Inactivating PSMB5 Mutations and P-Glycoprotein (Multidrug Resistance-Associated Protein/ATP-Binding Cassette B1) Mediate Resistance to Proteasome Inhibitors: Ex Vivo Efficacy of (Immuno)Proteasome Inhibitors in Mononuclear Blood Cells from Patients with Rheumatoid Arthritis

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ABSTRACT

Bortezomib (BTZ), a registered proteasome inhibitor (PI) for multiple myeloma, has also been proposed as a potential antirheumatic agent. Its reported side effects, however, make it unappealing for long-term administration, and resistance may also develop. To overcome this, second-generation PIs became available. Here, we investigated whether a novel class of peptide epoxyketone-based PIs, including carfilzomib, N-(S)-3-methoxy-1-(((S)-3-methoxy-1-((S)-1-(2-methylxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl) amino)-1-oxopropan-2-yl)-2-methylthiazole-5-carboxamide (ONX0912), and (S)-3-(4-methoxyphenyl)-N-((S)-1-(S)-2-methylxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)-2-(2-(2morpholinoacetamido)propanamido)propanamide (ONX0914), might escape two established BTZ-resistance mechanisms: 1) mutations in the proteasome β5 subunit (PSMB5) targeted by these PIs, and 2) drug efflux mediated by ATP-binding cassette transporters. THP1 myeloid sublines with acquired resistance to BTZ (54- to 235-fold) caused by mutations in the PSMB5 gene displayed marked cross-resistance but less pronounced cross-resistance to carfilzomib (9- to 32-fold), ONX0912 (39- to 62-fold), and ONX0914 (27- to 97-fold). As for ATP-binding cassette transporter-mediated efflux, lymphoid CEM/VLB cells with P-glycoprotein (Pgp)/multidrug resistance 1 overexpression exhibited substantial resistance to carfilzomib (114-fold), ONX0912 (23-fold), and ONX0914 (162-fold), whereas less resistance to BTZ (4.5-fold) was observed. Consistently, β5 subunit-associated chymotrypsin-like proteasome activity was significantly less inhibited in these CEM/VLB cells. Ex vivo analysis of peripheral blood mononuclear cells from therapy-naive patients with rheumatoid arthritis revealed that, although basal Pgp levels were low, P-glycoprotein expression compromised the inhibitory effect of carfilzomib and ONX0914. However, the use of P121 (reversin 121), a Pgp transport inhibitor, restored parental cell inhibitory levels in both CEM/VLB cells and peripheral blood mononuclear cells. These results indicate that the pharmacologic activity of these PIs may be hindered by drug resistance mechanisms involving PSMB5 mutations and PI extrusion via Pgp.

Introduction

The ubiquitin-proteasome system plays a central role in maintaining cellular homeostasis by controlling the timely breakdown of many key proteins. This includes the regulation of cell cycle, activation of transcription factors (e.g., nuclear factor-κB), and induction of apoptosis (Hershko and Ciechanover, 1998). As such, the ubiquitin-proteasome system has been recognized as an attractive target for cancer therapeutic intervention by proteasome inhibitors (PIs). Currently available PIs target at least one of three β-subunits within the 20S core of the proteasome that harbor catalytic activity: the β5 subunit (chymotrypsin-like activity), the β1 subunit (caspase-like activity), and the β2 subunit (trypsin-like activity) (Kisselev et al., 2006). Upon stimulation by proinflammatory stimuli, e.g., interferon-γ or tumor necrosis...
factor α, these constitutive proteasome subunits can be replaced by immunoproteasome subunits β5i (LMP7), β2i (MECL1), and β1i (LMP2). This is followed by their assembly into immunoproteasomes that have specialized functions in facilitating antigen presentation via major histocompatibility complex-I (Hershko and Ciechanover, 1998; Kloetzel, 2004), or, as indicated recently (Seifert et al., 2010), to preserve complex-I (Hershko and Ciechanover, 1998; Kloetzel, 2004), facilitating antigen presentation via major histocompatibility into immunoproteasomes that have specialized functions in stress.

Bortezomib (BTZ) (1R)-3-methyl-1-((2S)-3-phenyl-2-[[pyrazin-2-y]carbonyl]amino)propanoyl]amino)butyl]boronic acid), a boron-containing dipeptide that primarily targets the β5 subunit of the proteasome, was the first clinically approved proteasome inhibitor for the treatment of therapy-refractory multiple myeloma (Orlowski and Kuhn, 2008). Emergence of resistance to this drug (Oerlemans et al., 2008; Ruschak et al., 2011) as well as side effects such as peripheral neuropathy (Orlowski and Kuhn, 2008) initiated the search for novel PIs that would be devoid of these limitations. These efforts have resulted in a second generation of PIs that selectively target either β subunits within the constitutive proteasome (Demo et al., 2007; Zhou et al., 2009; Chauhan et al., 2010; Ruschak et al., 2011) or the immunoproteasome (Muchamuel et al., 2009).

Beyond the successful application of PIs in cancer chemotherapy, there is a growing body of data that these agents may also hold promise as drugs for the treatment of (chronic) autoimmune diseases, e.g., rheumatoid arthritis and ulcerative colitis (Elliott et al., 2003; Bennett and Kirk, 2008). In fact, both BTZ and second-generation PIs displayed clear therapeutic efficacy in animal models of rheumatoid arthritis or lupus-like disease (Neubert et al., 2008; Lee et al., 2009; Muchamuel et al., 2009). These effects are in part mediated by their ability to suppress the production of nuclear factor κB-inducible proinflammatory cytokines from immune competent cells (van der Heijden et al., 2009). Whenever it comes to the clinical application of PIs for the treatment of autoimmune diseases, the issue of retaining long-term efficacy is particularly important because this type of patient would face chronic drug treatment during which drug resistance modalities may emerge. Little is known about the underlying mechanisms of intrinsic or acquired resistance to second-generation PIs. For a prototypal epoxyketone-based proteasome inhibitor such as epoxomicin it has been reported that cellular extrusion via the ABC transporter ABCB1 (Pgp/MDR1) could confer drug resistance (Gutman et al., 2009). Whether or not cellular extrusion by ABC pumps other than ABCB1 (Pgp/MDR1) can also contribute to these second-generation epoxyketone-based PIs is still unknown.

In the present study we explored whether mutations in the PSMB5 gene, encoding for the β5 subunit of the proteasome, and cellular extrusion via drug efflux transporters (Gillet et al., 2007; van de Ven et al., 2009) may interfere with the efficacy of three second-generation epoxyketone-based PIs, i.e., carfilzomib (formerly named PR-171) ([S]-4-methyl-[(S)-1-((S)-4-methyl-1-(R)-2-methylxiran-2-yl)-1-oxopentan-2-yl]amino)-1-oxo-3-phenylpropan-2-yl)-2-[(S)-2-(morpholinooacetamido)-4-phenylbutanamido] pentanamide) (Demo et al., 2007), N-((S)-3-methoxy-1-((S)-3-methoxy-1-((S)-1-((F)-2-methylxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)-2-methylthiazole-5-carboxamide (ONX0912; formerly named PR-047) (Zhou et al., 2009; Chauhan et al., 2010), and the immunoproteasome inhibitor (S)-3-(4-methoxyphenyl)-N-((S)-1-((S)-2-methylxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)-2-((S)-2-(2-morpholinooacetamido)propanamido)propanamide (ONX0914; formerly named PR-957) (Muchamuel et al., 2009). Our data provide evidence that ABCB1 (Pgp/MDR1), but none of the other ABC transporters, harbors the ability to extrude these drugs and can confer drug resistance in a cell line model with high ABCB1 (Pgp/MDR1) overexpression. However, in an ex vivo setting with peripheral blood mononuclear cells (PBMCs) from healthy controls and patients with rheumatoid arthritis basal ABCB1 (Pgp/MDR1) activities were modest and still allowed for the retention of proficient proteasome inhibitory capacity by the three epoxyketone-based drugs.

Materials and Methods

Proteasome Inhibitors and Other Chemicals. Carfilzomib, ONX0912, and ONX0914 were provided by Onyx Pharmaceuticals, Inc. (South San Francisco, CA). BTZ was provided by Millennium Pharmaceuticals (Cambridge, MA). The chemical structures of these compounds are depicted in Fig. 1. The P-glycoprotein inhibitor reversin-121 (P121) was obtained from Alexis Benelux (Zandhoven, Belgium). Syto16 was purchased from Invitrogen (Breda, The Netherlands).

Cell Lines. Human monocytic-macrophage THP1 cells with various levels of acquired resistance to BTZ were selected as described previously (Oerlemans et al., 2008). Multiple human cell lines were selected based on their known expression of ABC transporters, P-glycoprotein/MDR1 (CEM/VLB) (Zamora et al., 1988), multidrug resistance-associated protein 1 (CEM/CHQ, 2008/MRP1) (Scheffer et al., 2000; Oerlemans et al., 2006), MRP2 (2008/MRP2) (Scheffer et al., 2000), MRP3 (2008/MRP3) (Scheffer et al., 2000), MRP4 (HEK293/MRP4) (Wielinga et al., 2002), MRP5 (HEK293/MRP5) (Wielinga et al., 2002), and breast cancer resistance protein (BCRP) (CEMSZS and MCF7/MR) (Taylor et al., 1991; van der Heijden et al., 2004). Cell cultures were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 1000 U/ml penicillin/streptomycin. Cells were routinely cultured at an initial density of 3 × 10^5 cells/ml (for suspension cells) and 5 × 10^3 cells/cm² for adherent cells, all in 25-cm² culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) at 37°C in a humidified 5% CO₂ atmosphere.

Patients/Healthy Controls and Isolation of Blood Mononuclear Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy controls (n = 22) and newly diagnosed untreated patients with RA. Samples for this study were collected within the COBRA-light study, a randomized trial comparing two combination schemes in early rheumatoid arthritis. Consent informed was obtained from all patients, and the study was approved by the Ethical Review Board of the VU Medical Center. Blood was collected at baseline before initiation of the therapy protocol with methotrexate and prednisolone. Patients eligible to be included in this study ful-
filled the following criteria: RA diagnosis according to the ACR 1987 criteria (http://www.rheumatology.org/practice/clinical/classification/ra/ars.asp), with disease duration < 2 years, age > 18 years, active RA with > six swollen joints and > six painful joints, and an erythrocyte sedimentation rate of > 28 mm of visual analog scale (global health) > 10. PBMCs were isolated by Ficoll-Paque (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) density gradient centrifugation (15 min at 1000g at room temperature) according to the manufacturer’s instructions. The interphase was collected and washed three times with phosphate-buffered saline supplemented with 1% bovine serum albumin. PBMCs were then counted and resuspended in RPMI 1640 culture medium supplemented with 10% fetal calf serum.

**Assessment of Cell Growth Inhibition by Proteasome Inhibitors.** The growth inhibitory effects of BTZ, carfilzomib, ONX-0912, and ONX-0914 were analyzed essentially as described previously (Shafran et al., 2005; Oerlemans et al., 2006). In brief, for suspension cell cultures, 1.25 × 10^6 cells/ml were plated in individual wells of a 24-well cell culture plates containing an increasing concentration of each proteasome inhibitor. For adherent cells, a plating density of 1 × 10^4/cm^2 was used and drugs were added 24 h after plating. Inhibition of cell growth was determined after 72-h drug incubation by counting viable cells (for adherent cells after trypsinization) on the basis of trypan blue exclusion. Eight drug concentrations (in duplicate) were used covering a 100- to 200-fold concentration range. The proteasome inhibitor concentration required to inhibit cell growth by 50% compared with control growth is defined as the IC_{50}.

**Intact Cell-Based Assay for Chymotrypsin-Like, Caspase-Like, and Trypsin-Like Proteasome Activities.** An intact cell-based assay to measure basal and proteasome inhibitor-induced suppression of each of three types of proteasome activities (chymotrypsin-, caspase-, and trypsin-like) was performed by using a Proteasome-Glo assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. Before determination of proteasome activity, cells were incubated with the proteasome inhibitors for 1 h at 37°C in a flat, white-bottomed 96-well plate (NUNC A/S, Roskilde, Denmark) at a density of 15,000 cells per well in 50 μl. After drug exposure, a luminogenic substrate specific for chymotrypsin-like, caspase-like, or trypsin-like protease activity was added to the intact cell suspension. After 10-min incubation at 37°C, luminescence was measured on untreated cell lysates as described previously (Parlati et al., 2009). Luminescence was measured by using a GENios-Basic plate reader (Tecan Austria GmbH, Grödig, Austria). Data were statistically analyzed with XLfit Excel Add-In (ID Business Solutions Limited, Guildford, UK).

**Assessment of Pgp/MDR1 Functional Activity by Flow Cytometry.** Measurement of Pgp functional activity was performed essentially as described previously (Toornvliet et al., 2006). The assay was validated by using the Pgp-overexpressing human cell line CEM/VLB. In brief, cells were incubated in a total volume of 500 μl at a cell density of 3 × 10^6 cells/ml for 60 min in a 37°C water bath in the presence of a fluorescent chromophore and Pgp substrate Syto16 (10 nM) in the absence or presence of the specific Pgp transport inhibitor P121 (final concentration: 7.5 μM). After incubation, cells were washed twice with ice-cold phosphate-buffered saline supplemented with 0.1% bovine serum albumin and kept on ice protected from light for 30 min. Flow cytometric analysis was performed using a FACS Scan (BD Biosciences, San Jose, CA) with a 530-nm (FL1) laser. CellQuest software (BD Biosciences) was used for data acquisition and analysis. Pgp transporter activity was expressed as activity index described by the following formula: mean fluorescence level in the presence of antagonist nonloaded cells/mean fluorescence level in the absence of antagonist nonloaded cells.

An index of ≥1.10 (i.e., 10% above background, set to 1) is representative for functional Pgp activity.

**Statistical Analysis.** For comparison between groups a two-sided paired Student’s t test was used. Differences were considered to be significant at p < 0.05.

**Results**

**Bortezomib-Resistant THP1 Cells with Acquired Mutations in PSMB5 Display Cross-Resistance to Epoxyketone-Based Proteasome Inhibitors.** Human THP1 cells with acquired resistance to BTZ cells due to a mutation residing in
a highly conserved BTZ-binding pocket of the PSMB5 protein (Oerlemans et al., 2008) were evaluated for cross-resistance to epoxyketone-based PIs. Two BTZ-resistant THP1 selectants were studied. One subline (THP1/BTZ50) isolated after stepwise selection up to 50 nM BTZ that had a single Ala49Thr mutation in the PSMB5 protein exhibited 54-fold resistance to BTZ. The other subline (THP1/BTZ500) that was established by gradual selection up to 500 nM BTZ harbored two PSMB5 mutations (Ala49Thr and Met45Ile) and was 325-fold BTZ-resistant (Tables 1 and 2). Both THP1/ BTZ50 and THP1/BTZ500 cells displayed marked cross-resistance to the epoxyketone-based PIs ONX0912 (39- and 62-fold, respectively) and ONX0914 (27- and 97-fold, respectively). Cross-resistance levels to carfilzomib were moderate in THP1/BTZ50 cells (9-fold), elevated (32-fold) in THP1/ BTZ500 cells, but still 10-fold less than for BTZ (Table 1). Part of the cross-resistance may be associated with a 2- to 3-fold increase in constitutive proteasome subunit β5, β2, and β1 expression (Table 2). Together, these results suggest that mutations in PSMB5 induced by BTZ lead to drug resistance to different classes of PIs that bind both the β5 and the β5i subunit of the proteasome (Muchamuel et al., 2009; Zhou et al., 2009; Chauhan et al., 2010).

### Role of Drug Efflux Transporter (Pgp/MDR1) in Resistance to Epoxyketone-Based PIs

Previous evidence indicated that epoxomicin, a prototypical epoxyketone-based PI, is a substrate for the multidrug efflux transporter Pgp/MDR1/ABCB1 (Gutman et al., 2009). We explored whether or not carfilzomib, ONX0912, and ONX0914 could also serve as substrates for Pgp/MDR1/ABCB1 and other drug efflux transporters of the ABC superfamily including MRPI–5/ ABCC1–5 and BCRP/ABCG2. To this end we examined the growth inhibitory effects of BTZ, carfilzomib, ONX0912, and ONX0914 in a panel of human cell lines differentially overexpressing these MDR efflux pumps (Table 3). The basal growth inhibitory potency was tested in a panel of four parental cell lines (CEM, 2008, HEK293, and MCF7) in which the greatest sensitivity was displayed for BTZ and carfilzomib, followed by ONX0912 and the immunoproteasome inhibitor ONX0914. For BTZ only human CEM T cell leukemia cells (CEM/VLB) overexpressing F gp displayed a modest level (4.5-fold) of cross-resistance. In contrast, CEM/VLB cells exhibited marked levels of resistance to ONX0914 (162-fold), carfilzomib (114-fold), and ONX0912 (23-fold) compared with parental cells. It is noteworthy that no appreciable level of resistance to BTZ, carfilzomib, ONX0912, and ONX0914 was noted for cell lines overexpressing MRPI–5 (Table 3). It is noteworthy that MCF7/MR cells with BCRP/ ABCG2 overexpression displayed ~3-fold greater sensitivity to carfilzomib, ONX0912, and ONX0914 than their parental MCF7 cells. However, this effect was not related to BCRP overexpression because Ko143, a potent transport inhibitor

### Table 1

**Effect of PSMB5 mutations on growth inhibitory effects of bortezomib and epoxyketone-based proteasome inhibitors**

Data presented are the mean of three to six separate experiments ± S.D. after 72-h drug exposure. Values in parentheses depict resistance factor [ratio IC50 selected cell line over parental (WT) cell line].

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PSMB5 Mutation, Amino Acid Shift</th>
<th>Bortezomib IC50</th>
<th>Carfilzomib IC50</th>
<th>ONX0912 IC50</th>
<th>ONX0914 IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP1/WT</td>
<td>None</td>
<td>3.5 ± 0.7 (1)</td>
<td>16.4 ± 3.9 (1)</td>
<td>69 ± 9 (1)</td>
<td>99 ± 11 (1)</td>
</tr>
<tr>
<td>THP1/BTZ50</td>
<td>Ala49Thr</td>
<td>218 ± 39 (54)</td>
<td>160 ± 4 (9)</td>
<td>2686 ± 318 (39)</td>
<td>2741 ± 428 (27)</td>
</tr>
<tr>
<td>THP1/BTZ500</td>
<td>Ala49Thr and Met45Ile</td>
<td>823 ± 65 (235)</td>
<td>501 ± 69 (32)</td>
<td>4395 ± 458 (62)</td>
<td>10,092 ± 1642 (97)</td>
</tr>
</tbody>
</table>

**Table 2**

**Effect of PSMB5 mutations on proteasome subunit composition**

Data presented are the mean of three separate experiments ± S.D.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>β5</th>
<th>LMP7</th>
<th>β2</th>
<th>MECL1</th>
<th>β1</th>
<th>LMP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP1/WT</td>
<td>2.5 ± 1.3</td>
<td>5.7 ± 1.8</td>
<td>5.7 ± 0.7</td>
<td>3.1 ± 0.7</td>
<td>5.6 ± 0.7</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>THP1/BTZ50</td>
<td>7.3 ± 2.4</td>
<td>4.9 ± 2.9</td>
<td>10.6 ± 1.9</td>
<td>2.9 ± 1.4</td>
<td>10.4 ± 1.6</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>THP1/BTZ500</td>
<td>5.7 ± 1.9</td>
<td>3.5 ± 1.7</td>
<td>11.1 ± 3.8</td>
<td>2.6 ± 1.7</td>
<td>11.7 ± 1.8</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 3**

**Effect of MDR drug efflux transporter expression on growth inhibitory effect of bortezomib and epoxyketone-based proteasome inhibitors**

Data presented are the mean of three to six separate experiments ± S.D. Values in parentheses depict the resistance factor [ratio IC50 values of selected cell line over parental (WT) cell line].

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MDR Phenotype</th>
<th>Reference</th>
<th>Bortezomib IC50</th>
<th>Carfilzomib IC50</th>
<th>ONX0912 IC50</th>
<th>ONX0914 IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM/WT</td>
<td>None</td>
<td></td>
<td>1.6 ± 0.7 (1)</td>
<td>0.84 ± 0.20 (1)</td>
<td>14.8 ± 1.1 (1)</td>
<td>46.6 ± 7.9 (1)</td>
</tr>
<tr>
<td>2008/WT</td>
<td>None</td>
<td></td>
<td>9.2 ± 1.6 (1)</td>
<td>10.6 ± 6.2 (1)</td>
<td>40.0 ± 6.9 (1)</td>
<td>377 ± 206 (1)</td>
</tr>
<tr>
<td>HEK293/WT</td>
<td>None</td>
<td></td>
<td>5.1 ± 1.8 (1)</td>
<td>24.9 ± 5.5 (1)</td>
<td>38.3 ± 6.7 (1)</td>
<td>330 ± 26 (1)</td>
</tr>
<tr>
<td>MCF7/WT</td>
<td>None</td>
<td></td>
<td>6.2 ± 1.3 (1)</td>
<td>5.2 ± 2.2 (1)</td>
<td>37 ± 10 (1)</td>
<td>156 ± 15 (1)</td>
</tr>
<tr>
<td>CEM/VLB</td>
<td>Pgp/MDR1 (+ + +)</td>
<td>Zamora et al. 1988</td>
<td>7.2 ± 1.9 (4.5)</td>
<td>102 ± 35 (114)</td>
<td>366 ± 43 (23)</td>
<td>6790 ± 1310 (162)</td>
</tr>
<tr>
<td>CEM/CHQ</td>
<td>MRPI1 (+)</td>
<td>Oerlemans et al., 2006</td>
<td>3.3 ± 0.4 (2.1)</td>
<td>1.9 ± 0.3 (2.3)</td>
<td>27.0 ± 2.0 (1.9)</td>
<td>83 ± 11 (1.9)</td>
</tr>
<tr>
<td>2008/MRPI</td>
<td>MRPI1 (+ + +)</td>
<td>Scheffer et al., 2000</td>
<td>8.3 ± 3.7 (0.9)</td>
<td>12.3 ± 4.5 (1.3)</td>
<td>45.7 ± 11.6 (1.1)</td>
<td>372 ± 70 (1.1)</td>
</tr>
<tr>
<td>2008/MRPI</td>
<td>MRPI2 (+ + +)</td>
<td>Scheffer et al., 2000</td>
<td>8.5 ± 3.5 (0.9)</td>
<td>10.8 ± 5.9 (1.0)</td>
<td>45.0 ± 7.9 (1.1)</td>
<td>290 ± 47 (0.9)</td>
</tr>
<tr>
<td>2008/MRPI</td>
<td>MRPI3 (+ + +)</td>
<td>Scheffer et al., 2000</td>
<td>7.6 ± 2.9 (0.9)</td>
<td>9.9 ± 4.2 (1.0)</td>
<td>40.3 ± 3.1 (1.0)</td>
<td>320 ± 44 (1.1)</td>
</tr>
<tr>
<td>HEK293/MRPI</td>
<td>MRPI4 (+ + +)</td>
<td>Wielinga et al., 2002</td>
<td>5.1 ± 1.7 (1.0)</td>
<td>34.3 ± 9.7 (1.3)</td>
<td>38.3 ± 5.0 (1.0)</td>
<td>321 ± 69 (1.0)</td>
</tr>
<tr>
<td>HEK293/MRPI</td>
<td>MRPI5 (+ + +)</td>
<td>Wielinga et al., 2002</td>
<td>3.1 ± 1.4 (0.7)</td>
<td>14.1 ± 4.3 (0.7)</td>
<td>19.5 ± 3.1 (0.6)</td>
<td>211 ± 75 (0.7)</td>
</tr>
<tr>
<td>CEM/SSZ</td>
<td>BCRP (+)</td>
<td>van der Heijden et al., 2004</td>
<td>1.7 ± 0.2 (1.1)</td>
<td>0.69 ± 0.14 (0.9)</td>
<td>8.0 ± 1.1 (0.6)</td>
<td>49.0 ± 7.6 (1.1)</td>
</tr>
<tr>
<td>MCF7/MR</td>
<td>BCRP (+ + +)</td>
<td>Taylor 1991</td>
<td>2.3 ± 1.0 (0.4)</td>
<td>1.5 ± 0.8 (0.3)</td>
<td>8.9 ± 2.5 (0.3)</td>
<td>60 ± 19 (0.4)</td>
</tr>
</tbody>
</table>
of BCRP activity, failed to alter this increased sensitivity (results not shown). These results demonstrate that Pgp, but not MRP1–5 or BCRP, may contribute to drug efflux and consequent resistance to epoxyketone-based PIs and BTZ.

**P-Glycoprotein Blockade Overcomes Resistance to Epoxyketone-Based PIs by Retention of Inhibition of Proteasome Activity.** To confirm the potential role of Pgp in conferring resistance to BTZ, carfilzomib, ONX0912, and ONX0914, we examined the growth inhibitory effects of these PIs after blockade of Pgp efflux activity with the specific inhibitor P121/reversin. Indeed, blocking Pgp activity in CEM/VLB cells nearly fully restored parental CEM cell sensitivity levels to PIs (Fig. 2).

We next assessed whether or not drug extrusion by Pgp would also diminish the ability of the proteasome inhibitors to inhibit chymotrypsin-like proteasome activity. As expected, markedly higher concentrations of carfilzomib (22.3-fold), ONX0912 (11.0-fold), ONX0914 (26.3-fold), and BTZ (1.4-fold) were required for 50% inhibition of intracellular chymotrypsin-like proteasome activity in CEM/VLB cells (Table 4). Furthermore, blocking Pgp function by P121 restored the capacity of carfilzomib, ONX0912, ONX0914, and BTZ to inhibit chymotrypsin-like proteasome activity at inhibitory concentrations obtained with parental CEM cells. Together, these data underscore a role for Pgp, in both attenuating the potency of proteasome inhibition and conferring resistance to carfilzomib, ONX0912, and ONX0914. BTZ function, in comparison to the epoxyketone-based PIs, is the least dependent on Pgp. Nevertheless, this effect could be readily counteracted by blocking Pgp function.

**Pgp Activity in PBMCs from Controls and Patients with RA Modestly Contributes to Diminished Proteasome Inhibitory Capacity of Epoxyketone-Based PIs.** Because high levels of Pgp overexpression underlie the marked resistance to epoxyketone-based PIs in CEM/VLB cells, we assessed whether or not basal physiological levels of Pgp activity in PBMCs would also compromise the efficacy of these PIs in comparison to BTZ. To this end, we first determined Pgp efflux activity in parental CEM/WT versus CEM/VLB cells and compared it with Pgp activity in PBMCs from healthy controls and patients with RA. CEM/VLB cells displayed a Pgp activity index of 17.3 ± 6.2, which is >80-fold higher than CEM/WT cells (activity index: 1.18 ± 0.13), which is just above a functionally detectable activity index of 1.10 ± 0.1. Functional Pgp activity in PBMCs from patients with RA and controls was slightly higher (activity index: 1.55 ± 0.36 and 2.08 ± 0.69) than in parental CEM/WT cells, but was still >30-fold lower than in Pgp-overexpressing CEM/VLB cells (Fig. 3). We next determined the effect of Pgp blockade by P121 on the efficiency of carfilzomib, ONX0912, ONX0914, and BTZ to inhibit chymotrypsin-like activity in PBMCs. Both carfilzomib- and ONX0914-induced inhibition of chymotrypsin-like activity was significantly enhanced (2- and 1.3-fold, respectively; p < 0.05) after blocking Pgp efflux activity in PBMCs from patients with RA (Fig. 4A). Similar findings were observed in the control PBMCs (Fig. 4A) where the Pgp function blockage only seemed to affect inhibitory capacity of carfilzomib and ONX0914 (1.9- and 1.4-fold, respectively). In contrast, in both RA and control PBMCs, no significant changes were observed in the inhibitory potential of ONX0912 and BTZ after Pgp blockade. It is noteworthy that the concentration of carfilzomib, ONX0912, ONX0914, and BTZ needed for 50% inhibition of the chymotrypsin-like proteasome activity in RA PBMCs was higher compared with control PBMCs. This effect seems to be caused by a higher chymotrypsin-like activity found in RA PBMCs compared with healthy controls (Fig. 4B).

These results suggest that further enhancement of the inhibition of chymotrypsin-like activity by carfilzomib and ONX0914 may be achieved by abolishing the Pgp-mediated cellular efflux of these PIs.

**Potency of Carfilzomib, ONX912, ONX914, and Bortezomib to Inhibit Chymotrypsin-Like Proteasome Activity in PBMCs from Controls and Patients with RA.** To further explore the mechanism by which the epoxyketone-based PIs function in PBMCs we examined whether or not, least dependent on Pgp. Nevertheless, this effect could be readily counteracted by blocking Pgp function.
apart from chymotrypsin-like activity, these PIs could also inhibit the caspase-like and trypsin-like proteasome activities in PBMCs from healthy controls and patients with RA. It is noteworthy that chymotrypsin-like activity was preferentially inhibited by all three epoxyketone-based PIs as well as by BTZ (Fig. 5). In a small series of PBMCs from healthy controls ($n=2$) and patients with RA ($n=4$), we noted that the relative potency (referring to drug concentrations required for 50% proteasome activity inhibition) to inhibit caspase-like and trypsin-like activity relative to chymotrypsin-like activity (set to 1) was 0.17 and 0.06 for bortezomib, 0.1 and 0.3 for carfilzomib, 0.02 and 0.06 for ONX0912, and 0.02 and 0.09 for ONX0914, respectively (data not shown).

**Discussion**

Here, we demonstrate that bortezomib shows a different resistance profile than the epoxyketone-based PIs carfilzomib, ONX0912, and ONX0914. An established resistance mechanism to bortezomib caused by mutations in the $PSMB5$ gene encoding the $\beta_5$ subunit of the proteasome results in cross-resistance to the novel compounds, albeit at a lower level. However, the novel PIs display resistance caused by drug efflux via Pgp in a human CEM cell line (CEM/VLB) with high Pgp overexpression. In PBMCs from healthy controls and patients with RA with basal levels of Pgp, blocking Pgp drug efflux enhanced proteasome inhibition.

Notwithstanding the established therapeutic activity of BTZ (Velcade) as the first approved PI, emergence of drug resistance phenomena and the side effects of BTZ, such as neuropathy, called for the design and (pre)clinical evaluation of second-generation PIs that can overcome these drawbacks (Dick and Fleming, 2010). Epoxyketone-based PIs such as carfilzomib, ONX0912, and ONX0914 differ from BTZ in their mode of action by the fact that they bind irreversibly to active-site amino acid Thr1 within the binding pocket of the $\beta_5$ subunit of the proteasome. In contrast, BTZ binds reversibly to this active site (Groll et al., 2006; Demo et al., 2007). ONX0914 was designed as a more selective inhibitor of the immunoproteasome over the constitutive proteasome (Muchamuel et al., 2009). We (Oerlemans et al., 2008) and others have shown previously that acquired BTZ resistance led to single amino acid substitutions in the highly conserved sub-
strate- and BTZ-binding pocket of the β5 subunit (Lü et al., 2009; Ri et al., 2010). These mutations in the PSMB5 gene conferred resistance to the parent drug BTZ as well as cross-resistance to other aldehyde-based PIs including ALLN, MG132, MG262, and a hexapeptide 4A6 (Oerlemans et al., 2008). Therefore we investigated whether the epoxyketone-based PIs such as carfilzomib, ONX0912, and ONX0914 confer cross-resistance in BTZ-resistant cell lines with PSMB5 mutations: THP1/BTZ 50 cells (Ala49Thr) and the highly BTZ-resistant THP1/BTZ500 cells (Ala49Thr and Met45Ile mutation). It is noteworthy that marked cross-resistance was observed for these drugs in cell lines harboring β5-subunit mutations, although to a lesser extent, thus suggesting that these acquired mutations confer on cells reduced binding capacity for a broad spectrum of PIs. It should be mentioned, however, that to date, β5-subunit mutations have not been identified in clinical specimens that are refractory to BTZ treatment; hence, the clinical relevance of the in vitro PSMB5 mutation data in multiple BTZ-resistant cell line models (Lü et al., 2009; Li et al., 2010; Ri et al., 2010) is still not clear.

Inherent or acquired drug-induced overexpression of ABC drug efflux transporters is a common resistance modality that can result in the multidrug resistance phenotype (Gillet et al., 2007). Pgp/MDR1 is the best-known family member of MDR efflux transporters facilitating not only cellular extrusion of neutral/hydrophobic small-molecule drugs but also several types of peptides (cyclic/linear and neutral/hydrophobic), including leupeptin, pepstatin A, dolastatin 10, and PIs such as the tripeptide N-acetyl-leucyl-leucyl-norleucinal and expoxomicin (Sharma et al., 1992; Eytan et al., 1994; Borgnia et al., 1996; Gutman et al., 2009). Therefore it may not seem surprising that peptide epoxyketone-based PIs may be recognized by Pgp as transport substrates. This was established by several lines of evidence: 1) marked cross-resistance of Pgp-overexpressing cells to these PIs, 2) requirement for higher proteasome inhibitor concentrations in Pgp-overexpressing cells compared with parental cells to inhibit intracellular chymotrypsin-like proteasome activity, and 3) full reversal of both cross-resistance and chymotrypsin-like proteasome inhibition after Pgp blockade with the established Pgp transport inhibitor P121. BTZ, a boron-containing peptide, proved to be a poor Pgp substrate, consistent with previous studies (Minderman et al., 2007; Oerlemans et al., 2008).

The notion that the orally active proteasome inhibitor ONX0912 was also recognized as a Pgp transport substrate, albeit less prominently than carfilzomib and ONX0914, may be of relevance for its bioavailability, which was found to be 39% in rodents and dogs (Zhou et al., 2009), given the fact that intestinal Pgp is a major determinant of drug efflux and absorption (van de Ven et al., 2009). Hence, evading the Pgp-dependent mucosal epithelium barrier may further enhance ONX0912 bioavailability. The present study also provided evidence that carfilzomib, ONX0912, and ONX0914 were not substrates for other multidrug efflux transporters including MRP1–5 and BCRP. It is noteworthy that BCRP-overexpressing MCF7/MR cells even displayed 3- to 4-fold greater sensitivity for BTZ, carfilzomib, ONX0912, and ONX0914. This hypersensitivity is probably caused by an intrinsic property of MCF7/MR cells acquired after the establishment of resistance to the topoisomerase inhibitor mitoxantrone. Ogiso et al. (2002) reported that under these conditions increased accumulation of the proteasome occurs at the nucleus with concomitantly decreased proteasome levels in the cytoplasm. This might have a more prominent inhibition of cytoplasmic proteasome activity, resulting in hypersensitivity to PIs.

To explore the potential in vivo impact of Pgp in drug resistance to peptide epoxyketone-based PIs, we assessed the efficiency of proteasome inhibition in PBMCs isolated from patients with RA and healthy controls. We (van der Heijden et al., 2009) and others (Agarwal et al., 2009) have reported that Pgp expression on lymphocytes from patients with RA correlated with disease activity. Here, we showed that the low basal Pgp activity in ex vivo isolated PBMCs partially
Reduced the potency (i.e., nanomolar) inhibition of the chymotrypsin-like proteasome activity by carfilzomib, ONX0912, ONX0914, and BTZ. However, for carfilzomib and ONX0914, the two PIs that displayed the highest resistance-levels in Pgp-overexpressing CEM cells, up to 2-fold further enhancement of chymotrypsin-like proteasome activity inhibition could be achieved by blocking Pgp-mediated drug efflux, thereby underscoring the role of Pgp in resistance to these PIs. It is of interest to note that the ranking of potency of chymotrypsin-like proteasome inhibition in PBMCs from patients with RA and healthy controls (carfilzomib > ONX0914 > ONX0912) is different from their ranking for growth inhibitory potency (carfilzomib > ONX0912 > ONX0914) observed for four different parental cell lines (CEM, HEK293, A2008, and MCF7 cells). This result may be caused by higher immunoproteasome content in ex vivo isolated PBMCs than in cell line cultures in vitro. As such, cell line models may underestimate the full potential of immunoproteasome-targeted drugs such as ONX0914, which may be more prominently displayed in immunocompetent cells in vivo.

In conclusion, single amino acid substitutions in the substrate/inhibitor binding site of the proteasome β5 subunit as well as overexpression of the drug efflux transporter Pgp were identified as modalities conferring resistance to the peptide epoxyketone-based (immuno)proteasome inhibitors carfilzomib, ONX0912, and ONX0914. However, targeting of immune cells harboring basal levels of Pgp did not show cross-resistance to ONX0912, whereas carfilzomib was affected only to a small extent. Hence, identifying the structural elements within peptide epoxyketone-based chemical structures that render them Pgp substrates may be an important strategy for evading Pgp-mediated drug efflux and overcoming drug resistance. In fact, we have shown that small chemical modifications can generate analogs of a class of topoisomerase drug inhibitors, i.e., imidazocarboxidiones, which were no longer substrates for the drug efflux transporter BCRP (Bräm et al., 2009). Indeed, Zhou et al. (2009) showed that modifications in the N-cap of the peptide epoxyketone backbone, in particular introducing (5-Me)-3-isoxazole or 2-(S)-tetrahydrofuran, retained chymotrypsin-like proteasome activity while Pgp substrate activity was abolished. Such considerations in future drug design may be crucial for the rational overcoming of established modalities of MDR.

Taken together, our data provide evidence that cellular extrusion via the drug efflux transporter ABCB1 (Pgp/MDR1), but not by other ABC transporters, can facilitate resistance to peptide epoxyketone-based proteasome inhibitors in Pgp-overexpressing model systems. In the PBMCs of controls and patients with RA, the presence of basal Pgp activity only modestly influenced the proteasome inhibitory potential by the epoxyketone-based drugs. Blocking of Pgp activity could further potentiate their activity, although caution for increased toxicity may also need to be exercised when these combinations are used in the clinic.

Authorship Contributions

Participated in research design: Verbrugge, Assaraf, de Grujil, and Jansen. Conducted experiments: Verbrugge, Al, Oerlemans, Chan, and Jansen.

Contributed new reagents or analytic tools: Scheffer, den Uyl, Oerlemans, Chan, Kirk, Peters, van der Heijden, and Jansen. Performed data analysis: Verbrugge, Chan, and Jansen.

Wrote or contributed to the writing of the manuscript: Verbrugge, Assaraf, Dijkmans, Scheffer, Kirk, Peters, de Grujil, Schepers, and Jansen.

References


Ri M, Iida S, Nakashima T, Miyazaki H, Mori F, Ito A, Inagaki A, Kusumoto S, Ishida T, Komasu H, et al. (2010) Bortezomib-resistant myeloma cell lines: a role for increased toxicity may also need to be exercised when these combinations are used in the clinic.
for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress. Leukemia 24:1506–1512.


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