Inactivating PSMB5 mutations and P-glycoprotein (MDR1/ABCB1) mediate resistance to proteasome inhibitors: ex vivo efficacy of (immuno) proteasome inhibitors in mononuclear blood cells from rheumatoid arthritis patients


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**Abstract**

Bortezomib (BTZ), a registered proteasome inhibitor (PI) for multiple myeloma, has also been proposed as a potential anti-rheumatic 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, ONX 0912 and ONX 0914, might escape two established BTZ-resistance mechanisms: (a) mutation(s) in the proteasome β5 subunit (PSMB5) targeted by these PIs, and (b) drug efflux mediated by ABC transporters. THP1 myeloid sublines with acquired resistance to BTZ (54-235 fold) due to mutations in PSMB5, displayed marked cross-resistance but less pronounced to carfilzomib (9-32 fold), ONX 0912 (39-62 fold) and ONX 0914 (27-97 fold). As for ABC transporter mediated efflux: lymphoid CEM/VLB cells with P-glycoprotein (Pgp/MDR1) overexpression exhibited substantial resistance to carfilzomib (114-fold), ONX 0912 (23-fold), ONX 0914 (162-fold) whereas less resistance to BTZ (4.5-fold) was observed. Consistently, the β5 subunit-associated chymotrypsin-like proteasome activity was significantly less inhibited in these CEM/ VLB cells. Ex vivo analysis of peripheral blood mononuclear cells (PBMCs) from therapy naive rheumatoid arthritis patients revealed that, although basal Pgp levels were low, it compromised the inhibitory effect of carfilzomib and ONX 0914. However, the use of P121, a Pgp transport inhibitor, restored parental cell inhibitory levels both in CEM/VLB cells as well as in PBMCs. 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 (UPS) 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. NF-κB) and induction of apoptosis. As such, the UPS has been recognized as an attractive target for cancer therapeutic intervention by proteasome inhibitors.
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). Upon stimulation by pro-inflammatory stimuli, e.g. interferon-γ or TNFα, 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 MHC-I, or, as indicated recently to preserve protein homeostasis after interferon-γ-induced oxidative stress. Bortezomib (BTZ), a boron-containing dipeptide which primarily targets the β5 subunit of the proteasome, was the first clinically approved proteasome inhibitor for the treatment of therapy-refractory multiple myeloma. Emergence of resistance to this drug, as well as side effects like peripheral neuropathy, initiated the search for novel generation 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 or the immunoproteasome.

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. In fact, both BTZ and second generation PIs displayed clear therapeutic efficacy in animal models of rheumatoid arthritis or lupus-like disease. These effects are in part mediated by their ability to suppress the production of NF-κB-inducible proinflammatory cytokines from immune competent cells. Whenever it comes to clinical application of PIs for the treatment of autoimmune diseases, the issue of retaining long term efficacy is particularly important as this type of patients 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 prototypical epoxyketone-based proteasome inhibitor like epoxomicin, it has been reported that cellular extrusion via the ABC transporter ABCB1 (Pgp/MDR1) could confer drug resistance. Whether or not cellular extrusion by ABC pumps other than ABCB1 (Pgp/MDR1) can also contribute to these second generation epoxyketone-based PIs is yet 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 may interfere with the efficacy of 3 second generation epoxyketone-based PIs i.e. carfilzomib (formerly named PR-171), ONX 0912 (formerly named PR-047), and the immunoproteasome inhibitor ONX 0914 (formerly named PR-957).\(^8\)–\(^{11,18,19}\) Our data provide evidence that only 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 (PMBCs) from healthy controls and rheumatoid arthritis patients, 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, ONX 0912, ONX 0914 were provided by Onyx Pharmaceuticals, Inc. (South San Francisco, USA). Bortezomib (BTZ)/Velcade was kindly provided by Millennium Pharmaceuticals (Cambridge, USA). The chemical structures of these compounds are depicted in Figure 1. The P-glycoprotein inhibitor Reversin-121 (P121) was obtained from Alexis Benelux, The Netherlands. 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.\(^6\) Multiple human cell lines were selected based on their known expression of ABC transporters; P-glycoprotein/MDR1 (CEM/VLB),\(^{20}\) Multidrug Resistance-associated Protein 1 (CEM/CHQ, 2008/MRP1),\(^{21,22}\) MRP2 (2008/MRP2),\(^{22}\) MRP3 (2008/MRP3),\(^{22}\) MRP4 (HEK293/MRP4),\(^{23}\) MRP5 (HEK293/MRP5)\(^{23}\) and BCRP (CEM/SSZ & MCF7/MR).\(^{24,25}\)
Cell cultures were maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 1000U/mL penicillin/streptomycin. Cells were routinely cultured at an initial density of $3 \times 10^5$ cells/mL (for suspension cells) and $5 \times 10^3$ cells/cm² for adherent cells, all in 25-cm² culture flasks (Greiner Bio-One, Frickenhansen, 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 RA patients. Samples for this study were collected within the COBRA-light study, a randomized trial comparing two combination schedules in early rheumatoid arthritis. Informed consent 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 prior to ini-
tiation of the therapy protocol with methotrexate and prednisolone. Patients eligible to be included in this study fulfilled the following criteria: RA diagnosis according to the ACR 1987 criteria, disease duration > two years, age > 18 years, active RA with > 6 swollen joints, > 6 painful joints and an erythrocyte sedimentation rate of > 28 mm of VAS (global health) > 2. PBMCs were isolated by Ficoll-Paque (Amersham Biosciences, Amersham, UK) density gradient centrifugation (15 minutes at 1000 x g at room temperature) according to manufacturer’s instructions. The interphase was collected and washed three times with phosphate-buffered saline (PBS) supplemented with 1% BSA. PBMCs were then counted and resuspended in RPMI-1640 culture medium supplemented with 10% FCS.

**Assessment of cell growth inhibition by proteasome inhibitors**

The growth inhibitory effects of BTZ, carfilzomib, ONX 0912, ONX 0914 were analyzed by methods essentially as previously described. Briefly, for suspension cell cultures, 1.25 x 10^5 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 x 10^4/cm² was used and drugs were added 24 hours after plating. Inhibition of cell growth was determined after 72 hours 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 100-200 fold concentration range. The proteasome inhibitor concentration required to inhibit cell growth by 50% compared with control growth is defined as the IC_{50} concentration.

**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 3 types of proteasome activities; chymotrypsin-, caspase-, and trypsin-like activity was performed using Proteasome-Glo™ assay kit (Promega, Madison, WI, USA) according to manufacturer’s instructions. Prior to determination of proteasome activity, cells were incubated with the proteasome inhibitors for 1 hour at 37°C in a flat, white-bottomed 96-well plate (Thermo Scientific, NUNC, 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 determined with an Infinite 200 pro microplate reader (Tecan, Giessen, The Netherlands). Negative controls consisted of wells with medium without cells. The potency of proteasome inhibitors is indicated by the drug concentration that elicits 50% inhibition of luminescence signal.

**Proteasome subunit determination with the ProCISE assay**

The ProCISE (proteasome constitutive/immunoproteasome subunit enzyme linked generated and immunosorbent) assay was performed on untreated cell lysates as was previously described.²⁷ Luminescence was measured using a GENios-Basic plate reader (Tecan Austria, GmBH). Data was statistically analysed with XLfit Excel Add-In (ID Business Solutions Limited).

**Assessment of Pgp/MDR1 functional activity by flow cytometry**

Measurement of Pgp functional activity was performed essentially as described previously by Toornvliet et al.²⁸ The assay was validated 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 x 10⁵ cells/mL for 60 minutes 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 PBS supplemented with 0.1% BSA and kept on ice protected from light for 30 min. Flow cytometric analysis was performed using a FACS Scan (Becton Dickinson) with a 530nm (FL1) laser. CellQuest software (Becton Dickinson) was used for data acquisition and analysis. Pgp transporter activity was expressed as Activity Index (AI) described by the following formula:

\[
\text{Mean fluorescence level in the presence of antagonist non loaded cells} - \text{Mean fluorescence level in the absence of antagonist non loaded cells}
\]
An index of $\geq 1.10$ (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 when $p < 0.05$.

**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 $\beta 5$ subunit of the proteasome (PSMB5) protein 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 235-fold BTZ resistant (Table 1). Both THP1/BTZ50 and THP1/BTZ500 cells displayed marked cross-resistance to the epoxyketone-based PIs ONX 0912 (39-fold and 62-fold, respectively) and ONX 0914 (27-fold and 97-fold, respectively). Cross-resistance levels to carfilzomib were moderate in THP1/BTZ50 cells (9-fold), but elevated (32-fold) in THP1/BTZ500 cells, but still 10-fold less than for BTZ (Table 1A). Part of the cross-resistance may be associated with a 2-3 fold increase in constitutive proteasome subunits $\beta 5$, $\beta 2$ and $\beta 1$ expression (Table 1B). Together, these results suggest that mutations in PSMB5 induced by BTZ lead to drug resistance to different classes of PIs which bind both the $\beta 5$ and the $\beta 5i$ subunit of the proteasome.$^9$–$^{11}$
**Table 1A.** Effect of PSMB5 mutations on growth inhibitory effects of Bortezomib and epoxyketone-based proteasome inhibitors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PSMB5 mutation (amino acid shift)</th>
<th>Bortezomib IC₅₀ (nM)</th>
<th>Carfilzomib IC₅₀ (nM)</th>
<th>ONX 0912 IC₅₀ (nM)</th>
<th>ONX 0914 IC₅₀ (nM)</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 &amp; Met45lle</td>
<td>823 ± 65 (235)</td>
<td>501 ± 69 (32)</td>
<td>4395 ± 458 (62)</td>
<td>10092 ± 1642 (97)</td>
</tr>
</tbody>
</table>

Data presented are the mean of 3-6 separate experiments ± SD after 72 hours drug exposure. Values between brackets depict Resistance Factor (ratio IC₅₀ selected cell line over parental (WT) cell line).

**Table 1B:** Effect of PSMB5 mutations on proteasome subunit composition

<table>
<thead>
<tr>
<th>Cell line</th>
<th>β5</th>
<th>β5i (LMP7)</th>
<th>β2</th>
<th>β2i (MECL1)</th>
<th>β1</th>
<th>β1i (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>

Data are presented as ng subunit/µg total protein and are the mean of 3 separate experiments ± SD.
**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 P-glycoprotein (Pgp)/MDR1/ABCB1.\textsuperscript{17} We explored whether or not carfilzomib, ONX 0912 and ONX 0914 could also serve as substrates for Pgp/MDR1/ABCB1 and other drug efflux transporters of the ABC-superfamily including multidrug resistance-associated protein 1-5 (MRP1-5/ABCC1-5) and breast cancer resistance protein (BCRP/ABCG2). To this end we examined the growth inhibitory effects of BTZ, carfilzomib, ONX 0912 and ONX 0914 in a panel of human cell lines differentially overexpressing these MDR efflux pumps (Table 2). The basal growth inhibitory potency was tested in a panel of 4 parental cell lines (CEM, 2008, HEK293 and MCF7) in which the greatest sensitivity was displayed for BTZ and carfilzomib, followed by ONX 0912 and the immunoproteasome inhibitor ONX 0914. For BTZ, only human CEM T cell leukemia cells (CEM/VLB) overexpressing Pgp displayed a modest level (4.5 fold) of cross-resistance. In contrast, CEM/VLB cells exhibited marked levels of resistance to ONX 0914 (162-fold), carfilzomib (114-fold) and ONX 0912 (23-fold), compared to parental cells. Notably, no appreciable level of resistance to BTZ, carfilzomib, ONX 0912 and ONX 0914 was noted for cell lines overexpressing MRP1-5 (Table 2). Interestingly, MCF7/MR cells with BCRP/ABCG2 overexpression displayed ~3-fold greater sensitivity to carfilzomib, ONX 0912 and ONX 0914, than their parental MCF7 cells. However, this effect was not related to BCRP overexpression as Ko143, a potent transport inhibitor 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.
Table 2: Effect of Multidrug Resistance (MDR) drug efflux transporter expression on growth inhibitory effect of Bortezomib and exoxyketone-based proteasome inhibitors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MDR phenotype</th>
<th>Reference</th>
<th>Bortezomib</th>
<th>Carfilzomib</th>
<th>ONX 0912</th>
<th>ONX 0914</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC\textsubscript{50} (nM)</td>
<td>IC\textsubscript{50} (nM)</td>
<td>IC\textsubscript{50} (nM)</td>
<td>IC\textsubscript{50} (nM)</td>
</tr>
<tr>
<td>CEM/WT</td>
<td>none</td>
<td>Zamora et al 1986</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>2008/WT</td>
<td></td>
<td>Oerlemans 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>HEK293/WT</td>
<td></td>
<td>Scheffer 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>MCF7/WT</td>
<td>none</td>
<td>Scheffer 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>CEM/VBl</td>
<td>Pgp/MDR1 (+++)</td>
<td>Wielinga 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></td>
<td>MRP1 (+)</td>
<td>vd Heijden 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>

Data presented are the mean of 3-6 separate experiments ± SD. Values between brackets depict the Resistance Factor (ratio of IC\textsubscript{50} values of selected cell line over parental (WT) cell line). Abbreviations: Pgp: P-glycoprotein/MDR1, MRP: Multidrug Resistance-related Protein, BCRP: Breast Cancer Resistance Protein.
P-glycoprotein blockade overcomes resistance to epoxyketo- tone-based PIs by retention of inhibition of proteasome activity.

To confirm the potential role of Pgp in conferring resistance to BTZ, carfilzomib, ONX 0912 and ONX 0914, 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/VELB cells nearly fully restored parental CEM cell sensitivity levels to PIs (Figure 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), ONX 0912 (11.0-fold), ONX 0914 (26.3-fold) and BTZ (1.4-fold) were required for 50% inhibition of intracellular chymotrypsin-like proteasome activity in CEM/VELB cells (Table 3). Furthermore, blocking Pgp function by P121 restored the capacity of carfilzomib, ONX 0912, ONX 0914 and BTZ to inhibit chymotrypsin-like proteasome activity at inhibitory concen-
trations obtained with parental CEM cells. Together, these data underscore a role for Pgp, both in attenuating the potency of proteasome inhibition and conferring resistance to carfilzomib, ONX 0912 and ONX 0914. 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.

**Table 3:** Inhibition of chymotrypsin-like (ChT-L) proteasome activity in CEM/WT cells vs. Pgp/MDR1-overexpressing CEM/VLB cells in the absence or presence of the Pgp-blocker P121

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Bortezomib</th>
<th>Carfilzomib</th>
<th>ONX 0912</th>
<th>ONX 0914</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM)</td>
<td>Resistance Factor</td>
<td>IC$_{50}$ (nM)</td>
<td>Resistance Factor</td>
</tr>
<tr>
<td>CEM/WT</td>
<td>no blocker</td>
<td>13 ± 1</td>
<td>1.0</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>+ P121</td>
<td>13 ± 1</td>
<td>1.0</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>CEM/VLB</td>
<td>no blocker</td>
<td>19 ± 4</td>
<td>1.4</td>
<td>148 ± 34</td>
</tr>
<tr>
<td></td>
<td>+ P121</td>
<td>15 ± 1</td>
<td>1.4</td>
<td>29 ± 4</td>
</tr>
</tbody>
</table>

IC$_{50}$ values (in nM) depict drug concentrations establishing 50% inhibition of ChT-L activity after 1 hour drug exposure. Data presented are the mean of 3 separate experiments ± SD. Resistance Factor depicts the ratio of IC50 values of selected cell line over parental (WT) cell line.

Pgp activity in PBMCs from controls and RA patients modestly contributes to diminished proteasome inhibitory capacity of epoxyketone-based PIs.

Since 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 peripheral blood mononuclear cells (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 vs. CEM/VLB cells and compared it with Pgp activity in PBMCs cells from healthy controls and RA patients. 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 RA patients and controls were slightly higher (activity index: 1.55 ± 0.36 and 2.08 ± 0.69) than in parental CEM/WT cells, but were still >30-fold lower than in Pgp-overexpressing CEM/VLB cells (Figure 3). We next determined the effect of Pgp blockade by P121 on the efficiency of carfilzomib, ONX 0912, ONX 0914 and BTZ to inhibit chymotrypsin-like activity in PBMCs. Both carfilzomib- and ONX 0914-induced inhibition of chymotrypsin-like activity was significantly enhanced (2-fold and 1.3-fold, respectively, *p < 0.05) after blocking Pgp efflux activity in PBMCs from RA patients (Figure 4A). Similar findings were observed in the control PBMCs (Figure 4A) where the Pgp function

**Figure 3: Pgp functional activity in PBMCs from healthy controls and RA patients vs CEM/VLB cells.**

*Cells were incubated with the chromophore Syto16 for 1 hour in the absence or presence of 7.5 µM of the Pgp-blocking peptide P121. The Pgp activity index is presented as ratio of Syto16 retention in cells incubated with P121 over cells without P121. Results for PBMCs are means ± SD of 4-8 experiments.*
blockage only seemed to affect inhibitory capacity of carfilzomib and ONX 0914 (1.9-fold and 1.4-fold, respectively; ""p = 0.001; ""p = 0.007). In contrast, both in RA as in control PBMCs, no significant changes were observed in the inhibitory potential of ONX 0912 and BTZ after Pgp blockade. It is noteworthy that the concentration of carfilzomib, ONX 0912, ONX 0914 and BTZ needed for 50% inhibition of the chymotrypsin-like proteasome activity in RA PBMCs is higher compared to control PBMCs. This effect seems to be due to a higher chymotrypsin-like activity found in RA PBMCs as compared to healthy controls (Figure 4B). These results suggest that further enhancement of inhibition of chymotrypsin-like activity by carfilzomib and ONX 0914 may be achieved by abolishing the Pgp-mediated cellular efflux of these PIs.

**Potency of carfilzomib, ONX 0912, ONX 0914 and BTZ to inhibit chymotrypsin-like proteasome activity in PBMCs from controls and RA patients.**

To further explore the mechanism by which the epoxyketone-based PIs function in PBMCs we examined whether or not, apart from chymotrypsin-like activity, these PIs could also inhibit the caspase-like and trypsin-like proteasome activities in PBMCs from healthy controls and RA patients. Notably, chymotrypsin-like activity was preferentially inhibited by all 3 epoxyketone-based PIs as well as by BTZ (Figure 5). In a small series of PBMCs from healthy controls (n=2) and RA patients (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 BTZ; 0.1 and 0.3 for carfilzomib; 0.02 and 0.06 for ONX 0912, and 0.02 and 0.09 for ONX 0914, respectively (not shown).
Figure 4: Effect of Pgp blocking on inhibition of chymotrypsin-like (ChT-L) proteasome activity in PBMCs from RA patients. A: PBMCs from RA patients were incubated for 1 hour with various concentrations of carfilzomib, ONX 0912, ONX 0914 and BTZ in the absence or presence of the Pgp-blocking peptide P121 (7.5 µM) and then analyzed for inhibition of chymotrypsin-like proteasome activity. Results depict drug concentrations required to inhibit chymotrypsin-like proteasome activity for 50% (IC50). Note: at the indicated concentration, P121 had no effect on chymotrypsin-like proteasome activity (not shown).
Figure 5: Inhibitory effects of carfilzomib, ONX 0912, ONX 0914 and bortezomib on chymotrypsin-like, caspase-like and trypsin-like proteasome activity in PBMCs from healthy controls and RA patients. PBMCs from healthy controls and RA patients were incubated for 1 hour with various concentrations of carfilzomib, ONX 0912, ONX 0914 and BTZ and then analyzed for inhibition of chymotrypsin-like (ChT-L) proteasome activity. Results depict individual (•) and group mean (----) drug concentrations required to inhibit each of these proteasome activities by 50% (IC$_{50}$). indicates that IC$_{50}$ value was not reached at the highest drug concentration tested.
Here we demonstrate that bortezomib shows a different resistance profile than the epoxyketone-based PIs carfilzomib, ONX 0912 and ONX 0914. An established resistance mechanism to BTZ due to mutations in the PSMB5 gene encoding the β5 subunit of the proteasome results in cross-resistance to the novel compounds, albeit at a lower level. However, the novel PIs display resistance due to drug efflux via Pgp, in a human CEM cell line (CEM/VLB) with high Pgp overexpression. In PBMCs from healthy controls and RA patients 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 side effects of BTZ such as neuropathy, called for the design and (pre-)clinical evaluation of second generation PIs that can overcome these drawbacks. Epoxyketone-based PIs like carfilzomib, ONX 0912 and ONX 0914 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 β5-subunit of the proteasome. In contrast, BTZ binds reversibly to this active site. ONX 0914 was designed as a more selective inhibitor of the immunoproteasome over the constitutive proteasome. We and others have previously shown that acquired BTZ resistance led to single amino acid substitutions in the highly conserved substrate- and BTZ-binding pocket of the β5-subunit. These mutations in the PSMB5 gene conferred resistance to the parent drug BTZ as well as cross-resistance to other peptide aldehyde-based PIs including ALLN, MG-132, MG-262 and a hexapeptide 4A6. Therefore we investigated whether the epoxyketone-based PIs like carfilzomib, ONX 0912 and ONX 0914 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). Notably, 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 upon 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 yet in clinical specimens which are re-
fractory to BTZ treatment; hence, the clinical relevance of the in vitro PSMB5 mutation data in multiple BTZ-resistant cell line models is still not clear.\textsuperscript{31–33}

Inherent or acquired drug-induced overexpression of ABC drug efflux transporters is a common resistance modality that can result in the multidrug resistance phenotype.\textsuperscript{18} 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, as well as PIs such as the tripeptide N-acetyl-leucyl-leucyl-norleucinal and expoxomycin.\textsuperscript{17,34–36} Therefore it may not seem surprising that peptide epoxoyketone-based PIs may be recognized by Pgp as transport substrates. This was established by several lines of evidence; (a) Marked cross-resistance of Pgp-overexpressing cells to these PIs, (b) requirement for higher proteasome inhibitor concentrations in Pgp-overexpressing cells compared to parental cells to inhibit intracellular chymotrypsin-like proteasome activity, and (c) 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.\textsuperscript{6,37}

The notion that the orally active proteasome inhibitor ONX 0912 was also recognized as a Pgp transport substrate, albeit less prominently than carfilzomib and ONX 0914, may be of relevance for its bioavailability, which was found to be 39% in rodents and dogs, given the fact that intestinal Pgp is a major determinant of drug efflux and absorption.\textsuperscript{9,19} Hence, evading the Pgp-dependent mucosal epithelium barrier may further enhance ONX 0912 bioavailability. The present study also provided evidence that carfilzomib, ONX 0912 and ONX 0914 were not substrates for other multidrug efflux transporters including MRP1-5 and BCRP. Interestingly, BCRP-overexpressing MCF7/MR cells even displayed 3-4 fold greater sensitivity for BTZ, carfilzomib, ONX 0912 and ONX 0914. This hypersensitivity is likely due to an intrinsic property of MCF7/MR cells acquired after establishment of resistance to the topoisomerase inhibitor mitoxantrone as Ogiso et al.\textsuperscript{38} 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 hence resulting in hypersensitivity to PIs.

In order 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 RA patients and healthy controls. We and others reported that Pgp expression on lymphocytes from RA patients correlated with disease activity. Here we showed that the low basal Pgp activity in ex vivo isolated PBMCs partially reduced the potent (i.e. nanomolar) inhibition of the chymotrypsin-like proteasome activity by carfilzomib, ONX 0912, ONX 0914 and BTZ. However, for carfilzomib and ONX 0914, the two PIs that displayed the highest cross-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 RA patients and healthy controls (carfilzomib > ONX 0914 > ONX 0912) is different from their ranking for growth inhibitory potency (carfilzomib > ONX 0912 > ONX 0914) observed for 4 different parental cell lines (CEM, HEK293, A2008 and MCF7 cells). This result may be due to 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 like ONX 0914, 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, ONX 0912 and ONX 0914. However, targeting of immune cells harboring basal levels of Pgp did not show cross resistance to ONX 0912 while carfilzomib was effected only to a small extent. Hence, to identify the structural elements within peptide epoxyketone-based chemical structures that render them Pgp substrates, may be an important strategy to evade Pgp-mediated drug efflux and to overcome drug resistance. In fact, we have recently shown that small chemical modifications can generate analogues of a class of topoisomerase drug inhibitors, i.e. imidazoacridinones, which were no longer substrates for the drug efflux transport-
Indeed, also Zhou et al. 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 PBMCs of controls and RA patients, 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, though caution for increased toxicity may also need to be exercised when these combinations would be used in the clinic.
References


