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CHAPTER 4

Anti-leukemic activity and mechanisms underlying resistance to the novel immunoproteasome inhibitor PR-924

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ABSTRACT

PR-924 is a novel prototypic immunoproteasome inhibitor bearing markedly enhanced specificity for the β5i immunoproteasome subunit, compared to the classical proteasome inhibitor bortezomib. Here, we assessed the growth inhibitory potential of PR-924 in three human hematologic malignancy cell lines (CCRF-CEM, THP1, and 8226) and their bortezomib-resistant sublines. Parental cells displayed equal sensitivity to PR-924 (IC$_{50}$: 1.5 µM ± 2.8 µM), whereas their bortezomib-resistant tumor lines displayed a 10-12 fold cross-resistance to PR-924. However, PR-924 cross-resistance factors for bortezomib-resistant sublines were markedly lower compared to the resistance factors to bortezomib. Proteasome inhibition experiments confirmed that PR-924 specifically inhibited β5i activity, even far below concentrations that exerted anti-proliferative activity. We further determined whether PR-924 activity might be compromised by acquisition of drug resistance phenomena. Indeed, CEM cells rendered stepwise resistant to 20 µM PR-924 (CEM/PR20) displayed 13-fold PR-924-resistance and 10-fold cross-resistance to bortezomib. CEM/PR20 cells were devoid of mutations in the PSMB8 gene (encoding β5i), but acquired Met45Ile mutation in the PSMB5 gene (encoding constitutive β5), consistent with β5 mutations observed in bortezomib-resistant cells. Furthermore, compared to parental CEM cells, CEM/PR20 cells exhibited 2.5-fold upregulation of constitutive proteasome subunit expression, whereas immunoproteasome subunit expression was 2-fold decreased. In conclusion, PR-924 displayed potent anti-leukemic activity including towards bortezomib-resistant leukemia cells. Despite the specificity of PR-924 to the β5i immunoproteasome subunit, its anti-leukemic effect required concentrations that blocked both β5 and β5i subunits. This is underscored by the emergence of mutations in PSMB5 rather than in PSMB8.

INTRODUCTION

In the past decade, multiple clinical trials combining conventional chemotherapeutics with proteasome inhibitors were initiated in patients with hematologic malignancies$^{1-5}$. In particular, multiple myeloma (MM) and mantle cell lymphoma patients clearly benefited from such therapeutic interventions containing the proteasome inhibitors bortezomib and carfilzomib, which expedited their FDA approval of these compounds$^{6,7}$. Proteasome inhibition by bortezomib is also recognized as an emerging treatment strategy for acute leukemia$^{8}$. However, the occurrence of bortezomib resistance and the recorded side-effects of this drug, including peripheral neuropathy, has triggered an ongoing research to develop next generation proteasome inhibitors lacking these untoward toxicity$^{8,10}$. Within this category, selective inhibitors of the immunoproteasome rather than the constitutive proteasome, received considerable attention given their potential in autoimmune diseases and inflammatory disorders$^{11-13}$. Whereas the constitutive proteasome is expressed in all cell types, the immunoproteasome is primarily expressed in immune-competent cells and is induced via interferon-gamma (IFN-γ) and TNF-α in other cell types$^{14}$. The immunoproteasome differs from the constitutive proteasome in its catalytically active β-subunits. The constitutive proteasome is comprised of β5, β1, and β2 subunits representing the chymotrypsin-like, caspase-like, and trypsin-like catalytic activities, respectively. During the assembly of the immunoproteasome, these are exchanged by the inducible immune-
subunits β5i, β1i, and β2i resulting in higher trypsin-like and chymotrypsin-like catalytic activities, but reduced caspase-like activity. Furthermore, two hybrid proteasomes exist, one containing β1, β2 and the inducible β5i subunit and the other containing β2 and inducible β1i and β5i subunits. All proteasome subtypes feature slightly different preferences of substrate protein cleavage, which facilitates a diversity of antigenic peptides to be presented on HLA Class I molecules. Bortezomib represents a prototypical reversible proteasome inhibitor that mainly targets the chymotrypsin-like and caspase-like proteasome activities. Carfilzomib and its oral analogue oprozomib are next generation epoxyketone proteasome inhibitors that selectively and irreversibly bind to the chymotrypsin-like activity of the proteasome. Moreover, two novel immunoproteasome inhibitors have recently entered preclinical testing; one of these, ONX 0914, was the first β5i-specific proteasome inhibitor, with 40-fold greater specificity for β5i over β5. ONX 0914 underwent preclinical evaluation for immunologic disorders, and it is also being evaluated for the possible treatment of hematologic malignancies. The latter was indicated by the fact that immunoproteasomes are highly expressed compared to constitutive proteasomes in cells of hematologic malignancies, including acute lymphocytic leukemia (ALL). ONX 0914 was selected based on the efficiency of its immunoproteasome inhibition in rats, PR-924 was designed and identified as a novel structure being more selective towards the human immunoproteasome. Notably, as an epoxyketone tripeptide compound, PR-924 is 130-fold more selective for β5i than β5. PR-924 has been shown to inhibit growth and elicit apoptosis in human MM cell lines and primary patient samples and has shown minimal cytotoxic effect towards normal PBMCs. However, with respect to its anti-leukemic properties, PR-924 is relatively unexplored. The aim of the current study was to examine the proteasome inhibitory capacity of PR-924, along with its anti-leukemic effect in human acute leukemia cell lines and their bortezomib resistant sublines. We also assessed whether or not prolonged exposure to PR-924 would provoke the onset of acquired resistance to this drug and if so, to delineate the molecular mechanism underlying drug resistance.

MATERIALS AND METHODS

CELL CULTURE AND DEVELOPMENT OF PR-924-RESISTANT CELL LINES
Human T-cell ALL CCRF-CEM cells, human myeloid leukemic THP1 cells, and human multiple myeloma RPMI-8226 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium containing 2 mM glutamine (Invitrogen/Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and 100 µg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 5% CO2 and 37°C. Cell cultures were seeded at a density of 3×10^5 cells/ml and refreshed twice weekly. Bortezomib-resistant sublines of these tumor cell lines were previously established. Development of PR-924-resistant cells was achieved by treatment with gradually increasing concentrations of PR-924 for a period of 3 months starting from 2 µM to up to 8 µM for 8226/PR8, 12 µM for THP1/PR12 and 20 µM for CEM/PR20 cells.

DRUGS
PR-924, carfilzomib, and ONX 0914 were provided by Onyx Pharmaceuticals, Inc. (South San Francisco, CA, USA). Bortezomib was provided by Millennium Pharmaceuticals (Cambridge,
CELL GROWTH INHIBITION ASSAYS
In vitro drug sensitivity was determined using the 4-day MTT cytotoxicity assay. Prior to these experiments, bortezomib-resistant cells and PR-924 resistant cells were cultured in drug-free medium for at least 4 days. Cells were then exposed to various concentrations of PR-924 (range: 0.04 µM –75 µM), bortezomib (1 nM - 2 µM), carfilzomib (0.007 nM - 15.6 nM), and ONX 0914 (8 nM–16 µM) for 4 days. The IC\textsubscript{50} value is defined as the drug concentration necessary to inhibit 50% of the cell growth compared to growth of the untreated control cells. For combination experiments, drugs were added simultaneously. Drug combinations were chosen starting from IC\textsubscript{10} concentrations (10% cell death) of both drugs and diluted in a constant ratio. CalcuSyn (Version 1.1.1 1996, Biosoft, Cambridge, UK) software was used to calculate a combination index (CI) based on the median-effect principle for each drug combination tested.

APOPTOSIS ASSAY
Apoptotic cell death was determined using the Apoptest\textsuperscript{TM}–FITC kit (VPS Diagnostics, Hoeven, the Netherlands) and 7-AAD (BD Via-Probe\textsuperscript{TM}, BD Bioscience, San Jose, CA, USA). Parental cells were exposed to 2.5 µM and 10 µM PR-924 and bortezomib-resistant cells to 25 µM and 100 µM PR-924 for 24 hours. Samples were analyzed using a BD Canto II flow cytometer and analyzed using BD FACSDiva software. PARP cleavage (antibody #9542 from Cell Signaling [Danvers, MA, USA]) was determined by Western blotting as previously described.

HLA CLASS I EXPRESSION
HLA Class I expression was determined using HLA-ABC FITC antibody (5 µg/ml) (eBioscience, San Diego, CA, USA) and mouse IgG2a antibody (5 µg/ml) as isotype control. Fluorescent cells were analyzed using a FACSDiva flow cytometer, using CELLQUEST software (BD Biosciences, San Jose, CA, USA).

PROTEASOME ACTIVITY
For measurement of specific β5, β5i, and β1i activities in cell extracts, the Ac-WLA-AMC, Ac-ANW-AMC, and Ac-PAL-AMC fluorogenic substrates were used, respectively. After 1 hour of exposure to 0.01-10 µM PR-924, cells were washed in ice-cold phosphate-buffered saline (PBS) and 5 mM ethylenediaminetetraacetic acid (EDTA) was added at pH 8.0 (Sigma Aldrich, St. Louis, MO, USA). Samples were then frozen at -80 °C until analysis. Samples were thawed and centrifuged at 10 000x g for 10 minutes at 4 °C. The supernatant was removed and assayed for protein content using the Bio-Rad Protein Assay following the manufacturer’s protocol (Bio-Rad, Hercules, CA, USA). Assays were performed at 37 °C in a final volume of 200 µL using 96-well black opaque plates (Greiner bio-one, Alphen a/d Rijn, Nederland). Protein extracts were diluted to 200 µg/mL in 5 mM EDTA at pH 8.0. Diluted protein extract aliquots (50 µL) were dispensed per well, resulting in 10 µg of protein extract per reaction. Reactions were initiated by addition of 100 µM peptide-AMC substrate in 20 mM N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES), pH 7.4, containing 0.5 mM EDTA (Sigma Aldrich, St. Louis, MO, USA). Peptidase activity was determined by kinetic monitoring of 7-amino-4-methylcoumarin (AMC) production over two hours with a Biotek plate reader (Winooski, VT, USA) and
analyzed by GraphPad Prism software (La Jolla, CA, USA) with linear regression analysis.

**DNA SEQUENCING**

DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Subsequently, *PSMB5* exon 2 and all *PSMB8* exons were amplified by PCR. The primers were designed using Vector NTI (Invitrogen) software and are as follows: *PSMB5* exon 2 forward: GTGAAGCTCGGGTGACA, reverse: GTAGTGTCAGCCCAAGA. *PSMB8* exon 1 E1 forward: CACAACTCACACCTCGCCCG, reverse: CTGGGGACCGCTTCTCTGCT. *PSMB8* exon 1 E2 forward: TCCCCGACCCCCATAACCG, reverse: TTGGGAGCCCCACCTCACCC. *PSMB8* exon 2 and 3 forward: TGCAATCTCTCACTCCC, reverse: TGCGCTATGAGTGGAGCACAG. *PSMB8* exon 4 forward: ACATCCACCTGGGGAGCA, reverse: GGGACATCTACAAACACATGCAA. *PSMB8* exon 5 forward: GGCATGGCTCTCTATGGG, reverse: CCGCGACTCTCCCAGGCA. *PSMB8* exon 6 forward: CAAAGGGAGAGTAGTAGGAGGA, reverse: TCATGGAGCAATTAGGCAGCC. PCR products were directly sequenced by the dideoxy chain-termination method using the ABI PrismTM BigDye Terminator kit (Perkin Elmer, Foster City, CA, USA) and analyzed with the ABI Prism Genetic Analyser 3100 automatic DNA sequencer (PerkinElmer, Waltham, MA, USA).

**PROTEASOME ACTIVE-SITE ELISA**

An ELISA-based assay (ProCISE) for quantitative assessment of active constitutive and immunoproteasome subunits was performed as previously described. Briefly, aliquots of cell lysate were incubated with a biotinylated proteasome active-site binding probe. Lysate was then denatured, and subunits bound to probe were isolated with streptavidin-conjugated sepharose beads. Individual subunits were probed with subunit-specific primary antibodies, followed by HRP-conjugated secondary antibodies. A chemiluminescent substrate was used to generate signal associated with HRP binding, which was read on a luminometer (Tecan, Grödig, Austria). Absolute values of nanograms of subunit per microgram of total cellular protein were based on a purified proteasome standard curve. Protein quantification was performed utilizing the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL, USA).

**DRUG EFFLUX VIA THE MULTIDRUG EFFLUX TRANSPORTER MDR/P-GLYCOPROTEIN (Pgp/ABCB1)**

The P-glycoprotein/MDR1 overexpressing cell line CEM/VBL was used as described previously in the absence or presence of the specific Pgp transport inhibitor Reversin P121 (Alexis Benelux, Zandhoven, Belgium), at a final concentration of 7.5 µM. Cells were subsequently exposed to PR-924 (range: 0.01 µM – 10 µM) for 4 days and in vitro drug sensitivity was determined using the MTT cytotoxicity assay. Pgp levels were determined by Western blot analysis, as previously described, using the Pgp antibody JSB1 (Enzo Life Sciences, Farmingdale, NY, USA).

**RESULTS**

**SENSITIVITY OF BORTEZOMIB-SENSITIVE AND BORTEZOMIB-RESISTANT HEMATOLOGIC TUMOR CELLS TO PR-924**

To assess the anti-leukemic activity of PR-924, its cell growth inhibitory effect was determined
in three human tumor cell lines of hematologic origin: the MM cell line RPMI-8226, the T-ALL cell line CCRF-CEM, and the acute myeloid leukemia (AML) cell line THP1, and their 41-153-fold bortezomib-resistant sublines 8226/BTZ100, CEM/BTZ200, and THP1/BTZ200, respectively. Although PR-924 displayed cytotoxic activity in the low micromolar range for all three tumor cell lines, parental CEM and THP1 cells displayed the same sensitivity to PR-924 (IC\textsubscript{50} values of CEM: 1.8 µM ± 0.4, THP1: 1.5 µM ± 0.2), whereas 8226/WT cells were slightly less sensitive to PR-924 (IC\textsubscript{50}: 2.8 µM ± 0.6). Notably, their bortezomib-resistant sublines displayed 9-12 fold cross-resistance to PR-924 (IC\textsubscript{50} 8226/BTZ100: 24.4 µM ± 3, CEM/BTZ200: 21.1 µM ± 0.5, THP1/BTZ200: 15.6 µM ± 1.6). Nonetheless, absolute PR-924 cross-resistance factors for bortezomib-resistant sublines were markedly lower compared to the resistance factors to bortezomib: 5-fold lower for 8226, 13-fold lower for CEM and 15-fold lower for THP1 (Table 1).

Based on these encouraging results, we next examined whether or not combination of PR-924 with conventional chemotherapeutic drugs such as dexamethasone for the ALL cell lines CEM/WT and CEM/BTZ200 as well as cytarabine for AML cell lines THP1/WT and THP1/BTZ200 would show synergistic effects; however, no synergistic effects were observed (results not shown). Flow cytometric analysis revealed that PR-924-induced cell death was mediated by induction of apoptosis, being similarly induced in all three parental cell lines after 24 hours of exposure to 2.5 µM PR-924 (range: 31-38% apoptotic cells) and 10 µM PR-924 (range 67-90% apoptotic cells; Figure 1A). In the bortezomib-resistant CEM and THP1 cell lines, induction of apoptosis was observed in less than 5% of cells after exposure to 25 µM PR-924, and in 15% and 31% of cells after treatment with 100 µM PR-924, respectively (Figure 1B).

For comparison, induction of apoptosis was noted in 26% and 78% of 8226/BTZ100 cells after exposure to 25 µM and 100 µM PR-924, respectively, as well as <3% of cells after exposure to 2.5-10 µM of PR-924 (results not shown). These apoptosis induction profiles were further verified and confirmed by PARP cleavage (Figure 1C). Lastly, exposure to 2.5-10 µM PR-924 for 24 hours resulted in an up to 50% reduction of cell surface expression of HLA Class I molecules for all parental and bortezomib-resistant cell lines, except for THP1/WT cells, in which HLA Class I expression was completely lost after exposure to 10 µM PR-924 (Figure 2).

Table 1. Sensitivity of hematologic tumor cell lines and their bortezomib-resistant and PR-924-resistant sublines to PR-924 and bortezomib.

<table>
<thead>
<tr>
<th>Wildtype cell lines</th>
<th>IC\textsubscript{50} PR-924 ± SD µM (RF)</th>
<th>IC\textsubscript{50} bortezomib ± SD nM (RF)</th>
<th>Mutations PSMB5 exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>8226/WT</td>
<td>2.8±0.6</td>
<td>2.9±0.3</td>
<td></td>
</tr>
<tr>
<td>CEM/WT</td>
<td>1.8±0.4</td>
<td>2.7±0.5</td>
<td></td>
</tr>
<tr>
<td>THP1/WT</td>
<td>1.5±0.2</td>
<td>2.6±0.6</td>
<td></td>
</tr>
<tr>
<td>Bortezomib-resistant sublines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8226/BTZ100</td>
<td>24.4±3 (9)</td>
<td>120±17 (41)</td>
<td>Ala49Thr</td>
</tr>
<tr>
<td>CEM/BTZ200</td>
<td>21.1±0.5 (12)</td>
<td>416±85 (152)</td>
<td>Ala49Val &amp; Cys52Phe</td>
</tr>
<tr>
<td>THP1/BTZ200</td>
<td>15.6±1.6 (10)</td>
<td>390±68 (153)</td>
<td>Ala49Thr</td>
</tr>
<tr>
<td>PR-924-resistant sublines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEM/PR20</td>
<td>22.1±1.8 (13)</td>
<td>28±3 (10)</td>
<td>Met45Ile</td>
</tr>
<tr>
<td>THP1/PR12</td>
<td>14±3.7 (10)</td>
<td>28±3.6 (10)</td>
<td>Ala49Thr</td>
</tr>
<tr>
<td>8226/PR8</td>
<td>10.1±1.6 (6)</td>
<td>14±0.2 (6)</td>
<td>Met45Val</td>
</tr>
</tbody>
</table>

SD: standard deviation, RF: resistance factor
Antileukemic effect and resistance to PR-924

Figure 1. Effect of PR-924 on the induction of apoptosis in parental and bortezomib-resistant hematologic tumor cell lines. Flow cytometric analysis of apoptotic (Annexin-V/7AAD positive) fraction of (A) the parental cell lines 8226/WT, CEM/WT, and THP1/WT after exposure to 2.5 µM and 10 µM PR-924 for 24 hours and, (B) bortezomib-resistant sublines 8226/BTZ100, CEM/BTZ200, and THP1/BTZ200 exposed to 25 µM and 100 µM PR-924 for 24 hours. Bars depict percentages of apoptotic cells as means (± standard deviation) of three independent experiments. (C) Representation of cleaved and full length poly (ADP-ribose) polymerase (PARP) determined by Western blot analysis after 24 hours exposure of parental cells to 2.5 µM PR-924 and bortezomib-resistant cells to 25 µM PR-924. Quantified mean (±SD) of three independent experiments is shown and PARP expression in PR-924 treated cells was normalized to PARP expression in untreated cells.

SUBUNIT-SPECIFIC PROTEASOME ACTIVITY INHIBITION PROFILE OF PR-924

To determine whether or not PR-924 catalytic activity relies solely on specific inhibition of β5i, inhibition experiments of proteasome catalytic activity were performed over a range of 0.01–10 µM PR-924. Notably, in parental cells, β5i catalytic activity was already inhibited by 9-17% at 10 nM PR-924 and by >95% in the presence of 100 nM PR-924 (Figure 3A). At higher PR-924 concentrations of 1-10 µM, near complete inhibition of both β5 and β1i catalytic activities was observed. No inhibition of the β5 catalytic activity was noted up to 10 µM PR-924 in the bortezomib-resistant sublines, whereas the inhibition profile of β1i was similar to that of the parental cell lines (Figure 3B). Inhibition of β5i catalytic activity by PR-924 in the bortezomib-resistant cells was less effective when compared to parental cell lines, with an average inhibition of 33% at 100 nM PR-924. However, β5i catalytic activity was almost completely abrogated at 10 µM PR-924 (Figure 3B). Of further notice was the observation that basal constitutive β5-associated catalytic activity was higher in bortezomib-resistant cells compared to parental cells; in contrast, immunoproteasome β5i and β1i catalytic activities were down-regulated compared to parental cells (Figure 3C).
Figure 2. Effect of PR-924 on HLA Class I molecule expression decrease in bortezomib-sensitive and bortezomib-resistant hematologic tumor cell lines. Impact on HLA Class I expression in (A) parental cell lines 8226/WT (MFI: 1894±35), CEM/WT (MFI: 389±29), and THP1/WT (MFI: 42±2) exposed to 2.5 µM and 10 µM PR-924 for 24 hours compared to untreated cells, and (B) their bortezomib-resistant sublines 8226/BTZ100 (MFI: 1293±69), CEM/BTZ200 (MFI: 330±64), and THP1/BTZ200 (MFI: 979±52). Mean (±SD) of three independent experiments is shown.

CHARACTERIZATION OF PR-924-RESISTANT CELLS

PROTEASOME INHIBITOR SENSITIVITY AND MUTATION STATUS OF PR-924 RESISTANT CELLS

Previous studies from our laboratory showed that continuous exposure of leukemic cells to bortezomib is associated with the onset of acquired resistance to this drug. However, it has not been established whether or not this would also hold true for an immunoproteasome inhibitor like PR-924. To this end, we provoked acquired resistance to PR-924 in parental CEM cells by treatment with step-wise increasing concentrations of PR-924 in cell culture, starting at the wild-type IC\textsubscript{50} concentration of 2 µM PR-924. Following a 3 month stepwise selection, CEM cells resistant to 20 µM PR-924 (CEM/PR20) exhibited an IC\textsubscript{50} value of 22.1 ± 1.8 µM PR-924 (resistance factor: 13; Table 1). Additionally, these PR-924-resistant cells displayed 10-fold cross-resistance to bortezomib, 10-fold to carfilzomib, and 38-fold to ONX 0914. To explore the molecular mechanism underlying PR-924 resistance, sequencing of the PSMB8 gene (encoding β5i) was performed. However, no mutations were found in PSMB8. Remarkably, a point mutation was identified in exon 2 of the PSMB5 gene (encoding the S1 pocket of β5), introducing an amino acid substitution (Met45Ile) at a position similar to those identified in bortezomib-resistant sublines (Table 1).

INDUCTION OF APOPTOSIS AND HLA CLASS I EXPRESSION IN PR-924 RESISTANT CELLS

We further examined whether or not a decreased capability to undergo apoptosis would contribute to PR-924 resistance in CEM/PR20 cells. Apoptosis induction was observed in 27% and 75% of cells upon 24 hours exposure to 25 µM and 100 µM PR-924, respectively (Figure 4A), hence exceeding apoptosis induction by PR-924 in bortezomib-resistant CEM cells exposed to similar concentrations (4% and 15% for 25 µM and 100 µM PR-924, respectively in CEM/BTZ200).
Antileukemic effect and resistance to PR-924

Figure 3. PR-924 inhibition of proteasome catalytic activity in parental and bortezomib-resistant hematologic cell lines. Subunit specific proteasome activity of β5, β5i, and β1i determined after 1 hour exposure to various PR-924 concentrations (0.01 – 10 µM) for (A) parental cell lines 8226/WT, CEM/WT, THP1/WT and their (B) bortezomib-resistant sublines 8226/BTZ100, CEM/BTZ200, and THP1/BTZ200. (C) Basal levels of subunit specific proteasome activity in parental cell lines compared to their bortezomib-resistant sublines. Results represent the mean (± standard deviation) of three independent experiments.

Also, HLA Class I expression was reduced by 50% after exposure to 100 µM PR-924 for 24 hours, similar to that observed in bortezomib-resistant cell lines (Figure 4B).

PROTEASOME SUBUNIT CATALYTIC ACTIVITY AND EXPRESSION LEVELS IN PR-924 RESISTANT CELLS
Assessment of proteasome subunit catalytic activity in CEM/PR20 cells after 1 hour exposure to 0.01–10 µM PR-924, showed that β5i activity was consistently inhibited at 10 nM PR-924 as in CEM/WT and reached almost complete inhibition at 10 µM PR-924 (Figure 4C). In this concentration range, β1i and β5 catalytic activities were not appreciably inhibited by PR-924. Basal subunit catalytic activities in CEM/PR20 cells revealed slightly increased β5 activity (1.4-fold), and prominently reduced β5i (3.4-fold) and β1i (2-fold) activities in CEM/PR20 cells compared to parental cells (Figure 4D). Additionally, active proteasome subunit expression in CEM/PR20 cells was quantified by ProCISE analysis. Consistent with catalytic activity analysis, expression levels of constitutive proteasome subunits in CEM/PR20 cells were upregulated (up to 2.5-fold), compared to parental CEM cells, whereas immunoproteasome subunit expression was moderately decreased (up to 2-fold) in CEM/PR20 cells (Figure 4E).
A side-by-side comparison of bortezomib and PR-924 showed that except for β5i activity, β5 and β1i catalytic activities in CEM/PR20 cells were efficiently inhibited by bortezomib compared to PR-924 (Figure 4F). Apart from CEM cells, we also provoked resistance to PR-924 in two other hematologic tumor cell lines: THP1 and 8226. Cytotoxicity experiments showed that THP1 cells adapted to grow in 12 µM PR-924 (THP1/PR12; IC50 PR-924: 14±3.7 µM) were 10-fold resistant to PR-924 and 10-fold cross-resistant to bortezomib, relative to THP1/WT cells (Table 1). Moreover, THP1/PR12 cells also harbored a point mutation in PR5MB5 introducing an Ala to Thr substitution at amino acid 49 (Ala49Thr) in the β5 subunit. 8226 cells adapted to grow in 8 µM PR-924 (8226/PR8; IC50 PR-924: 10.1 µM, Table 1) displayed 6-fold resistance to PR-924 and 6-fold cross-resistance to bortezomib and also acquired a point mutation in in PSMB5 introducing a Met to Val substitution at amino acid 45 in the β5 subunit protein (Met45Val). Quantification of proteasome subunit expression in THP1/PR8 cells revealed a clear upregulation (up to 2.5-fold) of all constitutive proteasome subunits and a modest increase (up to 1.6-fold) of all immunoproteasome subunits (Figure 4G). A similar profile was noted in 8226/PR8 cells; upregulation of the constitutive subunits (up to 1.5-fold) with no alteration in immunoproteasome subunit expression (Figure 4H).

ROLE OF PGP IN CONFERRING RESISTANCE TO PR-924

Recent studies by our laboratory32 and other labs33 showed that cellular drug extrusion via the multidrug efflux transporter P-glycoprotein (Pgp)/MDR1/ABCB1 can serve as a resistance modality for peptide-based proteasome inhibitors. To determine whether or not Pgp expression may confer resistance to PR-924, cell growth inhibitory effects of PR-924 were tested in a Pgp-overexpressing cell line CEM/VLB32. Indeed, CEM/VLB cells exhibited a marked level of resistance to PR-924 compared with parental CEM cells (IC50: 7.0 µM vs. 0.8 µM, respectively), indicating that PR-924 is a bona fide Pgp substrate. This was further corroborated by the fact that blocking Pgp efflux activity with the inhibitor P121, largely restored parental CEM cell sensitivity to PR-924 (IC50: 1.9 µM) (Figure 5A). However, Western blot analysis of Pgp revealed that CEM/PR20 cells appeared to lack Pgp expression, indicating that upregulation of Pgp is not involved in acquired resistance to PR-924 in CEM/PR20 cells (Figure 5B).

DISCUSSION

PR-924 was rationally designed to be a more selective inhibitor of the β5i subunit than previously developed proteasome inhibitors, and was therefore proposed to be more selective in targeting hematologic malignant cells, which abundantly express the immunoproteasome19,24–26. Towards this end we showed in the present study that (a) PR-924 displays anti-leukemic activity, but only at concentrations that inhibit the catalytic activities of both immunoproteasome β5i and constitutive β5 subunits, (b) Despite a low level of cross-resistance, PR-924 retains appreciable pharmacologic activity against three different bortezomib-resistant leukemia cell lines, and (c) Leukemia cells with acquired resistance to PR-924 share a common molecular mechanism of resistance to bortezomib, i.e. acquisition of point mutations in the constitutive β5 subunit at a well-defined site involved in proteasome inhibitor binding. Consistent with studies by Singh et al25 demonstrating that PR-924 displayed growth inhibitory effects against a panel of MM cell lines at low micromolar drug
Figure 4. Characteristic features of the PR-924-resistant cell lines CEM/PR20, THP1/PR12 and 8226/PR8. (A) Ability to undergo apoptosis as shown by percentage of Annexin-V/7AAD positive cells and (B) impact on HLA Class I expression in CEM/PR20 cells exposed to 25 µM and 100 µM for 24 hours compared to untreated cells. (C) Subunit specific proteasome activity of β5, β5i, and β1i determined in cell extracts of CEM/PR20 cells after 1 hour exposure to 0.01-10 µM PR-924. (D) Basal levels of subunit specific proteasome activity for CEM/PR20 compared to parental CEM cells. (E) Constitutive and immunoproteasome subunit expression of CEM/PR20 cells relative to CEM/WT cells as determined by ProCISE analysis. (F) Subunit specific proteasome activity of β5, β5i, and β1i determined in cell extracts of CEM/PR20 cells after 1 hour exposure to 0.01 – 10 µM bortezomib. (G) Proteasome subunit expression of THP1/PR12 as percentage of THP1/WT expression as well as (H) proteasome subunit expression of 8226/PR8 as percentage of 8226/WT expression as determined by ProCISE analysis. All results are presented as means of at least 3 (± standard deviation) independent experiments.

concentrations (IC_{50}: 3-7 µM), the present study also revealed a comparable potency against the human leukemia cell lines CCRF-CEM and THP1 (IC_{50}: 1.5-1.8 µM). These concentrations were relatively high, as growth inhibitory effects against MM and leukemia cell lines by bortezomib usually require low nanomolar drug concentrations^{27,28}. This apparent discrepancy is unrelated to the capacity of PR-924 to completely inhibit immunoproteasome β5i catalytic activity, which occurred at PR-924 concentrations < 100 nM (Figure 3C), but rather points to the fact that β5i inhibition alone is not sufficient to elicit an anti-leukemic effect. This hypothesis was previously indicated by Parlati et al^{19}, who showed that a concomitant inhibition of constitutive β5 activity was required to achieve an anti-leukemic activity. These results were corroborated in our present studies hence showing that induction of apoptosis and cell growth inhibition by PR-924 paralleled a concomitant inhibition of β5 catalytic activity at drug concentrations > 1 µM. Notwithstanding this fact, recent studies, including some conducted in pediatric acute leukemia samples demonstrated that immunoproteasome levels, and more precisely an increased ratio of immunoproteasome over constitutive proteasome, do contribute to enhanced immunoproteasome inhibitor sensitivity^{24}. Likewise, upregulation of immunoproteasome levels following exposure to interferon-γ also markedly sensitized leukemic cell lines to immunoproteasome inhibitor activity^{14}. Consistently here, ratios of β5i/β5 expression in THP1 (i.e. 5.2), CCRF-CEM (i.e.
Figure 5. Role of Pgp in conferring resistance to PR-924. (A) IC50 values after PR-924 exposure of parental CEM cells and the P-glycoprotein/MDR1 overexpressing cell line CEM/VBL, in the absence or presence of the specific Pgp transport inhibitor P121 (7.5 µM) for 96 hours. (B) Pgp levels were determined by Western blot analysis in parental CEM cells, CEM/VBL cells, and CEM/PR20 cells. Quantified mean (±SD) of three independent experiments is shown.

1.2) and RPMI-8226 (i.e. 0.7) determined previously14 correlated with the ranking of PR-924 sensitivity in these tumor cells (IC50: 1.5 µM, 1.8 µM and 2.8 µM, respectively). Since the same drug efficacy ranking also applies for ratios of β5i/β5 catalytic activity, this parameter may deserve further exploration for future prediction of leukemia cell sensitivity to PR-924. Along with PR-924, several other immunoproteasome-specific inhibitors have been developed with the premise to improve on bortezomib with respect to efficacy, reduction of side effects and overcoming of bortezomib resistance21. Kuhn et al34 showed that the specific β1i-binding inhibitor IPSI-001 had anti-myeloma activity and could overcome resistance to bortezomib in addition to the synergistic activity of bortezomib combined with dexamethasone in these cells. However, this inhibitor and the β1i-specific inhibitor developed by Ho et al35 both appeared to preferentially inhibit β1i-associated chymotrypsin-like proteasome activity rather than the caspase-like activity of β115,16.

PR-924 displayed appreciable activity against human leukemia cell lines with acquired resistance to bortezomib27,28. Although cross-resistance levels to PR-924 were approximately 10-fold, this resistance factor was markedly lower than the 43 to 153-fold resistance to bortezomib. Since concomitant inhibition of b5 contributes to the growth inhibitory activity of PR-924, active site mutations in the b5 subunit may have a negative impact on the binding affinity of PR-924. Indeed, this suggestion gains support by the loss of PR-924 inhibition of b5-dependent catalytic activity in bortezomib-resistant cells (Figure 3B) versus parental cells (Figure 3A). The relatively low levels of cross-resistance to PR-924 in bortezomib-resistant leukemia cells implies that a substantial fraction of the bortezomib-resistance phenotype may be attributable to additional unknown factors other than β5 mutations. Resistance modalities often reveal critical mechanisms of drug action of anti-leukemic drugs. To this end, it was noteworthy that the acquisition of resistance to PR-924 in CEM cells had no impact on the expression levels of β5i and/or mutational status of PSMB8 as a primary target for PR-924. Surprisingly however, mutations in PSMB5 emerged as dominant mechanism in PR-924-resistant CEM/PR20 cells. This may be consistent with the fact that PR-924 concentrations used during the selection process (i.e. up to 20 mM) exert an inhibitory
pressure on β5 catalytic activity, although this would seem to be greatly outweighed by > 90% inhibition of β5i catalytic activity already by 100 nM PR-924 (Figure 4C). Mutations in PSMB8 have been reported which present by severe clinical manifestations of auto-inflammation and lipodystrophy. Surprisingly however, these mutations localize in exon 1 and exon 5 of the gene rather than in exon 2, which encodes for the substrate/proteasome inhibitor binding site. Mutations in exon 2 of PSMB5 in CEM/PR20 (Met45Ile), THP1/PR12 (Ala49Thr) and 8226/PR8 (Met45Val) in PR-924-resistant cells fully comply with a mutation cluster region in exon 2 of PSMB5, involving predominantly amino acids Met45 and Ala49, which were repeatedly observed in a large series of bortezomib-resistant leukemia cell lines. In this respect, the Met45Ile mutation was also described in bortezomib-resistant JY lymphoblastoid cells and THP1 cells, whereas the Ala49Thr mutation was identified in bortezomib-resistant THP1 and Jurkat cells. Consistent with bortezomib-resistant cells, PR-924-resistant CEM cells also displayed upregulation of the mutated b5 subunit, likely as a compensatory mechanism to sustain overall proteasome catalytic activity. Recently, deciphering the crystal structure of the immunoproteasome allowed 3D modeling of the inhibitor β5i-binding site towards the establishment of the binding specificity of the immunoproteasome inhibitor ONX 0914. This study showed the critical involvement of Met45 in binding of ONX 0914 to the β5 subunit of the proteasome by involving the rotation of the non-polar spatially flexible side chain Met45 residue, to increase the size of the S1 pocket of β5. Consistently, Met45 has also been shown to undergo a conformational change upon binding of bortezomib to the β5 subunit. Since PR-924 shares structural similarities with ONX 0914, PR-924 may also impact the Met45 side chain rotation upon binding to β5, thus enforcing diminished binding to the β5 subunit upon acquisition of a mutation in Met45 as seen in CEM/PR20 and 8226/PR8. Whether a mutation in Met45 leads to altered rotational properties of the side chain and thereby confers resistance to PR-924, warrants complementary modeling studies. As a preliminary account, PR-924 inhibition profiling of β5i catalytic activity in parental CEM cells (Figure 3A) vs. CEM/PR20 cells (Figure 4C) suggests that in the latter cells, PR-924 inhibition is less efficient at increasing drug concentrations above 1 mM. Along with Met45 amino acid substitutions, the Ala49Thr mutation observed in THP1/PR12 cells also represents a prominent mutation shown previously, next to several other acquired mutations in the S1 binding pocket of PSMB5 upon exposure to proteasome inhibitors. Taken together, these observations establish the β5 subunit as a key determinant in mediating drug resistance to proteasome inhibitors, even for immunoproteasome-targeted inhibitors, as impacting PSMB8 expression and/or mutational status would have more unfavorable consequences. In conclusion, the immunoproteasome-targeted drug PR-924 displayed significant anti-leukemic activity, but was found to heavily rely on essential complementary inhibition of both β5i and constitutive β5. The relevance of the latter finding is underscored by the fact that the emergence of acquired resistance to PR-924 is based on point mutations in PSMB5 rather than in PSMB8.

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REFERENCES

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