation extraction (PPE) and liquid–liquid extraction (LLE) were established and compared. Extracted bortezomib was quantified by positive electrospray tandem mass spectrometry using deuterated D_8-bortezomib as internal standard. The calibrated ranges were 0.5–2500 pg per sample. For LLE, overall accuracies varied between 99.2% and 112% (medium) and 89.9% and 111% (cells), while overall precision ranged from 1.13% to 13.0% (medium) and 2.80% to 12.7% (cells), respectively. Recovery rates (cells/medium) were >77%/>65% for LLE and >89%/63% for PPE. Matrix effects were generally lower for LLE compared to PPE. Regardless of the extraction method, retrievable amounts of bortezomib were considerably reduced after 24 h of incubation (0.2, 1, 5, and 25 nM). Revealing greater dependence on the extent of acidification, retrieval of bortezomib can be increased distinctly in acidified solution or acidified culture medium. Thus, particular attention needs to be paid to the occurring bortezomib degradation in neutral culture medium since correct quantification of intracellular bortezomib can only be achieved in relation to the corresponding extracellular concentration.

1. Introduction

Increasingly, pharmacologic characterization of drugs also implies cellular drug kinetics since an accurate prediction of the pharmacologic effect is only possible with profound knowledge on concentration and distribution within target cells [1,2]. Among novel agents in tumor therapy this concept also applies to the modified dipeptidyl boronic acid bortezomib (Velcade®), a reversible intracellular inhibitor of proteasome activity [3,4]. The resulting accumulation of ubiquitinated proteins originally designated for degradation leads to imbalance of cellular homeostasis, cell cycle arrest, and ultimately apoptosis [5,6]. Showing satisfying activity in patients with relapsed multiple myeloma [7,8], application of bortezomib was also expanded to previously untreated patients since its clinical approval [9,10]. Moreover, bortezomib might also be a therapeutic option for the veterinary oncologist, especially for the treatment of canine malignant melanoma [11].

Up to now, basic pharmacokinetic data about time-dependent plasma levels or tissue distribution of bortezomib have been gathered [12–14]. However, little is known about drug kinetics in targeted cells (*e.g.* myeloma cells), such as cellular uptake, concentration-time profiles, or possible enrichment. More profound knowledge might help to better understand the relation between cellular kinetics and drug efficacy in myeloma cell lines with different sensitivities [15]. With regard to clinically observable primary and secondary resistance to bortezomib [16,17], this

* Corresponding author. Tel.: +49 6221 56 36395; fax: +49 6221 56 5832.
E-mail address: juergen.burhenne@med.uni-heidelberg.de (J. Burhenne).
[1] These authors contributed equally.

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aspect seems to be of special importance for the controversially discussed relevance of drug transporters for the efficacy of bortezomib. For instance, some previous studies advocated the relevance of *P*-glycoprotein for the efflux of bortezomib in tumors [18], while other studies described more ambivalent results [19] or even denied a considerable importance of this transport process [20]. Thus, complementary data on intracellular drug concentrations has the potential to bridge the gap between possible resistance factors and cellular sensitivity as their surrogate [19].

For this purpose highly sensitive methods based on liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) are needed. In the literature several methods can be found for the analysis of bortezomib *e.g.* in human plasma [12,14,21], rat plasma and tissue [13], and cell lysates [22] with lower limits of quantification (LLOQ) down to 100 pg mL^{-1}. For sample extraction, these methods used either protein precipitation (PPE) with acetonitrile or liquid–liquid extraction (LLE) with methyl-*tert*-butylether (MTBE). A previous study made a comparison of bortezomib extraction by PPE or LLE and concluded that both methods reveal high recovery yields from spiked blank material, while LLE revealed advantages for the extraction from real study samples [23]. Another study presented a method for the simultaneous quantification of nine anticancer drugs using ultra performance liquid chromatography (UPLC) [14].

In our study, we present procedures based on UPLC/MS/MS, LLE, and PPE to obtain concentration-time profiles of bortezomib in myeloma cell systems at sub nanomolar levels resulting in the by far most sensitive bioanalytical methodology for bortezomib (LLOQ 0.5 pg per 100 μL sample or cell pellet (1×10^6 cells)). These procedures further include cell incubation, sampling, and processing of myeloma cells and respective media. In contrast to studies using plasma or tissue samples, our *in vitro* implementation not only allows more sensitive detection of intracellular and extracellular bortezomib but also constant monitoring of its total amount due to the closed experimental setting. As a result, it was necessary to calculate bortezomib balances, which revealed considerable instability of bortezomib in cell culture media. Having not yet been described, this novel observation represents a major challenge for any *in vitro* setting using bortezomib.

2. Materials and methods

2.1. Materials

Bortezomib was purchased from Absource Diagnostics (München, Germany) while the internal standard D_8-bortezomib was purchased from Toronto Research Chemicals (Toronto, Canada). RPMI-1640 medium, medium supplements, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal calf serum (FCS) was supplied by PAA (Pasching, Austria), dimethyl sulfoxide (DMSO) by AppliChem (Darmstadt, Germany). Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Invitrogen (Karlsruhe, Germany). Sodium hydroxide solution (NaOH) and acetonitrile (ACN) were obtained from Carl Roth GmbH (Karlsruhe, Germany) and saline solution (0.9% NaCl) from B. Braun Melsungen (Melsungen, Germany). Formic acid (FA), methyl-*tert*-butylether (MTBE), and hydrochloric acid (HCl) were from Merck KGaA (Darmstadt, Germany). Casy® ton and Casy® clean (isotonic and isoosmotic cell counter reagents) were from Roche Diagnostics (Mannheim, Germany). Cell culturing bottles, reaction tubes, and 6-well cell culture plates were purchased from Greiner (Frickenhausen, Germany). 96-well (300 μL) polystyrene microtiter plates and 96-well (500 and 2000 μL) polypropylene plates were supplied by Nunc (Wiesbaden, Germany). Glass vials were from VWR International (Darmstadt, Germany). Deionized water was obtained from a HP 6 UV/UF TKA ultra-filtration system (TKA GmbH, Niederelbert, Germany).

Table 1
Overview on the main characteristics of the utilized cell lines.

	Cell line		
	KMS-12-BM	LP-1	EJM
Cellular sensitivity			
IC_{50} of bortezomib (nM)	6.53 ± 0.34	5.07 ± 0.09	1.64 ± 0.07
Cell pellet parameters			
Mean single cell volume (fL)	1319 ± 210	2782 ± 402	1166 ± 162
Number of cells ($\times 10^6$)	0.93 ± 0.09	0.93 ± 0.12	0.76 ± 0.15
Mean cell pellet volume (μL)	1.24 ± 0.28	2.60 ± 0.53	0.90 ± 0.28

Comparison of the cellular sensitivity to bortezomib (IC_{50}) and the mean cell pellet parameters of all extracted samples.

2.2. Cell lines

Myeloma cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured as previously delineated [24]. All described analyses were performed using the non-adherent human multiple myeloma cell line KMS-12-BM [25]. For comparison with other cell lines, bortezomib mass balances (see Section 3.3) were exemplarily investigated in the partly adherently growing EJM and LP-1 cells [26,27]. Table 1 gives an overview on the main characteristics of these different cell lines. Following standard cell culture conditions, myeloma cells were cultured in RPMI-1640 medium (KMS-12-BM) or IMDM (EJM, LP-1), respectively, each supplemented with 10% FCS, 2 mM glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin sulfate.

2.3. Standard solutions

Bortezomib (5.08 mg) was weighed into volumetric flask (10 mL) and dissolved in ACN/H_2O (1/1, v/v + 0.01% FA), creating a stock solution (0.5 mg mL^{-1}) used for both incubation in experiments and calibration in UPLC/MS/MS and stored at −25 °C in darkness. Thereof, 100 μL were diluted volumetrically in another 10 mL ACN/H_2O (1/1, v/v + 0.01% FA) to prepare calibration stock solutions (5 μg mL^{-1}) for supernatant and myeloma cells. An aliquot of 394 μL was again diluted in 10 mL ACN/H_2O (1/1, v/v + 0.01% FA) to obtain the highest calibration point solution (197 ng mL^{-1}). Thereof, nine supplementary calibration solutions were prepared by diverse dilution with ACN/H_2O (1/1, v/v + 0.01% FA).

From independently weighed bortezomib (4.90 mg) a quality control (QC) stock solution was prepared according to equivalent dilution procedures. Hence three QC solutions in the lower (QC A), middle (QC B), and higher (QC C) calibration range for supernatant and myeloma cells were prepared by diluting with ACN/H_2O (1/1, v/v + 0.01% FA).

The internal standard (IS) D_8-bortezomib (1.00 mg) was transferred into volumetric flask (5 mL) and filled up with ACN/H_2O (1/1, v/v + 0.01% FA). The final concentration of IS solution (2.7 ng mL^{-1}) was obtained after quadruple dilution (1:10, 1:50, 1:50, 1:3) in ACN/H_2O (1/1, v/v + 0.01% FA) resulting in 66.7 pg IS per sample (100 μL supernatant or cell pellet (1×10^6 cells), respectively).

2.4. Calibration and QC samples

Calibration curves for intracellular and extracellular quantification of bortezomib were obtained by spiking duplicates of either blank (bortezomib-free) myeloma cells (1×10^6) or blank