A Phase I/II Trial of Belinostat in Combination with Cisplatin, Doxorubicin, and Cyclophosphamide in Thymic Epithelial Tumors: A Clinical and Translational Study

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Abstract

Purpose: This phase I/II study sought to determine the safety and maximum tolerated dose (MTD) of a novel schedule of belinostat, a histone deacetylase inhibitor (HDAC) administered before and in combination with cisplatin (P), doxorubicin (A), and cyclophosphamide (C) in thymic epithelial tumors (TET). Antitumor activity, pharmacokinetics, and biomarkers of response were also assessed.

Experimental Design: Patients with advanced, unresectable TET received increasing doses of belinostat as a continuous intravenous infusion over 48 hours with chemotherapy in 3-week cycles. In phase II, belinostat at the MTD was used.

Results: Twenty-six patients were enrolled (thymoma, 12; thymic carcinoma, 14). Dose-limiting toxicities at 2,000 mg/m² belinostat were grade 3 nausea and diarrhea and grade 4 neutropenia and thrombocytopenia, respectively, in two patients. Twenty-four patients were treated at the MTD of 1,000 mg/m² with chemotherapy (P, 50 mg/m² on day 2; A, 25 mg/m² on days 2 and 3; C, 500 mg/m² on day 3). Objective response rates in thymoma and thymic carcinoma were 64% (95% confidence interval, 30.8%-89.1%) and 21% (4.7%-50.8%), respectively. Modulation of pharmacodynamic markers of HDAC inhibition and declines in regulatory T cell (Treg) and exhausted CD8⁺ T-cell populations were observed. Decline in Tregs was associated with response (P = 0.0041) and progression-free survival (P = 0.021). Declines in TIM3⁺ CD8⁺ T cells were larger in responders than nonresponders (P = 0.049).

Conclusion: This study identified the MTD of belinostat in combination with PAC and indicates that the combination is active and feasible in TETs. Immunomodulatory effects on Tregs and TIM3⁺ CD8⁺ T cells warrant further study.

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Belinostat plus Chemotherapy for Thymic Epithelial Tumors

Translational Relevance
Belinostat is a histone deacetylase (HDAC) inhibitor that inhibits both HDAC class I and II enzymes in nanomolar concentrations in vitro. Belinostat’s antitumor activity is documented in previously treated patients with thymoma with minimal toxicity. In preclinical studies, belinostat potentiated antitumor activity of platinum-containing chemotherapy. In this phase I/II trial, belinostat was administered as a continuous intravenous infusion before and in combination with cisplatin, doxorubicin, and cyclophosphamide (PAC) treatment in patients with advanced or recurrent thymic epithelial tumors (TET). This trial identified the maximum tolerated dose (MTD) of belinostat in combination with PAC and demonstrated that the combination is active and feasible in TETs. Modulation of pharmacodynamic markers of HDAC inhibition and declines in regulatory T cell (Treg) and exhausted CD8+ T-cell populations were observed with belinostat. Declines in Tregs and TIM3+ CD8+ T cells were associated with responses. The immunomodulatory effects of this novel schedule of belinostat administration need further evaluation.

Belinostat has been shown to potentiate the antitumor activity of platinum-containing agents in vitro (12). Potentiation of chemotherapy by HDAC inhibition is likely secondary to altered gene expression patterns allowing increased chemotherapy sensitivity (13). Chromatin decondensation could also facilitate increased access to DNA-damaging agents and target drug-tolerant subpopulations which evolve via an altered chromatin state in response to stressful exposures such as chemotherapy (14).

Here, we report results of a phase I/II study of belinostat in combination with PAC in the first-line treatment of advanced or recurrent TETs. We also evaluated the pharmacokinetics of belinostat when administered with chemotherapy and pharmacodynamic effects in peripheral blood.

Materials and Methods

Patients
Eligible patients were those more than 18 years of age with histologically confirmed advanced (Masaoka stage III or IV) TETs who had not received prior systemic therapy for advanced disease. Eastern Cooperative Oncology Group (ECOG) performance status score 0 or 1, life expectancy more than 3 months, and adequate organ function. Patients at increased cardiac risk were excluded, including those with unstable angina, myocardial infarction within the previous 12 months, baseline prolongation of QT/QTc interval as demonstrated by repeated QTc interval > 500 ms and long QT syndrome. Other exclusion criteria included: resectable disease, untreated brain metastases, radiotherapy or chemotherapy within 3 weeks before study drug administration, or positive serology for HIV.

The study was approved by the National Cancer Institute Institutional Review Board. The study was overseen by a Safety Monitoring Committee. All patients provided written informed consent to participate in the study before undergoing any study-related procedures. This trial was registered with ClinicalTrials.gov with the identifier NCT01100944.

Study design
Patients received 250 or 500 mg/m² of belinostat via 4 consecutive 12-hour continuous intravenous infusions (CIVI) for 48 hours starting on day 1. At dose levels 1 and 2, patients received the same doses of doxorubicin (25 mg/m² on days 2 and 3, intravenous push over 3–5 minutes), cisplatin (50 mg/m² over 60 minutes intravenous on day 2 after doxorubicin), and cyclophosphamide (500 mg/m² over 60 minutes intravenous on day 3 after doxorubicin). Combination of chemotherapy and belinostat was repeated every 21 days for a total of 6 cycles unless there was evidence of disease progression or intolerance of the study treatment.

The study was divided into 2 phases, a dose-escalation phase (phase I) and an expansion phase (phase II). The phase I portion of the trial consisted of a stepwise increase in
belinostat and chemotherapy using a standard phase I design with 4 planned dose levels to determine the safety and tolerability of this combination. A conventional 3 + 3 dose-escalation design was used with up to 3 additional patients added if one patient exhibited a dose-limiting toxicity (DLT). Dose escalation was halted if at least 2 of a maximum of 6 patients within a cohort exhibited a DLT. Intrapatient dose escalations were not allowed. Supplementary Table S1 contains the dose-escalation schema. Criteria for defining DLT and MTD are in Supplementary Methods.

During phase II, additional patients were enrolled to evaluate antitumor activity, safety, pharmacokinetics, and pharmacodynamics. In this phase, patients were treated at the maximum tolerated dose (MTD). Treatment was discontinued in the event of progressive disease (PD) or unacceptable toxicity.

Patients with non-PD after combination therapy could receive maintenance belinostat every 4 weeks until disease progression or unacceptable toxic effects developed. If, however, disease was felt to be resectable, they were offered multimodality therapy including surgical resection and radiation. Patients with resectable disease did not receive maintenance belinostat.

Safety evaluations
Safety assessments included monitoring for treatment-related adverse events, clinical laboratory tests, vital signs, physical examinations, and 12-lead electrocardiograms (ECG). Adverse events were evaluated in accordance with National Cancer Institute Common Terminology Criteria for Adverse Events, Version 4.0, and were followed to a satisfactory resolution or a clinically stable endpoint.

Pharmacokinetic evaluations
During cycle 1, plasma samples were drawn pretreatment, 0.5, 1, and 2 hours post-start of belinostat infusion on day 1; immediately before, immediately following, and 1-hour post-cisplatin on day 2; immediately before, immediately following, and 1-hour post-cyclophosphamide on day 3, as well as immediately following end of 48-hour belinostat infusion, and 5, 15, 30 minutes, 1, 2, 4, 6, 8, 12, and 24 hours after completion of belinostat infusion. This allowed for steady-state pharmacokinetics and the elimination of belinostat following the end of the 48-hour CITI. Details of pharmacokinetic analyses are in Supplementary Methods.

Pharmacodynamic evaluations
Whole-blood samples were collected in cell preparation tubes (CPT) with sodium citrate (BD Biosciences) on days 1 to 3 of cycle 1 and before treatment on day 1 of cycle 2. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation and viable frozen until analysis. All analyses were performed by multiparametric flow cytometry (MACSQuant, Miltenyi Biotec) and flow cytometric data were analyzed using FlowJo (Tree Star Inc.) software.

PBMCs were analyzed for global protein acetylation, tubulin acetylation (11), and γH2AX expression (15). Regulatory T cells (CD4+CD25hiFoxp3+ and CD8+T cells were evaluated for an immunosuppressive/exhausted phenotype (CTLA-4, T-cell immunoglobulin- and mucin domain-containing molecule 3, TIM3; programmed death-1, PD-1, p21). Details of pharmacodynamic analyses are in Supplementary Methods.

Efficacy evaluations
Baseline tumor assessments were conducted by CT scan within 4 weeks of starting study treatment, and efficacy was evaluated by repeat imaging after every 6 weeks during both combination and maintenance treatments. Tumor assessments were conducted using RECIST version 1.1 (16). Confirmatory scans were obtained 6 weeks following initial documentation of an objective response.

Statistical considerations in design and analysis
The primary objective of phase I was to determine the MTD of belinostat when combined with PAC. The phase I dose-escalation portion of the trial used a standard 3 + 3 design to determine MTD over 4 dose levels of belinostat with varying doses of cytotoxic chemotherapy.

The primary objective of phase II was to determine whether this drug combination was associated with ORRs exceeding those historically seen with PAC in metastatic or recurrent thymomas (7). The phase II used a Simon 2-stage optimal design to rule out an unacceptably low 50% ORR [PR + complete response (CR)], in favor of a moderately high ORR of 75%. With α = 0.10 and β = 0.10, this portion of the study was to initially enroll 12 evaluable patients with thymoma and if 7 or more of the first 12 had a response, then accrual would continue until a total of 28 patients were enrolled in the phase II portion. Patients who were treated at the MTD during phase I were to be included among the initial 12 patients constituting the first stage. If there were 7 to 17 responses in 28 patients, this would be an uninterestingly low ORR, whereas if there were 18 or more responses, it would be sufficiently interesting to warrant further study in later trials. Under the null hypothesis (50% ORR), the probability of early termination was 61%.

Patients with thymic carcinoma, which is less common than thymoma, were to be treated using the identified phase II doses, but they were not to be included in the 2-stage design. Patients with thymic carcinoma were to have a separate ORR determined and reported with 95% confidence interval (CI).

The safety analysis included all patients who received at least one dose of belinostat. Descriptive statistics were used to summarize the pharmacokinetic and pharmacodynamic data and safety. The expected accrual rate was up to 2 patients per month and it was anticipated that the trial would be completed in 2 to 3 years. Additional details of statistical analyses are in Supplementary Methods.

Results
Patient disposition and baseline characteristics
Between April 2010 and June 2013, 26 patients were enrolled in the study. Baseline demographic characteristics
are summarized in Table 1. Eight patients were treated in the dose-escalation phase of the protocol (phase I) which explored 2 dose levels (3 patients at dose level 1, 2 patients at dose level 2, and the last 3 patients at dose level 1) and 18 patients were treated on phase II. In total, 24 patients were treated at the recommended phase II dose (MTD).

All 26 patients received at least one cycle of treatment. The median number of combination treatment cycles administered was 6 (range, 1–6); 20 (77%) patients received the planned 6 cycles. After 5 cycles, treatment was discontinued in both patients who were treated at dose level 2 due to adverse events. Adverse events precluded treatment beyond 4 cycles in 2 more patients. One patient discontinued treatment after one cycle due to treatment delay secondary to viral meningoencephalitis. One patient had PD after 2 cycles in 2 more patients. One patient discontinued treatment because of treatment delay secondary to viral meningoencephalitis. One patient had PD after 2 cycles.

Of 20 patients who completed all 6 cycles of combination treatment and were eligible, only 4 (20%) proceeded to receive maintenance belinostat. As of October 15, 2013, treatment and were eligible, only 4 (20%) proceeded to receive maintenance belinostat. As of October 15, 2013, patients remain on treatment and have received 1 and 3 cycles of belinostat.

After enrolling 26 patients including the first 12 patients with thymoma to complete the first-stage of the 2-stage design, the study was closed because of slower-than-projected accrual.

Efficacy

Table 2 shows responses by histologic cohort. Twenty-five of 26 (96%) patients were evaluable for response. One patient with thymoma, who was treated at dose level 1, was hospitalized for viral meningoencephalitis during the first cycle and was not evaluable for response.

Both patients who were treated at dose level 2, one each with thymoma and thymic carcinoma, had PRs. Among the 23 evaluable patients who were treated at dose level 1, one patient (4%) had a CR, 7 (30%) had PRs, 14 (61%) had SD, and one patient (4%) had PD.

In the overall population of 25 evaluable patients, one patient (4%) had a CR, 9 (36%) had PRs, 14 (56%) had SD, and one (4%) patient had PD. The ORR in the overall population was 40% (95% CI, 21.1%–61.3%) and disease control rate (DCR = SD + PR + CR) of 96% (95% CI, 79.7%–99.9%). The median DOR was 7.4 months (95% CI, 5.2 months to undefined upper limit). After a median potential follow-up of 20.5 months, the median OS was 28.5 months and median progression-free survival (PFS) 9 months.

Among 11 evaluable patients with thymoma which included 10 who were treated at the MTD, one patient (9%) had a confirmed and durable CR: a 61-year-old woman with B2 thymoma who had undergone surgical resection 3 years prior with recurrent disease involving bilateral lung parenchyma achieved a CR after 4 cycles of treatment which lasted for 21 months. There were 6 PRs (55%) and 4 SD (36%). The ORR for thymoma was 64% (7 of 11; 95% CI, 30.8%–89.1%) and DCR was 100% (95% CI, 71.5%–100.0%). The median DOR, PFS, and OS for thymoma were not reached (Fig. 1A and B).

Among 14 patients with thymic carcinoma which included 11 who were treated at the MTD, there were no CRs. Three of 14 patients (21%) had PRs and 10 of 14 (71%) patients had SD. The ORR for thymic carcinoma was 21% (3 of 14; 95% CI, 4.7%–50.8%) and DCR was 93% (13 of 14; 95% CI, 66.1%–99.8%). The median DOR for thymic carcinoma was 7.4 months (95% CI, 4.2–8.5 months). The median PFS and OS were 7.2 and 21.4 months, respectively (Fig. 1A and B). The 6-month probability of PFS was 75%.

Additional treatments

Patients with non-PD after at least 4 cycles of combination chemotherapy were evaluated for multimodality therapy including surgical resection of residual tumor and radiation. Of the 23 patients who were assessed, 12 (52%) were deemed unresectable due to the following reasons: presence of extrathoracic disease (n = 6), co-morbidities (n = 2), myocardial and intracardiac tumor involvement (n = 1), extensive mediastinal involvement (n = 1), poor

Table 1. Demographic and baseline patient characteristics (n = 26)

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Median (range)</th>
<th>53 (23–76)</th>
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</thead>
<tbody>
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<td>Sex, n (%)</td>
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</tr>
<tr>
<td>Women</td>
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<td></td>
</tr>
<tr>
<td>Men</td>
<td>13 (50)</td>
<td></td>
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<tr>
<td>Race, n (%)</td>
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<td>19 (73)</td>
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<td>Black</td>
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<td>0</td>
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</tr>
<tr>
<td>1</td>
<td>8 (31)</td>
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<tr>
<td>Tumor type, n (%)</td>
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<td></td>
</tr>
<tr>
<td>Thymoma</td>
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</tr>
<tr>
<td>B1</td>
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<td>B2</td>
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<td>B3</td>
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<td>Thymic carcinoma</td>
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</tr>
<tr>
<td>Stage at enrollment</td>
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</tr>
<tr>
<td>IVA</td>
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<td></td>
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<tr>
<td>IVB</td>
<td>14 (54)</td>
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<td>Prior surgery*</td>
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</tr>
<tr>
<td>Radical</td>
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</tr>
<tr>
<td>Debunking</td>
<td>2 (8)</td>
<td></td>
</tr>
<tr>
<td>Prior radiation</td>
<td>7 (27)</td>
<td></td>
</tr>
<tr>
<td>Prior neoadjuvant or adjuvant chemotherapy</td>
<td>4 (16)</td>
<td></td>
</tr>
</tbody>
</table>

*A total of 11 patients had radical surgery. Of that number, 2 underwent debunking subsequently.
cardiac function \( n = 1 \), and PD \( n = 1 \). Eleven (48%) patients underwent surgery: 1 patient was found to be inoperable intra-operatively due to extensive epicardial tumor involvement; 1 patient had extensive fibrotic changes which were nonmalignant on intra-operative biopsy; 5 patients had complete resection of all visible tumors with negative margins (R0); 4 patients had complete resection of all visible tumors, but with positive margins (R1). In total, 9 of 23 (39%) patients underwent R0 or R1 resection including 6 patients with thymic carcinoma and 3 patients with thymoma. Eight of these patients received postoperative radiation therapy. Three patients had disease-free intervals of 6 weeks, 4 months, and 18 months after surgery. Six of 23 (26%) patients have no evidence of disease at the time of reporting with follow-up intervals of 4, 6, 10, 15, 20, and 23 months from surgery. Supplementary Table S2 shows the disease and treatment characteristics and outcomes of patients who underwent multimodality therapy.

### Safety

All patients who received at least one cycle of treatment \( n = 26 \) were assessed for toxicity. All treatment-related grade 3/4 adverse events for each dose level and for the overall trial population are listed in Table 3. DLTs were encountered at dose level 2: grade 3 nausea and diarrhea and grade 4 neutropenia and thrombocytopenia, respectively, in 2 patients. Four patients discontinued treatment due to adverse events: 2 patients, both of whom were treated at dose level 2, after cycle 5 due to grade 4 thrombocytopenia and grade 4 neutropenia; one patient who had previously received cisplatin postoperatively, after 4 cycles due to grade 3 hearing loss; one patient after cycle 4 due to grade 3 decrease in left ventricular ejection fraction (LVEF) from 60% at baseline to 30% before cycle 4. The latter, a 76-year-old lady with hypertension, had already required chemotherapy dose reduction of 25% for febrile neutropenia and pulmonary embolism after cycle 1.

Nine (35%) patients required dose reductions of PAC due to toxicities, which included febrile neutropenia \( n = 3 \), thrombocytopenia \( n = 2 \), renal insufficiency \( n = 1 \), pulmonary embolism \( n = 1 \), diarrhea \( n = 1 \), and nausea \( n = 1 \). Six (23%) patients required dose reductions of belinostat for QTc prolongation \( n = 5 \) and bradycardia \( n = 1 \). One patient had persistent QTc prolongation despite 2 dose reductions of belinostat of 25% each during cycles 3 and 4. Hence, further administration of belinostat was discontinued. QTc prolongation was observed in 9 (35%) patients and included one grade 3 event (>500 milliseconds). 2 of these patients had electrolyte abnormalities and dehydration, which could have contributed to QTc prolongation. Adriamycin was discontinued in 2 patients after 3 cycles, as they had received cumulative doses of 450 mg/m².

### Pharmacokinetic evaluations

Supplementary Fig. S1 depicts the mean plasma concentration versus time curve at the 250 mg/m² belinostat dose.

### Table 2. Efficacy

<table>
<thead>
<tr>
<th>Histology</th>
<th>Thymoma</th>
<th>Thymic carcinoma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of evaluable patients</td>
<td>11</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>CR, n (%)</td>
<td>1 (9)</td>
<td>0</td>
<td>1 (4)</td>
</tr>
<tr>
<td>PR, n (%)</td>
<td>6 (55)</td>
<td>3 (21)</td>
<td>9 (36)</td>
</tr>
<tr>
<td>SD, n (%)</td>
<td>4 (36)</td>
<td>10 (72)</td>
<td>14 (56)</td>
</tr>
<tr>
<td>PD, n (%)</td>
<td>0</td>
<td>1 (7)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>ORR, % (95% CI)</td>
<td>64 (30.8–89.1)</td>
<td>21 (4.7–50.8)</td>
<td>40 (21.1–61.3)</td>
</tr>
<tr>
<td>DCR, % (95% CI)</td>
<td>100 (71.5–100.0)</td>
<td>93 (66.1–99.8)</td>
<td>96 (79.7–99.9)</td>
</tr>
<tr>
<td>Median PFS, mo</td>
<td>Not reached</td>
<td>7.2</td>
<td>9.0</td>
</tr>
<tr>
<td>Median OS, mo</td>
<td>Not reached</td>
<td>21.4</td>
<td>28.5</td>
</tr>
</tbody>
</table>

Figure 1. Kaplan–Meier plot showing PFS (A) and OS (B) of patients with thymoma and thymic carcinoma.
During the 48-hour infusion, the plasma concentration of belinostat remained around 1 \( \text{mmol/L} \). Supplementary Table S4 shows belinostat pharmacokinetic data following a 48-hour CIVI on cycle 1. The mean half-life of belinostat (\( n = 25 \)) was 0.47 hours (range, 0.2–0.91 hours; 40% interpatient variability). The mean clearance of belinostat was 133 L/h (range, 41–790 L/h; 106% interpatient variability). After excluding a patient with normal renal and liver function tests who had a clearance of 790 L/h, a statistical outlier, the mean clearance was 106 L/h (range, 41–201 L/h; 36% interpatient variability). The only patient with high serum creatinine (1.43 mg/dL; normal range, 0.5–1.16 mg/dL), indicating mildly impaired kidney function, had a normal belinostat total systemic clearance of 106 L/h.

With only 2 dose levels, and only 1 patient with adequate sampling at dose level 2, conclusions could not be drawn regarding dose proportionality or linearity of pharmacokinetic parameters. There were no statistically significant differences in \( C_{\text{MAX}} \), \( AUC_{\text{INF}} \), clearance, and half-life between different genders and races.

**Pharmacodynamic evaluations**

Histone acetylation. Figure 2A–C show relative changes in total protein hyperacetylation of CD3\(^+\) T cells and CD14\(^+\) monocytes and tubulin-specific hyperacetylation.
of total PBMCs with treatment. Total protein hyperacetylation of CD3+ cells increased significantly after belinostat (\(P < 0.0001\)). Increase ranged between 1.4- and 14.3-fold (median, 2.5-fold) and were observed in 21 of 23 (91%) patients. Although total protein hyperacetylation of CD14+ cells was higher than CD3+ cells at baseline, the magnitude of response to treatment was low. Total protein hyperacetylation of CD14+ cells increased only marginally after belinostat (\(P = 0.026\)). PBMC acetylated tubulin increased significantly after belinostat (\(P < 0.0001\)) and following belinostat plus chemotherapy (\(P = 0.0001\)). Changes in hyperacetylation with treatment were not associated with response or survival.

\(\gamma\)H2AX expression in CD14+ cells increased after belinostat alone (\(P = 0.019\)) and following belinostat plus chemotherapy (\(P < 0.0001\)). Although these changes were not associated with responses, higher \(\gamma\)H2AX expression in CD14+ cells post-belinostat monotherapy, was associated with a trend toward improved OS (\(P = 0.074\)) and PFS (\(P = 0.0058\); results not shown).

**T-cell subsets and expression of immune markers.** Relative changes in the number of Tregs with treatment are shown in Fig. 3A. Tregs decreased significantly following belinostat alone (\(P = 0.0009\)) and following belinostat plus chemotherapy (\(P < 0.0001\)). Declines in Tregs with treatment were significantly larger in patients who responded to treatment than in those who did not (\(P = 0.0041\)). Furthermore, patients with greater decline in Tregs post-combination therapy had improved PFS compared with those who had lesser decreases (median PFS: not reached vs. 7.3 months; \(P = 0.021\); Fig. 3B). There were no significant differences in Treg numbers between thymoma and thymic carcinoma. Although the total Treg numbers declined with treatment, the fraction of CTLA4-expressing Tregs increased significantly after belinostat alone (\(P = 0.077\); Supplementary Fig. S2A). Patients with higher pretreatment CTLA4-expressing Tregs had inferior OS compared with those who had lower CTLA4-expressing Tregs (median OS: 18.8 vs. 37.5 months; \(P = 0.03\); Supplementary Fig. S2B).

Relative changes in TIM3-expressing CD8+ T cells, a phenotype of exhausted CD8+ T cells, with treatment are shown in Fig. 3C. Compared with baseline, TIM3+ CD8+ T cells decreased significantly following belinostat alone (\(P < 0.0001\)) and following belinostat with chemotherapy (\(P < 0.0001\); Supplementary Fig. S2B). Patients with higher pretreatment CTLA4-expressing Tregs had nonstatistically significant improvements in PFS compared with those who had lesser decreases (median PFS: not reached vs. 7.8 months; \(P = 0.081\); Fig. 3D). PD-1+ CD8+ T cells decreased significantly following belinostat alone compared with baseline (\(P = 0.01\); results not shown). No statistically significant associations were noted between PD-1+ CD8+ T-cell changes and response or survival.

**DNA damage.** Supplementary Fig. S3A and S3B show relative changes in \(\gamma\)H2AX expression on CD3+ and CD14+ cells before and after treatment. \(\gamma\)H2AX expression on CD3+ cells increased after belinostat alone (\(P = 0.033\)) and following belinostat plus chemotherapy (\(P = 0.01\)). \(\gamma\)H2AX expression on CD14+ cells did not increase significantly after belinostat alone (\(P = 0.41\)) but was increased after belinostat plus chemotherapy (\(P = 0.012\)). \(\gamma\)H2AX expression was not associated with responses or survival. Supplementary Fig. S3C shows representative \(\gamma\)H2AX foci formation in PBMCs by microscopy.

**Discussion**

This is the first clinical trial to evaluate combination of chemotherapy with an epigenetic modifier in TETs. In this phase I/II study, we evaluated the combination of a novel schedule of belinostat administered as a CIVI before and in
combination with PAC in 3-week cycles in patients with advanced TETs. We established belinostat 1,000 mg/m² over 48 hours administered in combination with PAC as the MTD. The DLT of this regimen were consistent with known side effects of PAC and belinostat and included nausea, diarrhea, neutropenia, and thrombocytopenia.

Responses to monotherapy with HDACi have been observed in advanced hematologic malignancies including T-cell lymphoma, Hodgkin lymphoma, and myeloid malignancies. Solid tumors, in general, have proven refractory to monotherapy with HDACi. Available preclinical and clinical data suggest that most HDACi are optimally used in combination with chemotherapies (12,17), targeted therapies (18), radiation (19), or other epigenetic modifiers (20) rather than as single agents.

Unlike previous studies which used a 30-minute intravenous infusion (days 1–5 on a 21-day cycle), we administered belinostat over 48 hours (21–24). The rationale for CIVI was derived from in vitro studies with belinostat and other HDACi which showed markedly increased gene expression and activity with continuous exposure of 24 or 48 hours. We hypothesized that given the short half-life of belinostat, the daily 30-minute infusions would lead to significant reversal of effects following drug clearance and that a 48-hour CIVI would allow prolonged exposure of belinostat and increased pharmacologic effects. Elimina-
margins in 56% of patients who underwent surgery. Although complete surgical resection is the mainstay of treatment of TETs and is the most important predictor of long-term survival (29), debulking is also reported to provide palliation and prolong survival for selected patients incapable of being rendered completely disease-free (30). Considering that all our patients had surgically unresectable disease at enrolment including many who had disseminated disease and prior radical surgeries, the disease-free survival rate of 26% with multimodality therapy is noteworthy and has not been reported previously. Nevertheless, the benefit, if any, of combining belinostat with chemotherapy in improving resectability is unclear.

Although dose reductions were required in 35% of patients, a median of 6 cycles was administered in patients treated at the MTD. Adverse events were consistent with known toxicities of PAC and belinostat. Dose and schedule of administration-dependent ECG changes including mild-to-moderate QTc prolongation are known class effects of HDACi (10). Although the frequency of QTc interval prolongation seen in this study is comparable to our previous study of single-agent belinostat in TETs (11), it occurred more frequently than in previous studies of belinostat (31). Concurrent risk factors for QTc prolongation in our patient population included drugs associated with QT prolongation (e.g., ondansetron), chemotherapy, and anorexia-related electrolyte changes, prior exposure to anthracyclines (in the adjuvant setting) and radiation. Furthermore, high rates of subclinical cardiac involvement of TETs can by itself predispose to increased cardiac toxicity (32). Close cardiac monitoring is needed when HDACi are administered intravenously in combination with chemotherapy, and patient selection is crucial to minimize concurrent risk factors.

Histone acetylation is the basic epigenetic mechanism whereby HDACi regulate growth arrest, apoptosis, and differentiation. A marked and significant increase in total protein hyperacetylation in CD3+ and CD14+ cells and tubulin-specific hyperacetylation in PBMCs was observed after belinostat and after belinostat plus chemotherapy. Because HDACi induce hyperacetylation of many histone and non-histone proteins, we used a multiparameter flow cytometric assay that detects total protein acetylation rather than just histone acetylation. Increase in tubulin-specific hyperacetylation is indicative of the pan-HDACi effect of belinostat, which is similar to other hydroxamate HDACi such as vorinostat and panobinostat. Although histone acetylation was seen in peripheral blood cells, this did not correlate with clinical response.

Immunosuppressive cells such as Tregs play a major role in maintaining an immunosuppressive milieu in cancer. A number of chemotherapeutic agents including cyclophosphamide have been described to selectively suppress inhibitory cell subsets including Tregs (33). Class I/II HDACi have generally been reported to enhance Treg number and function (34). However, we found a marked and significant decrease in Tregs after belinostat monotherapy. This discrepancy between our observations and previous reports is possibly due to dose-dependent effects of pan-HDACi on Tregs. In vitro data suggest that pan HDACi may target class I HDACs at low doses and impair Treg function, whereas at higher doses, they may target class II HDAC and show a dominant Treg-promoting effect (35).

We found that patients with larger declines in Tregs with treatment were more likely to respond to treatment and had longer PFS. Although previous studies have shown that thymomas have low numbers of Tregs, which are functionally defective (36), the predictive role of Tregs in TETs has not been described previously. Significant decreases after belinostat monotherapy were also observed in TIM3+ and PD-1+ T cells, which exhibit a severe exhausted phenotype as defined by failure to proliferate and produce IL2, TNFα, and IFNγ (37). Blockade of both TIM3 and PD-1 pathways is reported to be more effective in controlling tumor growth than targeting either pathway alone (38). Furthermore, declines in TIM3+ CD8+ T cells with treatment were significantly larger in patients who responded to treatment. Taken together, a beneficial impact of belinostat on Treg and exhausted CD8+ T cells may play an important role in the prognosis of TET. The data presented here suggest that TETs may be amenable to immunomodulatory strategies, including therapeutic effects on Tregs and effector T cells, and that clinical efficacy may be enhanced by a combinatorial immunomodulatory strategy.

The cyclin-dependent kinase inhibitor, p21, promotes cell-cycle arrest in response to many stimuli, and several studies have shown that HDACi strongly activate the expression of p21 (39). The prognostic role of p21 expression in radically resected thymoma has been described by Mineo and colleagues who found an association between low tumoral expression of p21 and reduced disease-free survival (40). Although our data indicate a possible predictive role of p21 expression, given the small sample sizes, the significance of this finding will have to be further evaluated in larger samples. Detection and visualization of γH2AX, the phosphorylated form of the H2AX protein, provides a useful assessment of DNA damage (41). γH2AX expression increased in both CD3+ and CD14+ cells as expected following concurrent chemotherapy and belinostat.

This study was closed before the primary objective of phase II, that is, to improve ORR in thymoma compared with historical controls, was met. Nevertheless, among the patients who enrolled, the ORR was comparable to those of historic controls. In the absence of a randomized comparison, the questions as to whether the observed responses can be entirely explained by chemotherapy alone will remain. In conclusion, this phase I/II trial identified the MTD of PAC and belinostat in combination with PAC and indicates that the combination is active without additive toxicities. In one of the largest series of patients with TETs, this study demonstrates the effectiveness of multimodality therapy in rendering select patients disease-free and suggests the possible predictive role of T-cell populations such as Tregs and TIM3+ CD8+ T cells in TETs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Belinostat Plus Chemotherapy for Thymic Epithelial Tumors

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References


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