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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}: 1.5–2.8 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 toward 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*.

1. 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 [9,10]. Within this category, selective inhibitors of the immunoproteasome rather than the constitutive proteasome, received considerable attention given their potential in auto-

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immune 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 [14–16]. 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 [17]. 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 [18].

Bortezomib represents a prototypical reversible proteasome inhibitor that mainly targets the chymotrypsin-like and caspase-like proteasome activities. Carfilzomib [19] and its oral analogue oprozomib [20] are next generation epoxyketone proteasome inhibitors that selectively and irreversibly bind to the chymotrypsin-like activity of the proteasome [21]. 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 [22]. ONX 0914 underwent preclinical evaluation for immunologic disorders [23], and it is also being evaluated for the possible treatment of hematologic malignancies [24]. 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) [19,24–26]. Whereas 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 toward the human immunoproteasome. Notably, as an epoxyketone tripeptide compound, PR-924 is 130-fold more selective for β5i than β5 [19]. 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 toward normal PBMCs [25]. 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.

2. Materials and methods

2.1. 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% CO_2 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 [27,28]. 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.

2.2. 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, MA, USA).

2.3. Cell growth inhibition assays

In vitro drug sensitivity was determined using the 4-day MTT cytotoxicity assay [29]. 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: 0.04–75 μM), bortezomib (1 nM–2 μM), carfilzomib (0.007–15.6 nM), and ONX 0914 (8 nM–16 μM) for 4 days. The IC_{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_{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 [30].

2.4. Apoptosis assay

Apoptotic cell death was determined using the Apoptest™–FITC kit (VPS Diagnostics, Hoeven, The Netherlands) and 7-AAD (BD Via-Probe™, 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 h. Samples were analyzed using a BD Canto II flow cytometer and analyzed using BD FACSDiva software. PARP cleavage (antibody no. 9542 from Cell Signaling [Danvers, MA, USA]) was determined by Western blotting as previously described [28].

2.5. 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).

2.6. 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 [31]. After 1 h 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 10 000 × *g* for 10 min 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