Pralatrexate Is Synergistic with the Proteasome Inhibitor Bortezomib in In vitro and In vivo Models of T-Cell Lymphoid Malignancies

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Abstract

Purpose: Pralatrexate (10-propargyl-10-deazaaminopterin) is an antifolate with improved cellular uptake and retention due to greater affinity for the reduced folate carrier (RFC-1) and folyl-polyglutamyl synthase. Based on the PROPEL data, pralatrexate was the first drug approved for patients with relapsed and refractory peripheral T-cell lymphoma. Bortezomib is a proteasome inhibitor that has shown some activity in patients with T-cell lymphoma.

Experimental Design: Assays for cytotoxicity including mathematical analysis for synergism, flow cytometry, immunoblotting, and a xenograft severe combined immunodeficient-beige mouse model were used to explore the in vitro and in vivo activities of pralatrexate alone and in combination with bortezomib in T-cell lymphoid malignancies.

Results: In vitro, pralatrexate and bortezomib exhibited concentration- and time-dependent cytotoxicity against a broad panel of T-lymphoma cell lines. Pralatrexate showed synergism when combined with bortezomib in all cell lines studied. Pralatrexate also induced potent apoptosis and caspase activation when combined with bortezomib across the panel. Cytotoxicity studies on normal peripheral blood mononuclear cells showed that the combination was not more toxic than the single agents. Western blot assays for proteins involved in broad growth and survival pathways showed that p27, NOXA, HH3, and RFC-1 were all significantly modulated by the combination. In a severe combined immunodeficient-beige mouse model of transformed cutaneous T-cell lymphoma, the addition of pralatrexate to bortezomib enhanced efficacy compared with either drug alone.

Conclusion: Collectively, these data suggest that pralatrexate in combination with bortezomib represents a novel and potentially important platform for the treatment of T-cell malignancies.

Malignancies derived from mature (post-thymic) T cells and natural killer cells, collectively referred to as peripheral T-cell lymphomas (PTCL), encompass a variety of rare and often challenging diseases. PTCLs represent 10% to 15% of all non–Hodgkin’s lymphomas worldwide (1), accounting for 6,000 to 9,000 cases annually in the United States. CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) and CHOP-like chemotherapy programs are still the most commonly used regimens despite the suboptimal outcomes. Overall, CHOP-based chemotherapies achieve overall response rates of 30% to 60% with an overall survival at 5 years of approximately 15% to 20%. Efforts to improve on these approaches with more dose-intense combination chemotherapy regimens (2, 3) have failed to show a benefit (62% versus 56%, respectively). Given the often dismal results seen in patients with PTCL compared with other subtypes of non–Hodgkin’s lymphoma, there is an urgent need to identify new agents with demonstrable activity in these T-cell neoplasms. Over the past few years, a number of promising new drugs have emerged, which seem to have marked single-agent activity in T-cell lymphomas, including gemcitabine (4–7), alemtuzumab (8, 9), and the histone deacetylase (HDAC) inhibitors (10). More recently, pralatrexate, a novel antifol with improved cellular uptake and retention, has become the first drug approved by the U.S. Food and Drug Administration for the treatment of relapsed/refractory PTCL.

The results of the PROPEL registration study based on 109 evaluable patients showed an overall response rate...
of 29% in patients with very heavily treated disease. Subgroup analyses have shown that the activity of pralatrexate was equivalent irrespective of the amount of prior therapy, age, prior autologous stem cell transplant, or subtype of PTCL. Pralatrexate belongs to the class of folate analogues known as 10-deazaaminopterin, which have shown markedly superior antitumor effects compared with methotrexate in severe combined immunodeficient (SCID)-beige xenograft models of lymphoma (11, 12). Pralatrexate was designed to have greater affinity than methotrexate for the natural folate and antifolate principal transporters reduced folate carrier-1 (RFC-1; an oncofetal protein) and folyl-polyglutamyl synthase, leading to enhanced intracellular accumulation and polyglutamylation in tumor cells.

Recent preclinical studies by our group have established that pralatrexate synergizes with other agents active in PTCL, including gemcitabine. These preclinical data (13) have been confirmed in a phase 1 clinical trial exploring the schedule-dependent activity of pralatrexate and gemcitabine in patients with non-Hodgkin's lymphoma. In addition to PTCL, pralatrexate seems to be very active in patients with drug-resistant cutaneous T-cell lymphoma (CTCL), producing an overall response rate of 35% in patients who received a median of 6 prior regimens (range, 1-25; ref. 14). Collectively, these data suggest that pralatrexate exhibits marked activity across different subtypes of T-cell neoplasms and could combine favorably with a host of other agents known to be active in T-cell lymphoma. The observation that bortezomib has now been shown to exhibit significant activity across both CTCL and PTCL raises the interesting prospect that these agents could also be combined in an effort to define a new platform for these diseases that is not CHOP dependent. For example, Zinzani et al. (15) have reported that bortezomib produces an ORR of 67% in patients with relapsed or refractory CTCL and PTCL, including a complete response rate of 17%.

Given the relatively poor prognosis of patients with T-cell malignancies and the relatively poor outcomes associated with conventional chemotherapy regimens such as CHOP, there is now a strong rationale to begin to combine and integrate these targeted agents into novel combination treatment regimens for patients with T-cell malignancies that are not CHOP based. Based on this rationale and the available clinical data, we sought to explore the preclinical activity of pralatrexate and bortezomib in models of T-cell lymphoma.

**Materials and Methods**

**Cells and cell lines**

H9 (16) and HH (17) are CTCL cell lines, obtained from the American Type Culture Collection. P12, PF382, and CEM (18) are T-acute lymphoblastic leukemia (T-ALL) lines resistant to γ-secretase inhibitors, and KOKT-K1 (19), DND-41, and HPB-ALL (20) are T-ALL lines sensitive to γ-secretase inhibitors. All leukemic cell lines were provided by the laboratory of Dr. Adolfo Ferrando (Columbia University, New York, NY). Peripheral blood mononuclear cells (PBMC) from healthy donors were purchased from AllCells. All cell lines were grown as previously described (21).

**Materials**

All reagents for Western blotting were obtained from Bio-Rad Laboratories and Pierce Biotechnology, Inc.; DMSO was obtained from Sigma. Pralatrexate was from Allos Therapeutics, and bortezomib from Millennium.

**Cytotoxicity assays**

For all in vitro assays, cells were counted and resuspended at an approximate concentration of 3 × 10^6 per well in a 96-well plate (Becton Dickinson Labware) and incubated at 37°C in a 5% CO2 humidified incubator for up to 72 hours. Pralatrexate was added at concentrations from 100 pmol/L up to 200 nmol/L, whereas bortezomib was tested at concentrations from 1 to 100 nmol/L to determine growth inhibition curves for all cell lines. In combination experiments, pralatrexate was added at concentrations of 2 to 5.5 nmol/L, and bortezomib at 3 to 6 nmol/L. These concentrations were selected to approximate the IC_{50,50} (inhibitory concentration of 25-50% cells for each drug) for up to 72 hours. Following incubation at 37°C in a 5% CO2 humidified incubator, 100 μL from each well were transferred to a 96-well opaque-walled plate; CellTiter-Glo reagent (Promega Corporation) was used according to the manufacturer’s instruction. The plates were allowed to incubate at room temperature for 10 minutes before recording luminescence with a Synergy HT Multi-Detection Microplate Reader (Biotek Instruments, Inc.). Each experiment was done in triplicate and repeated at least twice.

**Flow cytometry for apoptosis**

H9, HH, P12, and PF382 cells were seeded at a density of 3 × 10^5/mL and incubated with pralatrexate (2-5.5 nmol/L) and bortezomib (2-6 nmol/L) alone or in combination for 48 or 72 hours. A minimum of 1 × 10^5 events were acquired from each sample. To quantitate apoptosis, Yo-Pro-1 and propidium iodide (PI) were used (Vybrant apoptosis assay kit #4, Invitrogen) according to the manufacturer’s instruction. The fluorescence signals acquired by a FACSCalibur System were resolved by detection in the conventional FL1 and FL3 channels. Cells were considered early apoptotic if Yo-Pro-1 positive but PI negative, late apoptotic if Yo-Pro-1 and PI positive, and dead if only PI positive.

**Caspase activation assays**

H9 cells were seeded at a density of 1 × 10^5/mL and incubated with pralatrexate and bortezomib alone or in combination at 4 to 6 nmol/L. After incubation for up...
to 24 hours, aliquots of 300 μL were obtained from each sample and transferred into Eppendorf tubes. One micro-liter of FITC-IETD-FMK (CaspGLOW Fluorescin Active Caspase-8, Biovision) and 1 μL of Red-LEHD-FMK (CaspaseGLOW red Active Caspase-9, Biovision) were added to each tube. A negative control, the caspase inhibitor Z-VAD-FMK (1 μL/mL), and a positive control, etoposide (100 nmol/L), were also evaluated in each experiment. The protocol followed the manufacturer’s instruction. All data from flow cytometry were analyzed by FlowJo software. Each experiment was done at least in duplicate and repeated at least twice. Data are presented as average ± SD.

Western blot analysis

Cells were incubated with the same concentrations of pralatrexate and/or bortezomib used in the apoptosis and caspase assays under normal growth conditions for up to 24 hours. Proteins from total cell lysates were resolved on 4% to 20% SDS-PAGE and transferred onto nylon membranes. Membranes were blocked in PBS, 0.05% Tween 20 containing 5% skim milk powder and were then probed overnight with specific primary antibodies. Antibodies were detected with the corresponding horseradish peroxidase–linked secondary antibodies. Blots were developed using SuperSignal West Pico chemiluminescent substrate detection reagents. The membranes were exposed to X-ray films for various time intervals. The images were captured with a GS-800 calibrated densitometer (Bio-Rad), and the ratios were quantified by densitometric analyses within the linear range of each captured signal. The following monoclonal and polyclonal antibodies were used: p27, p100/p52, and acetylated histone H3 (from Cell signaling monoclonal and polyclonal antibodies were used: p27, p100/p52, and acetylated histone H3 (from Cell Signaling Technology), Noxa (Imgenex Corp.), RFC-1 (SLC19A1 antibody from abcam), and acetylated α-tubulin (from Sigma).

Mouse xenograft models

In vivo experiments were done as follows: 5- to 7-week-old SCID-beige mice (Charles River Laboratories) were injected s.c. with 20 × 106 HH cells on the flank. When tumor volumes approached 50 mm³, mice were separated into treatment groups of 9 to 10 mice each. Tumors were assessed using the two largest perpendicular axes (l, length; w, width) measured with standard calipers. Tumor volume was calculated using the formula (4/3)πr³, where r = (l + w) / 4. Tumor-bearing mice were assessed for weight loss and tumor volume at least twice weekly. Animals were sacrificed when one-dimensional tumor diameter exceeded 2.0 cm or after loss of >10% body weight in accordance with institutional guidelines. Complete response (CR) was defined as nonpalpable tumor. Both pralatrexate and bortezomib were given by i.p. injection. In the combination experiments, pralatrexate was administered at 15 mg/kg (1/4 of the maximum tolerated dose) on days 1, 4, 8, and 11; bortezomib (B) was administered at 0.5 mg/kg on days 1, 4, 8, and 11. Control groups were treated with the vehicle solution alone.

Statistical analysis

For the different in vitro experimental groups, permutation tests were done to determine whether any of the experimental groups was superior to a control group. The analysis entailed comparing groups based on repetitions (typically 3) using ANOVA after a normalizing transformation. Because multiple hypotheses were simultaneously tested, all P values were adjusted using the Dunnett method (22). For each cell line, the IC50 (refs. 23, 24) and the drug-drug interaction in terms of synergism, additivity, or antagonism were computed using the CalcuSyn software (Biosoft; ref. 25) for the cytotoxicity assay. A combination index (CI) of <1 defines synergism, CI = 1 additivity, and CI > 1 antagonism. For the apoptosis data, the drug-drug interactions were computed using the relative risk ratio (RRR) analysis (GraphPad; http://www.graphpad.com) with RRR < 1 defining synergism, RRR = 1 additivity, and RRR > 1 antagonism. For the mouse experiments, the tumor volumes and areas under the curve were log transformed and evaluated using ANOVA for four-way comparison and Wilcoxon test for pairwise comparisons. Median absolute deviation was used as a measurement of variability.

Results

Pralatrexate interacts synergistically with bortezomib in T-cell lymphoma lines

The IC50 values for pralatrexate alone at 48 and 72 hours were generally in the low nanomolar range. Specifically, these values (nmol/L) at 48 and 72 hours, respectively, are as follows: H9, 1.1 and 2.5; P12, 1.7 and 2.4; CEM, 3.2 and 4.2; PF-382, 5.5 and 2.7; KOPT-K1, 1 and 1.7; DND-41, 97.4 and 1.2; and HPB-ALL, 247.8 and 0.77. HH was relatively resistant after 48 hours of exposure, with the IC50 at 72 hours being 2.8 nmol/L (Table 1; Fig. 1A). Two CTCL lines (H9 and HH) and two T-ALL cell lines (P12 and PF-382) were selected for combination experiments with bortezomib. Generally, the range of IC50s for bortezomib in these cells was more similar. For example, the IC50s for bortezomib alone at 48 hours were as follows: H9, 6 nmol/L; HH, 3.9 nmol/L (after 72 hours of incubation); P12, 4.7 nmol/L; and PF382, 2.2 nmol/L. In the cytotoxicity assays, the combination of pralatrexate and bortezomib showed synergism in all cell lines: H9, CI ≤ 0.38; P12, CI ≤ 0.513; PF382, CI ≤ 0.352; and HH, CI ≤ 0.4 (Fig. 1A and B). These data support the mathematical synergy of these two agents in vitro.

Pralatrexate plus bortezomib enhances apoptosis in T-cell lymphoma

Treatment with the combination of pralatrexate and bortezomib for 48 hours exhibited significant induction of apoptosis in H9, P12, PF382, and HH cell lines. For example, treatment of the CTCL line H9 with bortezomib alone at 5 nmol/L induced apoptosis in up to 28.6% of the population. When H9 cells were treated with pralatrexate at 3 or 4 nmol/L (IC50, 46%) and 58% of the cells,
respectively, became apoptotic. The combination of pralatrexate (3 or 4 nmol/L) and bortezomib (5 nmol/L) produced more than 70% induction of apoptosis, and the RRR for the combination of pralatrexate (3 nmol/L) and bortezomib was ≤0.8. Analysis of various pralatrexate and bortezomib concentrations revealed that the RRR seemed to be more sensitive to the relative concentration of bortezomib. For example, 53% of H9 cells were apoptotic when treated with 6 nmol/L bortezomib, whereas 46% and 58% of H9 cells were apoptotic when treated with 3 and 4 nmol/L of pralatrexate, respectively. Treatment of H9 with 6 nmol/L bortezomib and the highest concentrations of pralatrexate (4 nmol/L) produced nearly 90% induction of apoptosis. Furthermore, the RRR for the latter combination was <0.6. Importantly, all combinations studied were statistically significant, favoring the combination over the control and any single-agent exposure (Fig. 2A; Table 2). In one of the more resistant cell lines, the CTCL HH line, the combination was superior to any single agent (Fig. 2A; Table 2). In the cytotoxicity and apoptosis assays described above and confirmed that caspase activation with pralatrexate and bortezomib concentrations revealed that the RRR made in the cytotoxicity assays demonstrated that caspase activation with the combination groups did not show any significant increase in apoptosis compared with the bortezomib-alone group. Importantly, all combinations studied were statistically significant, favoring the combination over the control and any single-agent exposure (Fig. 2A; Table 2).

Table 1. Cytotoxicity of pralatrexate and methotrexate in eight T-cell lymphoma and leukemia cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subtype</th>
<th>Pralatrexate (nmol/L)</th>
<th>Methotrexate (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 48 h (c.i.)</td>
<td>IC50 72 h (c.i.)</td>
<td>IC50 48 h (c.i.)</td>
</tr>
<tr>
<td>H9</td>
<td>CTCL</td>
<td>1.13 (0.1-8.9)</td>
<td>2.54 (0.5-12.9)</td>
</tr>
<tr>
<td>HH</td>
<td>N/A</td>
<td>2.8 (0.3-29.2)</td>
<td>N/A</td>
</tr>
<tr>
<td>P12</td>
<td>T-ALL (resistant to GSI)</td>
<td>1.73 (1.7-1.74)</td>
<td>2.43 (1.6-3.6)</td>
</tr>
<tr>
<td>CEM</td>
<td>T-ALL (sensitive to GSI)</td>
<td>3.23 (1.6-6.5)</td>
<td>4.16 (3.8-4.6)</td>
</tr>
<tr>
<td>PF382</td>
<td>T-ALL</td>
<td>5.47 (4.3-6.9)</td>
<td>2.72 (1.9-3.9)</td>
</tr>
<tr>
<td>KOPT-K1</td>
<td>T-ALL</td>
<td>1 (0.8-1.3)</td>
<td>1.69 (1.3-2.2)</td>
</tr>
<tr>
<td>DND-41</td>
<td>CTCL</td>
<td>97.37 (25.38-368.2)</td>
<td>1.21 (1-1.3)</td>
</tr>
<tr>
<td>HPB-ALL</td>
<td></td>
<td>247.78 (60.6-1012.9)</td>
<td>0.77 (0.5-1.1)</td>
</tr>
</tbody>
</table>

NOTE: The table presents the IC50 values with the confidence interval for pralatrexate and methotrexate after 48 and 72 h of incubation. In most of the cell lines, the IC50 values at 48 and 72 h of incubation are in the low nanomolar range. There is minimal cytotoxic activity appreciated at 24 h in all the cell lines studied.

Abbreviations: c.i., confidence interval; GSI, γ-secretase inhibitor.

Pralatrexate enhances the activity of bortezomib in inducing caspase-8 and caspase-9 activation

To evaluate the effect of the combination on the extrinsic and intrinsic arms of the apoptotic cascade, we explored the effect of pralatrexate and bortezomib on the activation of caspase-8 and caspase-9 in the CTCL line H9. Incubation of H9 with pralatrexate alone at 4 nmol/L, bortezomib alone at 6 nmol/L, and the combination for 24 hours resulted in activation of caspase-8 in 14%, 10%, and 37% of cells, respectively. Similarly, activation of caspase-9 was seen in 13%, 5%, and 39% of H9 cells, respectively (Fig. 4A). These data are again concordant with the observations made in the cytotoxicity and apoptosis assays described above and confirmed that caspase activation with the combination was also synergistic.

Pralatrexate does not increase the toxicity of bortezomib in PBMCS from healthy donors

To assess the toxicity of the combination in normal PBMNC, we also investigated the induction of apoptosis of PBMCS from healthy donors. We explored the effects of bortezomib at 3 and 5 nmol/L, pralatrexate at 2, 3, and 4 nmol/L, and the combination at all the possible permutations after 48 hours of exposure. Importantly, all combination groups did not show any significant increase in apoptosis compared with the bortezomib-alone group or between the combination group and pralatrexate (P > 0.8). Pralatrexate also did not exhibit any increased cytotoxicity relative to the untreated controls (Fig. 4B).
Pralatrexate and bortezomib influence the expression of proteins belonging to different pathways in the intracellular network.

In an effort to identify discrete pathways influenced by the combination not markedly influenced by either drug alone, broad probes of the cell cycle and apoptotic machinery were analyzed by Western blot. Treatment of the H9 and P12 cell lines with bortezomib and pralatrexate revealed changes in a host of proteins known to be involved in cell cycle control, apoptosis, chromatin remodeling (histone acetylation), and cellular transport. Using precisely the same concentrations that effected synergy in the in vitro assays, we identified significant changes in p27, NOXA, HH3, and RCF-1 in H9 after exposure to 5 nmol/L bortezomib, 3 nmol/L pralatrexate, and the combination (after 16, 24, and 48 hours of exposure). These data (Supplementary Data A) showed that bortezomib increased the accumulation of p27, NOXA, and HH3 acetylation in the

![Graph](https://example.com/graph.png)

**Fig. 1.** A, cytotoxicity of pralatrexate in four T-cell lymphoma and leukemia cell lines. The plots graphically show the cytotoxicity curves for pralatrexate after 48 h of incubation in the cell lines selected for the combination studies. B, pralatrexate interacts synergistically with bortezomib in T-cell leukemia and lymphoma cell lines. Isobologram analysis of the interaction between pralatrexate and bortezomib in H9, HH, P12, and PF382. All data points below the red line define synergistic interaction between the two drugs (CI < 1).
CTCL cell line H9, as seen in the bortezomib-alone and the bortezomib plus pralatrexate combination groups. Pralatrexate alone had little to no effect on the accumulation of NOXA and HH3 acetylation, while clearly increasing p27 levels similar to that seen in the bortezomib-alone group. Moreover, pralatrexate seemed to increase the accumulation of RFC-1 in these cells, suggesting that the exposure to pralatrexate may induce an increase in its own intracellular accumulation. Supplementary Data B reveals the expression of p27, NOXA, and HH3 in the T-ALL cell line P12 after exposure to 5 nmol/L bortezomib, 2 and 3 nmol/L pralatrexate, and the possible combinations. These observations were similar to those in the H9 cell line, except that the increase in p27 seemed to be smaller. Whereas these drugs are likely to produce a number of effects on these cell lines at both the transcriptional and the

**Table 2. Range of apoptosis induction for the single drug compared with the combination of pralatrexate and bortezomib in the CTCL and T-ALL lines studied**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Bortezomib (% apoptosis)</th>
<th>Pralatrexate (% apoptosis)</th>
<th>Pralatrexate + bortezomib (% apoptosis – % expected)</th>
<th>RRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H9</td>
<td>5 nmol/L (26-31%)</td>
<td>3 nmol/L (40-51%)</td>
<td>63-73% (58%)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>6 nmol/L (43-63%)</td>
<td>3 nmol/L (40-51%)</td>
<td>73-92% (72%)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>6 nmol/L (43-63%)</td>
<td>4 nmol/L (51-63%)</td>
<td>83-94% (78%)</td>
<td>0.6</td>
</tr>
<tr>
<td>HH</td>
<td>2 nmol/L (28-34%)</td>
<td>3 nmol/L (38-41%)</td>
<td>50-55% (46%)</td>
<td>0.6</td>
</tr>
<tr>
<td>P12</td>
<td>5 nmol/L (50-67%)</td>
<td>2 nmol/L (21-27%)</td>
<td>65-81% (58%)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>5 nmol/L (50-67%)</td>
<td>3 nmol/L (42-55%)</td>
<td>84-92% (76%)</td>
<td>0.7</td>
</tr>
<tr>
<td>PF382</td>
<td>3 nmol/L (60-85%)</td>
<td>5.5 nmol/L (64-67%)</td>
<td>91-98% (89%)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

NOTE: The table displays the range of apoptosis induction for the drugs alone or in combination and the RRR value after the analysis. RRR < 1, synergy.
translational levels, the survey of these proteins suggests that the combination of bortezomib and pralatrexate likely exerts its effect by influencing both cell cycle and apoptotic pathways.

**Pralatrexate enhances the activity of bortezomib in a SCID-beige xenograft model**

The *in vivo* efficacy of pralatrexate combined with bortezomib was investigated in a xenograft model of CTCL (HH; Supplementary Data B). HH was selected because it was the most resistant line based on the *in vitro* assays. After 30 days from the beginning of the experiment, the results in the combination group treated with pralatrexate at a dose of 15 mg/kg (1/4 of the maximum tolerated dose) and bortezomib given on days 1, 4, 8, and 11 at a dose of 0.5 mg/kg were statistically significant compared with pralatrexate alone (*P* = 0.002), bortezomib alone (*P* = 0.001), and the control (*P* = 0.001; Supplementary Data C). Interestingly, CRs were observed only in the combination cohort, where 6 of 10 mice experienced CR in the combination cohort at day 18, with two of those CRs being maintained beyond day 30. Neither significant weight loss nor death was observed in any of the cohorts. These data support the *in vitro* experiments in establishing the superior efficacy of this combination in T-cell malignancies.

**Discussion**

PTCLs are, with few exceptions, considered to be aggressive diseases with a poor prognosis. During the last few years, the proteasome inhibitor bortezomib has been approved by the Food and Drug Administration for the treatment of multiple myeloma and mantle cell lymphoma and has been evaluated in a multicenter phase II clinical trial in patients with CTCL. Albeit a small study, an ORR of 67% including 2 CRs with a duration of response ranging from 7 to 14 months was reported (15). In addition to its single-agent activity, bortezomib has been found to synergize with innumerable agents, including HDAC inhibitors, Bcl-2–targeted agents (26), and conventional cytotoxic agents (27). Similarly, pralatrexate has been shown to exhibit marked activity across a panoply of B- and T-cell malignancies with unique activity in a subset...
of patients with T-cell lymphoma (28). While the precise mechanism for how these drugs complement each other is likely to be multifactorial, these agents seem to exhibit a synergistic interaction in T-cell–derived malignancies based on these preclinical data.

The experiments presented here support the potent activity of pralatrexate in a variety of T-cell lymphoma and leukemia lines. At present, there are no cell lines or xenograft models representative of PTCL. As a result, our studies are restricted to the wider range possible of cell lines of T-cell lineage, including T-ALL and transformed mycosis fungoides (MF).

The cytotoxicity assays suggested IC₅₀ values in the nanomolar range for the cutaneous lymphoma lines (H9 and HH) and the T-cell acute lymphoblastic leukemia lines (P12, PF382, and CEM; KOKPT K1, DND-41, and HPB-ALL) for both drugs. We also confirmed in this panel of T-cell lines that pralatrexate is at least a log more potent than methotrexate (Table 1). This study showed that the exposure of T-cell lines to even nanomolar concentrations of pralatrexate and bortezomib produced potent induction of apoptosis in these lines. For all the cell lines studied (H9, P12, PF382, and HH), the drug-drug interaction

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**Fig. 4.** Caspase-8 and caspase-9 activation in CTCL and lack of enhanced toxicity in PBMCs from healthy donors after treatment with pralatrexate ± bortezomib. A, the histograms show the percentage of cells with caspase-8 and caspase-9 activation after treatment with bortezomib (6 nmol/L) and/or pralatrexate (4 nmol/L) after 24 h of incubation. The histogram called “expected” reveals the degree of caspase activation assuming additivity of pralatrexate and bortezomib. The percentage of caspase-8/caspase-9 activation was calculated based on the percent of the cells with caspase activation in the untreated cells (“0” limit) versus the percentage of cells with caspase activation treated with etoposide (100 nmol/L). B, at the same dilution that showed synergistic effect, PMBCs were treated with bortezomib at 3 or 5 nmol/L and/or pralatrexate at 2, 3, and 4 nmol/L for 48 h. Each combination group was not significantly more cytotoxic than bortezomib given alone (*P > 0.87.*
was described as synergistic based on the calculation of the CI and the RRR. To determine if the two drugs affect their action through the extrinsic or intrinsic apoptotic pathways, flow cytometry was done to analyze the activation of caspase-8 and/or caspase-9–mediated apoptosis. Available published literature has shown that bortezomib itself is able to activate both the extrinsic (caspase-8) and the intrinsic (caspase-9) apoptotic pathways (29). Interestingly, pralatrexate seems to activate the extrinsic and intrinsic pathways of apoptosis as well. The combination of these agents was not only synergistic in conventional cytotoxicity assays but also seems to synergistically activate both pathways of apoptosis. To establish that the synergy appreciated in the tumor cell lines did not extend to normal lymphocytes, we explored the combination in PBMCs from healthy donors. The data showed that essentially the same and even higher concentrations of pralatrexate and bortezomib shown to be synergistic in tumor cell lines had no combined effect on normal PBMCs. The effect of the combination was not statistically more toxic against PBMCs than either drug alone (P > 0.8), although the concentration of bortezomib drove the toxicity against PBMCs.

A xenograft experiment of CTCL (HH) in SCID-beige mice with bortezomib combined with pralatrexate was done and showed a statistically significant advantage for the combination compared with the single agents and the control after 18 days (P ≤ 0.002). Bortezomib was given at a dose of 0.5 mg/kg and pralatrexate was administered at 15 mg/kg (1/4 of the maximum tolerated dose) on days 1, 4, 8, and 11 to maximize activity while minimizing toxicity (13). These doses were selected based on preliminary animal toxicology studies which established that higher doses of pralatrexate (30 and 60 mg/kg) in combination with bortezomib (0.5 mg/kg) were not tolerated. Interestingly, CRs (6 of 10 animals) were only observed in the combination cohort. No significant toxicity (weight loss in excess of 10% or toxic death) was seen in any of the dose cohorts. The combination of bortezomib and pralatrexate at 1/4 of the maximum tolerated dose produced significant activity with minimal toxicity in the xenograft model studied.

Proteasome inhibition is known to affect a diverse array of intracellular signaling pathways, including effects on NF-κB (impaired degradation of IB), cell cycle regulation (accumulation of p21/p27), modulation of Bcl-2 family members (upregulation of proapoptotic and BH3-only members), and accumulation of p53 (30–32). In two recent articles by our group (26, 27), AT-101, a small-molecule inhibitor of Bcl-2, Bcl-XL, and Mcl-1, potently synergized with the conventional agents (cyclophosphamide and rituximab) and the irreversible proteasome inhibitor carfilzomib in in vitro and in vivo models of mantle cell lymphoma and diffuse large B-cell lymphoma. In addition, the BH3-only mimic ABT-737 synergized with the proteasome inhibitor bortezomib, which was mechanistically attributed to an effect on both the relative balance between BAX/BAX binding to antiapoptotic proteins (Bcl-2, Bcl-XL, and Bcl-w) mediated by the BH3-only mimic and marked increases in the BH3-only proteins NOXA and PUMA mediated by bortezomib. It is likely that, whatever the ultimate mechanism of apoptosis induced by pralatrexate, the lowering of the apoptotic threshold with bortezomib serves to sensitize the T-cell lines to the antifol. Despite the multitude of hypotheses about the mechanism of action of proteasome inhibitors, it is difficult to be dogmatic regarding the relative contributions of one mechanism of action over another.

Furthermore, while pralatrexate has marked activity in patients with relatively drug-resistant T-cell lymphoma, the biological basis for the activity seen in T- over B-cell lymphomas remains unclear. In an effort to identify unique biomarkers of activity that might account for the synergy between these two agents, a series of Western blot analyses focused on proteins involved in cell cycle regulation and apoptosis were evaluated. Although there were no significant changes in most of these potential biomarkers, these data suggested that there were four proteins including p27, NOXA, RFC-1, and HH3 that seemed to be favorably modulated by these drugs used as single agents and in combination. Preliminary data have suggested that pralatrexate might actually lead to an increase in the expression of RFC-1 on the target cells, potentially leading to an "autocrine" increase in the intracellular uptake of pralatrexate. Although there was no evidence that bortezomib had any effect on RFC-1, these data support the observation that pralatrexate, at low concentrations, may be able to induce the expression of its own transporter. Pralatrexate treatment also leads to an accumulation of p27 (likely cell cycle arrest effect) seen predominantly in H9 cells, whereas bortezomib increases p27, NOXA, and HH3. Thus, the combination of pralatrexate and bortezomib leads to augmented accumulation of RFC-1, p27, NOXA, and, surprisingly, HH3.

Increase in NOXA is emerging as an interesting potential biomarker of effect for proteasome inhibitors. Its accumulation following exposure to combination therapy may explain potential synergistic effects, although admittedly, it has not shed light on the important upstream pathways. NOXA belongs to the group of Bcl-2 homology domain 3 (BH3) only proteins and seems to be crucial in fine-tuning cell death decisions by targeting the prosurvival molecule Mcl-1 for proteasomal degradation (33). This event seems to be critical for cell death induction in lymphoma cells treated with bortezomib and the combination. Pralatrexate itself has been shown to selectively induce apoptosis in human T-cell lymphotrophic virus 1–infected adult T-cell lymphoma/Pautrier microabcesses from a patient treated with a single dose of drug (34). Biopsies of the patient's skin revealed selective caspase activation and apoptosis only in the malignant cells and not in the normal surrounding keratinocytes. Although NOXA was not specifically evaluated in the tissue biopsies, these important observations confirm in a patient that pralatrexate seems to induce apoptosis via caspase activation selectively in malignant T cells.
The increase in HH3 acetylation is surprising, and suggests that bortezomib may have some properties as a HDAC inhibitor. Previous data by Marcucci et al. have established that bortezomib has an effect as a hypomethylating agent, and there is emerging data that hypomethylating agents may also increase histone acetylation (35). The observation of course raises the suspicion that this doublet of agents could be combined with HDAC inhibitors, which also have significant activity in T-cell lymphomas, but it does not provide specific insights into how this contributes to the cell kill noted.

An emerging number of studies are beginning to converge on the idea that proteasome inhibitors like bortezomib may mediate their effects through NOXA and p27 in T-cell malignancies. In one report by Ri et al. (36), bortezomib induced upregulation of NOXA at both the transcriptional and protein levels in a p53-independent manner in mature T cells (CTCL) and adult T-cell lymphoma/leukemia. Repression of NOXA by small interfering RNA rescued the CTCL cells. These data confirmed that the time-dependent binding of NOXA to Mcl-1 led to loss of the transmembrane mitochondrial potential, an observation we have similarly validated in mantle cell lymphoma as well. These data, coupled with the recent observation by Heider et al. (37) that both HDAC inhibitor and bortezomib led to the upregulation of p21 and p27 in CTCL cells, which synergistically induced apoptosis in these cells, are starting to suggest a possible mechanism for these drugs in T-cell malignancies.

Although the a priori rationale for combining drugs active in T-cell malignancies is at the moment largely focused on agents with clinically documented single-agent activity, it is becoming increasing clear that important themes in the underlying molecular pharmacology are emerging. In fact, it is likely that these themes appreciated at the molecular level and will become the immediate rationale for how to combine select classes of agents where other a priori rationales are limited. A convergence of data around the reproducible observation on the modulation of p21/p27, NOXA, Mcl-2, and, unique to pralatrexate, RFC-1 may begin to provide insights into potential biomarkers of activity and possibly response. Preclinical and clinical studies are now under way to evaluate how best to combine these agents in an effort to develop radically new platforms that are not CHOP-centric for T-cell lymphoma. At the moment, these efforts are oriented toward understanding the drug-drug interactions between pralatrexate, proteasome inhibitors, HDAC inhibitors, gemcitabine, forodesine, and Bcl-2–targeted agents. Phase I clinical trials are now being designed to determine the dose-limiting toxicity and maximum tolerated dose of pralatrexate given in combination with proteasome and HDAC inhibitors. Collectively, both these preclinical and available clinical data suggest that innovative and highly effective platforms can be developed for patients with aggressive T-cell lymphoma.

**Disclosure of Potential Conflicts of Interest**

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