Antitumor Activity of PR-171, a Novel Irreversible Inhibitor of the Proteasome

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Abstract
Clinical studies with bortezomib have validated the proteasome as a therapeutic target for the treatment of multiple myeloma and non-Hodgkin’s lymphoma. However, significant toxicities have restricted the intensity of bortezomib dosing. Here we describe the antitumor activity of PR-171, a novel epoxyketone-based irreversible proteasome inhibitor that is currently in clinical development. In comparison to bortezomib, PR-171 exhibits equal potency but greater selectivity for proteasome inhibitors are currently under development: (a) salinosporamide A (NPI-0052), a natural product related to lactacytin (20–22) and (b) PR-171, a modified peptide related to the natural product epoxomicin.

Epoxomicin was identified based on its in vivo antitumor activity (23) and subsequently shown to be a potent and selective inhibitor of the proteasome (24). Epoxomicin and its analogues are comprised of two key elements: a peptide portion that selectively binds in the substrate binding pocket(s) of the proteasome with high affinity and an epoxyketone pharmacophore that stereospecifically interacts with the catalytic threonine residue to irreversibly inhibit enzyme activity. X-ray crystallography has shown that epoxomicin forms a dual covalent morpholino adduct with the proteasome that requires the close juxtaposition of both the side chain hydroxyl and α-amino groups of the active site threonine residue (25). This unique mechanism imparts a high degree of specificity to the proteasome relative to the active sites of other protease classes.

Medicinal chemistry efforts focused on increasing the potency and chymotrypsin-like selectivity of epoxomicin resulted in the identification of YU-101 (26), a synthetic tetrapeptide epoxyketone analogue. PR-171, a derivative of YU-101 with improved pharmacoeutical properties, is currently under evaluation in phase I clinical...
trials in multiple myeloma and NHL. In the present study, we describe the in vitro characterization and preclinical pharmacology of PR-171. We show that PR-171 is a potent and selective inhibitor of the chymotrypsin-like activity of the proteasome, both in vitro and in vivo. In addition, we show that proteasome inhibition by PR-171 promotes apoptosis in a variety of tumor cell lines, and that daily dosing schedules that induce high levels of proteasome inhibition in vivo are well tolerated and result in antitumor activity in several xenograft models.

Materials and Methods

Materials. PR-171 was synthesized as described by Smyth and Laidig (27). [3H]-PR-171 was generated by Pd-catalyzed tritiation of a 2-Br-Phe analogue of PR-171. Bortezomib was purchased from a local pharmacy. PR-171 and bortezomib stock solutions were prepared in DMSO and were diluted 100-fold for 20S proteasome assays or 400-fold for cell treatments. 7-Amino-4-methylcoumarin (AMC)–conjugated fluorogenic proteasome substrates were acquired from Boston Biochem (succinyl-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-AMC or Biomol (Boc-Leu-Arg-Arg-AMC and Z-Val-Gly-Arg-AMC). Purified human 20S proteasome, 20S immunoproteasome, and clasto-lactacystin β-lactone were purchased from Boston Biochem. Tissue culture media and horse serum were from Mediatech and fetal bovine serum (FBS) was from HyClone. Primary antibodies recognizing the following proteins were purchased from commercial sources: β-catenin, p21, cyclin B1, hsp27 phospho-Ser82, and actin from Cell Signaling Technology; ubiquitin (7–9 weeks old) and BNX (5–7 weeks old) mice were purchased from Charles River Laboratories and housed for 1 week before experimentation. Animals. Male Sprague-Dawley rats (200–250 g) and female BALB/c (7–9 weeks old) and BNX (5–7 weeks old) mice were purchased from Charles River Laboratories and housed for 1 week before experimentation. For all experiments, animals had access to food and water ad libitum. All experiments were done under protocols approved by an institutional animal care and use committee. 20S proteasome assays. Protease chymotrypsin-like, caspase-like, and trypsin-like activities were determined using succinyl-Leu-Leu-Val-Tyr-AMC (10 μmol/L), Z-Leu-Leu-Glu-AMC (10 μmol/L), and Boc-Leu-Arg-Arg-AMC (50 μmol/L), respectively, with purified human 20S proteasome (2, 4, and 8.0 μmol/L, respectively) or HT-29 cell lysate (0.125, 0.25, and 0.25 μg protein/mL, respectively). Assay buffer consisted of 0.5 mL/L EDTA with (20S) or without (cell lysate) 0.03% SDS. Reactions were initiated by enzyme or lysate addition and monitored for AMC product formation at 27°C. For biodistribution experiments, PR-171 was given to rats (n = 4 per dose group) as an i.v. bolus (1 mL/kg) at 2, 4.5, and 9 mg/kg in a solution containing 5% (w/v) hydroxypropyl-β-cyclodextrin (Roquette) and 50 mmol/L sodium citrate (pH 3.5). Blood samples were collected at 2, 5, 10, 15, 30, 60, and 120 min postdose, and plasma PR-171 concentrations were measured in duplicate by LC/MS-MS. Pharmacokinetic analyses were done using WinNonlin (Pharsight Corp.). For pharmacodynamic studies, PR-171 was formulated in an aqueous solution of 10% (w/v) sulfobutylether-β-cyclodextrin (Cydex) and 10 mmol/L sodium citrate (pH 3.5) for administration to rats (0.1–9 mg/kg) and mice (5–10 mg/kg). Bortezomib was formulated in saline containing 10 mg/mL mannitol for administration to mice at 1 mg/kg. At selected time points after i.v. drug administration, tissue samples (adrenal, brain, heart, liver, lung, and tumors) were collected and frozen at −80°C. For imaging experiments, tissue samples were homogenized in two volumes of lysis buffer. Whole blood was collected by cardiac puncture into tubes containing sodium heparin. Rat spleenocytes (isolated by mechanical disruption) and bone marrow (flushed from the tibia) were washed with PBS and depleted of erythrocytes by hypotonic lysis (BD Pharm Lyse; BD PharMingen). Whole blood, spleenocytes, and bone marrow cells were washed twice with PBS and then lysed in lysis buffer and frozen at −80°C. Tissue homogenates and cell lysates were cleaved by centrifugation, and supernatants were collected for protein quantitation and proteasome activity determination using fluorogenic peptide substrates as described above.

Quantitative whole body autoradiography. Radiolabeled [3H]-PR-171 (250 μCi per animal) was given as an i.v. bolus to rats at a total dose of 2 mg/kg. Animals were sacrificed 0.5 h postdose and underwent whole-body perfusion with saline before being frozen. Sections were taken to identify organs of interest, and radioactivity levels in tissues were calculated using the AIB software (Imaging Research Corp.). These studies were done at Covance Laboratories. Xenograft studies. Tumors were established by s.c. injection of cell lines (passage number <9 and viability >95% at the time of implantation) in the right flank of BNX mice (n = 7 per group). For HT-29 and RL studies, cell suspensions containing 5 × 106 and 1 × 106 cells, respectively, in a volume
of 0.1 mL were injected. Mice were randomized into treatment groups and dosing initiated when tumors reached ~50 mm³ (HT-29) or ~90 mm³ (RL). For the HS-Sultan model, 0.2 mL containing 1 x 10⁶ cells in a 1:1 mixture with Matrigel (BD Discovery Labware) was injected. Tumor volume was measured 1 day after implantation, and mice were randomized to treatment groups and dosing initiated when the average tumor size exceeded the average size on Day 1 by ~100 mm³. PR-171 and bortezomib were given as described above for pharmacodynamic studies. In all treatment groups, tumors were measured thrice weekly by recording the longest perpendicular diameters and tumor volumes were calculated using the equation V = (length x width²)/2.

**Statistical analysis.** For comparisons of treatment groups, a one-way ANOVA followed by Bonferroni post hoc analysis using GraphPad Prism Software (version 4.01) was done. Statistical significance was achieved when P < 0.05.

**Results and Discussion**

PR-171 selectively inhibits proteasome chymotrypsin-like activity. The peptide epoxysketone YU-101 is a potent and selective inhibitor of the chymotrypsin-like activity of the 20S proteasome (26). However, the low aqueous solubility of this compound (<1 µg/mL) limits its utility in vivo. PR-171 is an analogue of YU-101 that exhibits improved aqueous solubility (>1000-fold) due to the introduction of an NH₂-terminal morpholino moiety (Fig. 1A). To evaluate the potency and selectivity of PR-171 for the three proteasome catalytic active sites, we monitored the rates of fluorogenic peptide substrate hydrolysis either by purified 20S proteasome or in a cell lysate. Like YU-101, PR-171 is potent and highly selective for the inhibition of the chymotrypsin-like activity of the proteasome (Table 1). PR-171 also inhibited the chymotrypsin-like activity of the immunoproteasome (IC₅₀ 33 nmol/L), an IFN-γ-inducible form of the proteasome (28). The ability of PR-171 to inhibit the proteasome in intact cells was also examined (Fig. 1B). Incubation of HT-29 colorectal adenocarcinoma cells with PR-171 for 1 h resulted in a dose-dependent inhibition of all three proteasome catalytic activities with the chymotrypsin-like activity exhibiting the greatest sensitivity (IC₅₀ 9 nmol/L). The caspase-like and trypsin-like activities were inhibited to a greater extent in the cellular assay (IC₅₀ values, 150–200 nmol/L) than in the isolated enzyme assay (IC₅₀ values, >1 µmol/L; Table 1). Whether this reflects differences in the proteasome (e.g., 26S versus 20S) or cellular environment (e.g., accumulation) remains to be determined. Similar dose-dependent suppression of proteasome activity was observed in other cell lines with PR-171 (data not shown).

Although PR-171, bortezomib, and salinosporamide A exhibit comparable potency on the proteasome chymotrypsin-like activity, bortezomib and salinosporamide A inhibit the caspase-like and trypsin-like activities, respectively, with greater potency than PR-171 (Table 1, Fig. 1B; refs. 20, 22). As a result, bortezomib and salinosporamide A may have a greater impact on overall protein turnover (29). Mechanistically, the epoxysketone pharmacophore of PR-171 is more selective for the unique NH₂-terminal threonine active site of the proteasome than either the boronic acid of bortezomib or the β-lactone of salinosporamide A (3). Both bortezomib and salinosporamide A have been shown to inhibit certain serine proteases with micromolar or submicromolar potencies (7, 20). In contrast, neither epoxomicin (24) nor PR-171 has significant activity on other protease classes at concentrations up to 10 µmol/L. The selectivity of PR-171 for the chymotrypsin-like activity of the proteasome as well as its weak activity on other protease classes may contribute to greater tolerability in vivo (see below).

**Cellular proteasome activity recovers following PR-171 treatment.** Although both PR-171 and bortezomib form covalent adducts with the proteasome (25, 30), the hydrolytic stability of the two inhibitor–enzyme complexes differ; the dual covalent morpholino adduct formed with PR-171 is irreversible, whereas the tetrahedral intermediate formed with bortezomib is slowly reversible (3). To evaluate the impact of this difference on the kinetics of proteasome activity recovery in cells, chymotrypsin-like activity was monitored over the course of 72 h following inhibitor treatment (Fig. 1C). Despite the irreversible binding of PR-171 to the proteasome, the rate of recovery of proteasome activity in cultured cells (t₁/₂ ~ 24 h) was only moderately slower than that observed with bortezomib. These results suggest that with both compounds, recovery of proteasome activity is due primarily to induction of mRNA transcription and de novo proteasome synthesis (31, 32).

**Brief exposure to PR-171 induces apoptosis and growth arrest in tumor cell lines.** The cytotoxic and proapoptotic effects of proteasome inhibitors are well established (3, 4, 10, 20, 33). However, most studies assessing the impact of proteasome inhibition on cells in culture have used extended treatment periods (24–72 h) that do not reflect the in vivo exposure that is achieved with either bortezomib or PR-171 due to their rapid clearance from plasma (see below and ref. 19). Therefore, we evaluated the cytotoxic effects of PR-171 and bortezomib on a panel of tumor cell lines and nontransformed cells treated for 1 h followed by a 72-h washout period (Table 2). Following brief exposure, PR-171 was more cytotoxic than bortezomib regardless of cell type, and both compounds were generally more cytotoxic to hematologic tumor lines than either solid tumor lines or nontransformed cells. No differences have been observed between PR-171 and bortezomib with regard to the onset of proteasome inhibition in a number of cell lines, eliminating the possibility that differences in cell penetration or proteasome accessibility are responsible for these observations. It is possible that the somewhat slower rate of proteasome activity recovery following PR-171 treatment results in greater cytotoxicity.

To evaluate the mechanisms underlying the cytotoxic effects of PR-171, the impact of brief compound exposure on the induction of apoptosis and growth arrest in tumor cells was examined. Following treatment with PR-171 for 1 h, apoptosis was rapidly induced in the two hematologic tumor cell lines (RPMI 8226 and HS-Sultan) with maximum annexin V staining detected by 24 h (Fig. 2A) and effector caspase activation detected as early as 5 h (Fig. 2B). In contrast, HT-29 cells initially growth arrest at 24 h, with an accumulation of cells in the G₂-M and S phases of the cell cycle (Supplementary Fig. S1) and only later undergo apoptosis (by 72 h). Brief treatments with PR-171 were more effective than equivalent concentrations of bortezomib at promoting both apoptosis and growth arrest.

**Brief exposure to PR-171 promotes accumulation of proteasome substrates and markers of apoptosis or stress response pathways.** To further investigate the pathways activated by PR-171, Western blot analysis was done on cell lysates prepared from either RPMI 8226 (Fig. 2C) or HT-29 cells (Fig. 2D) either 4 or 24 h after a 1-h treatment with compound. The markers examined included direct proteasome substrates as well as markers that

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accumulate as a functional consequence of activation of apoptotic, growth arrest, or stress response pathways. Each of the markers has been previously shown to accumulate in cells treated for extended periods with proteasome inhibitors (12, 31, 33–37). The effects of proteasome inhibition on the different markers varied by drug, cell line, and time point of analysis. Although accumulation of polyubiquitin chains was observed in both cell types within 4 h, the accumulation of other proteasome substrates was cell type specific, e.g., cyclin B1 in HT-29 and β-catenin in RPMI 8226. Although p21 levels increased in both cell types, the effect was more rapid in RPMI 8226 cells. The effects of PR-171 on accumulation of these markers was more pronounced than with bortezomib, consistent with the greater effects of brief PR-171 exposure on cell viability, apoptosis, and cell cycle progression. Heat shock protein induction is a common response to cellular stress, including perturbation of proteasome function and accumulation of misfolded proteins (31, 36, 38, 39). Both hsp27 and hsp70 were elevated at the 24-h time point in both cell lines and with both drugs, suggesting that this is a shared, but relatively delayed, response to proteasome inhibition. However, activation of hsp27 through mitogen-activated protein kinase-dependent phosphorylation of Ser82 (40) was only observed in RPMI 8226 cells.

The accumulation of cyclin B1 and p21 in HT-29 cells is consistent with the initial cell cycle arrest observed in these cells. However, in RPMI 8226 cells, the accumulation of p21 is apparently insufficient to maintain a growth arrest, presumably due to stabilization of other proteins and activation of dominant proapoptotic pathways. The precise mechanistic differences responsible for the greater sensitivity to proteasome inhibition and more rapid apoptotic induction seen in the hematologic tumor cell lines relative to solid tumor cell lines remain to be established.

**Prolonged exposure to PR-171 or bortezomib increases cytotoxicity.** Extending PR-171 and bortezomib treatment from 1 to 72 h (conditions that would be difficult to replicate in vivo due to their rapid clearance) resulted in greater cytotoxicity for both compounds and eliminated the differentials between the two molecules and between the different cell types (Table 2). Extended treatment with bortezomib or PR-171 has also been shown to have potent antiproliferative and proapoptotic effects on primary human acute myeloid leukemia cells (41). Suppression of proteasome activity recovery could be responsible for the increases in cytotoxicity observed with extended exposure. Alternatively, because both PR-171 and bortezomib are covalent inhibitors, a time-dependent increase in the degree of proteasome inhibition could contribute to greater cytotoxicity. We have found that extending the length of compound exposure from 1 to 6 h results in greater total proteasome inhibition with both compounds, particularly on the trypsin-like and caspase-like active sites.1 The inhibition of multiple proteasome active sites suppresses total cellular protein turnover to a greater extent than inhibition of the chymotrypsin-like site alone (29) and may therefore result in greater cytotoxicity.

**PR-171 is cleared rapidly but promotes a widespread pharmacodynamic response in rodents.** In rats, PR-171 was rapidly cleared from the plasma compartment following i.v. administration. Noncompartmental analysis of PR-171 pharmacokinetics revealed an average terminal plasma half-life of ~15 min. The estimated steady-state volume of distribution was much larger than blood volume, suggesting extensive penetration of PR-171 into peripheral tissues. To determine the pharmacodynamics of PR-171, blood and tissues isolated from rats 1 h after drug administration were analyzed for proteasome chymotrypsin-like activity. PR-171 induced a dose-dependent suppression of activity in all tissues examined except the brain (Fig. 3A), suggesting that despite rapid entry into cells, the compound does not readily cross the blood-brain barrier. Liver was relatively insensitive to the pharmacodynamic effects of PR-171 perhaps due to the competition between drug metabolism and target binding. As expected, the same tissues...
that showed chymotrypsin-like inhibition also showed extensive penetration by $[^3H]$-PR-171 (Fig. 3B). A similarly rapid clearance (19) and widespread tissue distribution (33, 42) has been reported for bortezomib. Although the pharmacokinetics and tissue pharmacodynamics of salinosporamide A have not been thoroughly characterized, its instability in aqueous environments (42) is likely to result in brief exposure in animals as well. Unlike PR-171 and bortezomib, i.v. administered salinosporamide A inhibits proteasome activity in the brain (42).

The in vivo proteasome active site selectivities of PR-171 and bortezomib were examined in mice 1 h after i.v. drug administration (Fig. 3C). At doses that induced >80% inhibition of chymotrypsin-like activity in blood, bortezomib, but not PR-171, was able to mediate pronounced inhibition of the caspase-like activity. Neither compound had significant activity on the trypsin-like activity. These results are consistent with the active site selectivity profiles observed in cells (Fig. 1B). Chauhan et al. (20) have reported similar results with bortezomib and have also shown that salinosporamide A, at a dose that fully inhibits proteasome chymotrypsin-like activity in blood, can also partially suppress the trypsin-like and caspase-like activities.

**Proteasome activity recovers in tissues following PR-171 administration.** To evaluate the recovery of proteasome activity in animals, tissues were examined at varying time points after i.v. PR-171 administration. With the exception of whole blood, proteasome activity recovered by 50% to 100% within 24 h following PR-171 dosing in all tissues examined in both mice (Fig. 3D) and rats (Supplementary Fig. S2 and data not shown). In whole blood, tissues were examined at varying time points after i.v. PR-171 and bortezomib, i.v. administered salinosporamide A inhibits proteasome activity in the brain (42).

**Table 1. Selective inhibition of the proteasome chymotrypsin-like activity by PR-171**

<table>
<thead>
<tr>
<th>Active site</th>
<th>Chymotrypsin-like</th>
<th>Caspase-like</th>
<th>Trypsin-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitory parameter</td>
<td>$k_{\text{max}}/K_i$ (M$^{-1}$ s$^{-1}$)</td>
<td>1 h $IC_{50}$ (nmol/L)</td>
<td>$k_{\text{max}}/K_i$ (M$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>Proteasome preparation</td>
<td>20S</td>
<td>20S</td>
<td>Cell lysate</td>
</tr>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR-171</td>
<td>33,000</td>
<td>6 ± 2</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>38,000</td>
<td>7 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>C也要-lactacystin</td>
<td>ND</td>
<td>62 ± 18</td>
<td>24 ± 44</td>
</tr>
<tr>
<td>β-lactone</td>
<td>ND</td>
<td>3.5 ± 0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

**NOTE:** Proteasome activity was measured spectrofluorometrically by monitoring the hydrolysis of Leu-Leu-Val-Tyr-AMC (chymotrypsin-like), Leu-Leu-Arg-Arg-AMC (trypsin-like) substrates. Values reported are the mean ± SD from ≥4 determinations.

*IC$_{50}$ values are the mean ± SD from ≥4 determinations.

**Table 2. Cytotoxicity profiles of PR-171 and bortezomib**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell line</th>
<th>Origin</th>
<th>Cell viability IC$_{50}$ (nmol/L)</th>
<th>Cell viability IC$_{50}$ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 h treatment/72 h washout</td>
<td>72 h treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PR-171</td>
<td>Bortezomib</td>
</tr>
<tr>
<td>Hematologic tumor</td>
<td>RPMI 8226</td>
<td>Multiple myeloma</td>
<td>71 ± 22</td>
<td>303 ± 52</td>
</tr>
<tr>
<td></td>
<td>HS-Sultan</td>
<td>B-cell lymphoma (Burkitt’s)</td>
<td>135 ± 30</td>
<td>454 ± 60</td>
</tr>
<tr>
<td></td>
<td>Molt4</td>
<td>Acute lymphoblastic leukemia</td>
<td>31 ± 17</td>
<td>126 ± 52</td>
</tr>
<tr>
<td></td>
<td>RL</td>
<td>B-cell lymphoma (NHL)</td>
<td>164 ± 92</td>
<td>814 ± 476</td>
</tr>
<tr>
<td>Solid tumor</td>
<td>HT-29</td>
<td>Colorectal adenocarcinoma</td>
<td>350 ± 84</td>
<td>2,190 ± 390</td>
</tr>
<tr>
<td></td>
<td>MiaPaCa-2</td>
<td>Pancreatic carcinoma</td>
<td>1,110 ± 240</td>
<td>&gt;6,500</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>Lung carcinoma</td>
<td>1,200 ± 900</td>
<td>4,600 ± 1,900</td>
</tr>
<tr>
<td>Nontransformed</td>
<td>NHDF</td>
<td>Normal skin</td>
<td>389 ± 128</td>
<td>2,460 ± 1,120</td>
</tr>
<tr>
<td></td>
<td>HUVEC</td>
<td>Normal umbilical vein</td>
<td>455 ± 45</td>
<td>3,510 ± 390</td>
</tr>
</tbody>
</table>

**NOTE:** Cell viability was measured with CellTiter-Glo reagent either after continuous compound exposure for 72 h or with 1 h compound exposure followed by a 72 h washout period. Values reported are the mean ± SD from ≥3 determinations.
a much slower rate of proteasome activity recovery was observed following PR-171 treatment (<50% recovery after 1 week), highlighting the impact of an irreversible mechanism of action in a cell type (erythrocytes) that cannot recover activity by making new proteasomes. Salinosporamide A, due to its irreversible mechanism (43), also induces proteasome inhibition in blood that recovers slowly (20). In contrast, complete recovery of proteasome activity in blood was achieved by 48 h following bortezomib dosing (Fig. 3D and ref. 20) due to its slowly reversible mechanism of action. In tissues, recovery from bortezomib-mediated inhibition was comparable to PR-171 (t1/2 ~ 24 h), but faster than blood recovery, suggesting that new proteasome synthesis, and not the slow reversibility of bortezomib, plays a dominant role in proteasome activity recovery in tissues other than whole blood.

In rats, proteasome inhibition and recovery was also examined following two (QDx2) or five (QDx5) consecutive daily doses of PR-171 (Supplementary Fig. S2). There were no significant differences in either the level of inhibition or rate of recovery of chymotrypsin-like activity in bone marrow following QDx2 dosing or QDx5 dosing as compared with a single dose. In contrast, a cumulative inhibition of chymotrypsin-like activity was evident in blood following QDx2 dosing which was even more pronounced following QDx5 dosing of PR-171. This cumulative inhibition is likely due to the inability of erythrocytes to recover activity between doses.

**Daily dosing of PR-171 is well tolerated.** Despite the sustained proteasome inhibition in erythrocytes and repeated suppression of proteasome activity in other tissues achieved with the QDx5 schedule, no changes in hematocrit or hemoglobin concentration were observed, and no significant weight loss was noted in animals at doses up to 2 mg/kg (data not shown). These results show that repeated administration of PR-171 in rodents for up to 5 consecutive days is well tolerated even at doses resulting in peak inhibition of the proteasome chymotrypsin-like activity in excess of 80% in blood and most other tissues (see Fig. 3A). Daily dosing schedules that prevent full recovery of proteasome activity between doses are more intensive than the recommended clinical dosing schedule for bortezomib and the preclinical dosing schedule tested with salinosporamide A (biweekly day 1/day 4; refs. 8, 18, 20). As noted above, it is possible that the greater biochemical selectivity of PR-171 for the chymotrypsin-like activity of the proteasome may contribute to greater tolerability in vivo. Other factors, including biodistribution differences, could also contribute to distinct safety profiles for the various proteasome inhibitors.

![Figure 2](image-url)
Intensive PR-171 dosing results in improved antitumor efficacy in human tumor xenograft models. The antitumor activity of PR-171 was evaluated and compared with bortezomib in BNX mice bearing established human tumor xenografts derived from three tumor cell lines: HT-29 (colorectal adenocarcinoma; Fig. 4A), RL (B cell lymphoma; Fig. 4B), and HS-Sultan (Burkitt's lymphoma; Fig. 4C). All PR-171 dosing schedules (up to 5 mg/kg delivered weekly QDx2) were tolerated in the tumor-bearing animals, resulting in weight loss of <10% (data not shown). The bortezomib dose (1 mg/kg on a biweekly day 1/day 4 schedule) was determined to be the maximum tolerated dose in this mouse strain (see also ref. 44). In all three models, 5 mg/kg PR-171 delivered i.v. on a weekly QDx2 schedule was more efficacious than 1 mg/kg bortezomib delivered i.v. on its standard clinical schedule (biweekly day 1/day 4). When the same 5 mg/kg dose of PR-171 was given on a day 1/day 4 schedule or combined into a single weekly 10 mg/kg dose, efficacy was eliminated in the HT-29 model. In addition, lowering the PR-171 dose to 3 mg/kg on the weekly QDx2 schedule reduced efficacy in both the RL and HS-Sultan models. These results show that the activity of PR-171 is dose and schedule dependent. Both bortezomib and salinosporamide A are efficacious in other xenograft models, including multiple myeloma models (20, 21, 33, 44), but neither has been tested with a weekly QDx2 dosing schedule.

At the doses used in our efficacy studies, both PR-171 and bortezomib suppressed proteasome activity in blood and adrenals to an equivalent extent (Fig. 4D). This suggests that the improved antitumor activity of PR-171 delivered on the weekly QDx2 schedule (relative to biweekly day 1/day 4 dosing of either PR-171 or bortezomib) may be due to suppression of proteasome activity recovery between doses. Antitumor efficacy was achieved with PR-171 despite the fact that proteasome activity was inhibited...
in HS-Sultan tumors to a lesser extent than other tissues (Fig. 4D).

Similar observations of low tumor proteasome inhibition have been reported with both bortezomib (33, 44) and salinosporamide A (42). This raises the possibility that mechanisms other than direct cytotoxicity to the tumor cells may contribute to antitumor efficacy. One such mechanism could be an antiangiogenic activity, as has been observed with bortezomib (13). Alternative explanations for the low tumor pharmacodynamic response at efficacious
doses include enhanced sensitivity of the tumor cells in vivo to proteasome inhibition or high local proteasome inhibition within critical subdomains of the tumor (due to heterogeneous penetration by the drug; ref. 45).

Taken together, the results described in the present study show that the novel proteasome inhibitor PR-171 has several in vitro and in vivo properties that distinguish it from bortezomib and salinosporamide A. Most importantly, PR-171 can be delivered with intensive daily dosing schedules that inhibit proteasome activity by >80% in most tissues without excessive toxicity. Furthermore, our xenograft studies show that more intensive dosing schedules can yield greater efficacy in both solid and hematologic tumor models. Supported by the preclinical results presented here, two phase I clinical trials in multiple myeloma and NHL patients have been initiated with PR-171 comparing two dose-intensive schedules (QD×2 and QD×5). The extent to which the distinct in vitro and in vivo properties of PR-171, salinosporamide A, and bortezomib impact clinical efficacy or safety will be determined in these ongoing or upcoming trials.

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