Clinical Activity and Safety of Combination Therapy with Temsirolimus and Bevacizumab for Advanced Melanoma: A Phase II Trial (CTEP 7190/Mel47)

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Abstract

Purpose: A CTEP-sponsored phase II trial was conducted to evaluate safety and clinical activity of combination therapy with CCI-779 (temsirolimus) and bevacizumab in patients with advanced melanoma.

Experimental Design: Patients with unresectable stage III to IV melanoma were treated intravenously with temsirolimus 25 mg weekly and bevacizumab 10 mg every 2 weeks. Adverse events were recorded using CTCAE v3.0. Tumor response was assessed by Response Evaluation Criteria in Solid Tumors and overall survival was recorded. Correlative studies measured protein kinases and histology of tumor biopsies and immune function in peripheral blood.

Results: Seventeen patients were treated. Most patients tolerated treatment well, but 2 had grade 4 lymphopenia and 1 developed reversible grade 2 leukoencephalopathy. Best clinical response was partial response (PR) in 3 patients [17.7%, 90% confidence interval (CI) 5.0–39.6], stable disease at 8 weeks (SD) in 9 patients, progressive disease (PD) in 4 patients, and not evaluable in 1 patient. Maximal response duration for PR was 35 months. Ten evaluable patients had BRAFwt tumors, among whom 3 had PRs, 5 had SD, and 2 had PD. Correlative studies of tumor biopsies revealed decreased phospho-S6K (d2 and d23 vs. d1, P < 0.001), and decreased mitotic rate (Ki67+) among melanoma cells by d23 (P = 0.007). Effects on immune functions were mixed, with decreased alloreactive T-cell responses and decreased circulating CD4+ FoxP3+ cells.

Conclusion: These data provide preliminary evidence for clinical activity of combination therapy with temsirolimus and bevacizumab, which may be greater in patients with BRAFwt melanoma. Mixed effects on immunologic function also support combination with immune therapies. Clin Cancer Res; 19(13); 1–10. ©2013 AACR.

Introduction

Targeted therapies directed at commonly overexpressed pathways in melanoma have induced clinical responses. The phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathway is constitutively activated in many melanomas, leading to increased cell growth, proliferation, and survival (3, 4). Melanoma also depends on growth factors, including insulin-like growth factor (IGF)-I, basic fibroblast growth factor, hepatocyte growth factor, and VEGF, which serve both autocrine and paracrine functions for cell proliferation and migration (5). VEGF blockade has antiangiogenic effects, and can induce intratumoral hypoxia, which modulates mTOR signaling (6). VEGF also provides autocrine growth stimulation to VEGFR2+ melanomas (5, 7, 8). Single-agent therapy with an irreversible mTOR inhibitor, temsirolimus,
Translational Relevance

This article reports on a phase II trial of combination therapy of advanced melanoma, with the mTOR inhibitor temsirolimus and the VEGF antibody bevacizumab. There was clinical activity of this regimen. Interestingly, all of the objective clinical responses were in patients whose tumors were BRAF wild-type, and all responding tumors also seemed to be wild-type for RAS. Thus, this study suggests a regimen with promise for the subset of patients who lack activating mutations in both BRAF and RAS, for whom targeted therapies are currently lacking. The study also includes an assessment of immunologic effects of this regimen and analysis of sequential tumor biopsies. There was significant downmodulation of phospho-S6 kinase in the tumor biopsies with treatment, confirming an effect of mTOR blockade in the tumor microenvironment. There was substantial preservation of immune function, suggesting that this regimen may be also combined with immunotherapy approaches.

Aims included toxicity assessment and correlative studies of mTOR signaling and histologic changes in tumor, as well as effects on immune function, to guide future combinations of molecular targeted therapy with immune therapy.

Materials and Methods

Patients

Patients with American Joint Committee on Cancer stage III to IV melanoma, with measurable disease, were eligible. Other inclusion criteria included age 18 or older, weight at least 110 pounds, Eastern Cooperative Oncology Group performance status 0–1, adequate hepatic, renal, and hematopoietic function (details in Supplementary Text), and ability to provide informed consent. Exclusion criteria included other therapy in the preceding 4 weeks, nitrosoureas or mitomycin C within 6 weeks, uncontrolled brain metastases, allergy to or prior treatment with temsirolimus or bevacizumab, other acute illness, clinically significant cardiovascular disease, pregnancy or nursing, HIV or hepatitis C infection, and uncontrolled diabetes. The study also required tumor accessible for biopsy at 3 time points but was modified after 11 patients were enrolled, to allow enrollment without biopsiable disease. Patients were studied after informed consent and with Institutional review board (#12471) and FDA approval (CTEP IND# 61010 & 7921). The trial was registered with ClinicalTrials.gov (NCT00397982).

Clinical trial design

The main objective of this study was to estimate the objective response (CR + PR) rate (ORR) in participants treated with CCI-779 (temsirolimus, 25 mg i.v. weekly) and bevacizumab (10 mg i.v. every 2 weeks). Secondary objectives included: to describe the adverse event profile, and to obtain preliminary assessments of pre- and posttreatment measurements of biomarkers and vascular and immune system parameters in these participants. Tumor biopsies were obtained pretreatment (cycle 1, day 1, C1D1, 0 hours), 24 hours after temsirolimus only (C1D2, at 24 hours), and 24 hours after treatment with both agents (C2D8, D23; Schema, Fig. 1). Treatment lasted up to 26 cycles (1 year). The study was designed to differentiate between ORRs of 5% and 25%, with a 2-stage design. For the first stage, 13 eligible participants were accrued. If no objective responses had been observed in the first 13 participants, accrual would halt, and the null hypothesis would be accepted. If 1 or more (≥8%) objective responses were observed, accrual

Experimental therapy – Mel47 Schema

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>8</th>
<th>15</th>
<th>22</th>
<th>23</th>
<th>29</th>
<th>36</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>PBMC</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Tumor biopsy</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T – Temsirolimus/CCI-779</td>
<td>Tumor/skin biopsies were obtained days 1, 2, 23.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B – Bevacizumab</td>
<td>Blood drawn 0, 6, 12, 24 hours after doses days 1, 22.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Clinical trial schematic. Drug treatment (top row) and tissue and blood collection (bottom row) are indicated. DLT, dose-limiting toxicity.
would continue to the second stage, with up to 7 additional eligible participants. If 3 or more (≥15%) objective responses were observed, it would be concluded that the data support the alternative hypothesis. With this design, if the true ORR was 5%, the probability of rejecting the null hypothesis is 0.074. If the true ORR is 25%, the probability of rejecting the null hypothesis is 0.903.

For changes in parameters over time, repeated measures linear mixed models were fit using the logarithm (base 10) of expression as the outcome measure and indicator variables representing the following time-points as covariates: day 1, 2, and 23. The reported P value is from the overall F test for comparing changes over time.

Patients were assessed every 8 weeks with computed tomography (CT), MRI, and physical exam. Clinical tumor responses were measured using Response Evaluation Criteria in Solid Tumors (RECIST v1.0), modified to allow tumor biopsies (Supplementary Text). All patients were observed for progression-free and overall survival.

Toxicity assessment and stopping rules

Adverse events (AE) were recorded using NCI CTCAE v3.0, and serious AEs were reported to CTEP using Adverse Event Expedited Reporting System (AdeERS). Comprehensive Adverse Events and Potential Risks Lists were used as a guide to expected toxicities. Toxicity data were captured in the UVA Cancer Center Clinical Trials Office database and submitted to CTEP quarterly through the Clinical Data Update System (CDUS). Patients were taken off study drugs if they experienced unacceptable adverse events and were not eligible for a dose delay or reduction. Detailed protocol instructions for managing toxicity are included in the Supplementary Text (online only).

Collection of PBMCs and tissue samples

Research peripheral blood (100 mL) was collected before administering study drug(s), and 80 mL was collected at 6, 12, and 24 hours for both C1D1 and C2D8 (D23, Fig. 1). Of each sample, serum was obtained from 20 mL, and viable peripheral blood mononuclear cells (PBMC) were isolated from the remainder.Normal tissue (two 3 mm punch biopsies) and tumor tissue (about 0.3 mm³; core or excisional biopsies) were collected at D1, D2, and D23. Details are in Supplementary Text and Fig. 1.

BRAF and NRAS testing

BRAF mutational analysis was conducted by our clinical laboratory. Methods for NRAS sequencing are in the Supplementary Text (online only).

Western blot and reverse-phase protein array analysis

Tumor samples snap-frozen in liquid nitrogen at excision were transferred to foil envelope packets covered with polyester labels, crushed to powder in foil envelopes under freezing conditions, and the powder transferred to microfuge tubes. During the process, all equipment and tumor fragments were kept cold by immersion or being in the vapor phase of liquid nitrogen. Lysis buffer was added to tubes based on tissue weight and kept on ice, with vortexing to solubilize tissue. Lysates were microfuged 10 minutes, and supernatants transferred to fresh tubes. Protein concentration was determined (BCA method, ThermoScientific) and lysates were stored at −80°C in small aliquots. Western blot analyses were conducted using 4%–12% NuPage gradient gels (Life Technologies) with transfer to polyvinylidene difluoride membranes (Millipore). Blocking, subsequent antibody incubations, washing, visualization, and analysis were conducted using protocols for the Odyssey near-infrared scanner (Li-Cor) per manufacturer’s instructions. Antibodies were used to: AKT, phospho-AKT, B-actin, e-NOS, phospho-e-NOS, GAPDH, p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK, P70S6K, S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236), Stat3, phospho-Stat3, and VEGFR2. Aliquots of lysates were submitted to reverse-phase protein array (RPPA) analysis (24–27); analysis for significance included adjustments for multiple comparisons. GAPDH and β-actin were used as controls. (Details in Supplementary Text, online only).

Immunologic function assays

Proliferation of PBMCs in response to mitogen or alloantigen was measured by [3H] thymidine incorporation in a 3-day or 5-day assay, respectively, reporting a stimulation index as fold increase over the mean background response, as described (28), with details in Supplementary Text Methods.

PBMC were immunophenotyped by flow cytometry for expression of CD3, CD4, CD8, CD13, CD16, CD45, CD56, CD14, CD19, and activation markers CD69, CD45RO, HLA-DR, CD127, and CD25. FoxP3 was detected intracellularly as described (28, 29).

IFN-γ ELISpot assays were conducted on PBMCs directly ex vivo after cryopreservation (direct ELISpot), as reported (30). Spots were enumerated on the Bioreader 4000 automated plate reader. Natural killer cell activity was determined in vitro using a 4-hour 51Cr release assay. Details are provided in Supplementary Text.

Immunohistochemistry

Sections of paraffin-embedded histologic tissues were evaluated for markers of cell proliferation, cell death, lymphocytes, dendritic cells, and VEGFR2 after antigen retrieval in citrate or EDTA (details are in Supplementary Text Methods, online only).

Results

The accrual goal was 20 patients but was limited by requirements for tumor biopsies during the grant funding period. At final analysis, a total of 17 eligible patients with unresectable stage III or IV melanoma were enrolled, from 5 August, 2007 to 2 August, 2011. There were 6 females (35%) and 11 males (65%), with median age 65 (range 23–81). Sixteen patients had stage IV melanoma (3 M1a, 4 M1b, 9 M1c) and 1 had unresectable stage IIIB melanoma; 5 (29%) had elevated serum lactate dehydrogenase (LDH). Four had
Table 1. Patient findings at study entry, clinical response, and BRAF mutation status

<table>
<thead>
<tr>
<th>Patient number</th>
<th>ECOG PS</th>
<th>Age, y</th>
<th>Sex</th>
<th>Primary site/M stage</th>
<th>Stage</th>
<th>Metastatic sites/M stage</th>
<th>LDH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Prior therapy&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BRAF mutation status</th>
<th>% Change in RECIST measures&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Time to progression (months)</th>
<th>Tumor biopsies on days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>80</td>
<td>F</td>
<td>Lower extremity/IIB</td>
<td></td>
<td>Intransit skin</td>
<td>.</td>
<td>None</td>
<td>V600E</td>
<td>−6.9%</td>
<td>5.5</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>66</td>
<td>M</td>
<td>Chest/IV M1c</td>
<td></td>
<td>Liver</td>
<td>1.6</td>
<td>None</td>
<td>V600E</td>
<td>+31.4%</td>
<td>1.7</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>50</td>
<td>F</td>
<td>Flank/L1v M1b</td>
<td></td>
<td>L flank, skin, lung</td>
<td>.</td>
<td>HD IL2</td>
<td>V600E</td>
<td>−11.0%</td>
<td>3.6</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>66</td>
<td>M</td>
<td>Back/L1v M1b</td>
<td></td>
<td>Lung</td>
<td>.</td>
<td>HD IL2</td>
<td>WT</td>
<td>−20.6%</td>
<td>3.7</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>63</td>
<td>F</td>
<td>Node, axilla/IV M1c</td>
<td></td>
<td>Bone, Nodes</td>
<td>.</td>
<td>Vaccine trial</td>
<td>V600E</td>
<td>−23.4%</td>
<td>9.4</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>55</td>
<td>M</td>
<td>Other: supraclavicular</td>
<td>IV M1c</td>
<td>Small bowel, nodes</td>
<td>.</td>
<td>HD IL2</td>
<td>V600E</td>
<td>−3.1%</td>
<td>3.9</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>67</td>
<td>F</td>
<td>Lower extremity/IV M1a</td>
<td></td>
<td>Soft tissue/skin, nodes</td>
<td>.</td>
<td>None</td>
<td>WT</td>
<td>−51.7%</td>
<td>32.7</td>
<td>1, 2</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>65</td>
<td>M</td>
<td>Ocular/ IV M1c</td>
<td></td>
<td>Skin, lung, liver, bone, brain, heart</td>
<td>.</td>
<td>DTIC, gamma knife</td>
<td>WT</td>
<td>+4.3%</td>
<td>1.1</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>34</td>
<td>F</td>
<td>Scapular skin/IV M1c</td>
<td></td>
<td>Soft tissue, Lung, liver, bone</td>
<td>5.3</td>
<td>HD IL2</td>
<td>V600E</td>
<td>+37.1%</td>
<td>1.7</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>72</td>
<td>M</td>
<td>Head/IV M1b</td>
<td></td>
<td>Skin, Lung, lymph node</td>
<td>.</td>
<td>HD IFN (adjuvant); TMZ</td>
<td>WT</td>
<td>−30.3%</td>
<td>12.5</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>69</td>
<td>M</td>
<td>Scalp/IV M1c</td>
<td></td>
<td>Lymph nodes</td>
<td>1.03</td>
<td>None</td>
<td>WT</td>
<td>NA</td>
<td>6.6</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>56</td>
<td>M</td>
<td>Lower extremity/IV M1a</td>
<td></td>
<td>Skin, lymph node</td>
<td>.</td>
<td>HD IFN (adjuvant)</td>
<td>WT</td>
<td>−12.9%</td>
<td>5.2</td>
<td>1, 2</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>63</td>
<td>F</td>
<td>Other: vulvar/IV M1c</td>
<td></td>
<td>Adrenal, Soft tissue</td>
<td>.</td>
<td>Vaccine trial</td>
<td>WT</td>
<td>−11.0%</td>
<td>5.3</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>23</td>
<td>M</td>
<td>Ocular/IV M1a</td>
<td></td>
<td>Nodes</td>
<td>−</td>
<td>HD IL2</td>
<td>WT</td>
<td>+5.9%</td>
<td>1.7</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>53</td>
<td>M</td>
<td>Upper extremity/IV M1c</td>
<td></td>
<td>Liver, pancreas, soft tissue, lymph node, spleen, bone</td>
<td>1.7</td>
<td>HD IL2</td>
<td>WT</td>
<td>−7.7%</td>
<td>4.6</td>
<td>1, 2, 23</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>74</td>
<td>M</td>
<td>Head/IV M1b</td>
<td></td>
<td>Lung</td>
<td>−</td>
<td>TMZ</td>
<td>WT</td>
<td>−33.3%</td>
<td>5.6</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>81</td>
<td>M</td>
<td>Upper extremity/IV M1c</td>
<td></td>
<td>Lung, lymph node, soft tissue, bone</td>
<td>1.1</td>
<td>None</td>
<td>WT</td>
<td>−2.7%</td>
<td>3.6</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>LDH reported as ratio of upper limit of normal (± within normal limits)

<sup>b</sup>HD IL2, high dose interleukin-2; HD IFN, high-dose IFN-α; TMZ, temozolomide; DTIC, dacarbazine; gamma knife, gamma knife radiosurgery for brain metastasis; vaccine trial, NCT00118274 (Mel44); adjuvant, therapy administered in the adjuvant setting before advanced melanoma.

<sup>c</sup>These patients were required to have tumor tissue available from a previous biopsy to satisfy the requirement of pretreatment tissue.

<sup>d</sup>Bold font highlights objective clinical response.
prior adjuvant systemic therapy [(high-dose IFN (2); vaccine trial (2)]; 10 had prior systemic therapy for advanced melanoma [high-dose IL-2 (7); cytotoxic chemotherapy (3)]; and one had prior gamma knife radiosurgery for brain metastasis (Table 1).

**Eligibility review**

All patients provided informed consent and were eligible. Four were taken off study drugs early for reasons other than progression: withdrawal of consent (1), noncompliance (1), unacceptable AEs (1), and unrelated health issues (1). The last of these was due to a soft tissue infection; that patient was not evaluable for clinical response assessment.

**Summary of treatment-related clinical toxicities and study discontinuation**

Combination therapy was well tolerated in most patients. However, one patient had grade 2 leukoencephalopathy, which was reversible but required discontinuation of therapy, and 2 had grade 4 lymphopenia. The 2 patients with severe lymphopenia had pretreatment lymphopenia, with lymphocyte counts of 910 (grade 1) and 480/mL (grade 3), respectively, at study enrollment and 640 (grade 2) and 300 (grade 3) on D1 (Supplementary Fig. S1, online only). Changes in other blood cell counts during treatment are shown in Supplementary Fig. S2. Toxicities were managed with dose reductions or delays in 14 patients: temsirolimus was delayed 22 times in 10 patients, and reduced 6 times in 5 patients; bevacizumab was delayed 5 times in 4 patients. Discontinuations for toxicity included temsirolimus (2, C2 and C8), bevacizumab (1, C23), and both (1, C5). There were no treatment-related deaths. Treatment-related grade 3 toxicities were hypokalemia, hypophosphatemia, and weight loss in 2 patients each, and decreased hemoglobin, anorexia, nausea, oral mucositis, lymphopenia, abdominal pain, hypertension, opportunistic infection, decreased CD4 count, and headache in 1 patient each (Supplementary Table S1). Other common treatment-related grade 1 and 2 toxicities included thrombocytopenia (82%); decreased hemoglobin, rash (76%); anorexia, dry skin (71%); fatigue (65%); hypercholesterolemia, taste alteration, oral mucositis, diarrhea (59%), weight loss, nausea, rigors/chills, and hypertriglyceridemia (53%).

Two patients were taken off study drugs early for reasons other than toxicity: noncompliance (1) and consent withdrawal (1).

**Clinical outcomes**

Patient and response data are shown in Table 1. Of the 16 patients evaluable for clinical response, 3 had objective partial responses (PR), 2 of which were confirmed, and one which was unconfirmed (patient 10, Table 1). The unconfirmed response manifested as a 30% decrease in tumor diameters at cycle 8 (17.5 weeks), 29% decrease at confirmation 8 weeks later, and subsequent lack of progression to one year (response duration 9 months, Fig. 2D). PRs
for the other 2 patients were durable for 3 and 35 months (patients 7 and 16). Nine patients had SD at 8 weeks, and 4 had progressive disease (PD) as best response (Table 1; Fig. 2A). Thus, ORR was 17.7% [3/17, 90% confidence interval (CI) 5.0%–39.6%], with SD in 53%. Images are shown in Fig. 3 for one patient with unresectable stage IV melanoma who had a near-complete response (PR) and continued without progression for 35 months.

**BRAF and NRAS mutational analysis**

We assessed whether clinical responses were associated with BRAF mutation status (Table 1). Six patients had BRAF<sup>V600E</sup>-mutated tumors, with no clinical responses. However, the 10 evaluable patients with BRAF<sup>WT</sup> tumors included all 3 RECIST responders (30%, 90% CI 8.7%–60.7%). Five additional BRAF<sup>WT</sup> patients had SD (50%), for an overall disease control rate of 80%. Waterfall plots of changes in tumor burden at time of overall response (ORR) are shown for all evaluable patients (Fig. 2A) and for BRAF<sup>WT</sup> patients (Fig. 2B). The study was not designed for precise survival estimates; however, Kaplan–Meier estimates show median survivals of 12.6 and 7.9 months, respectively, for BRAF<sup>WT</sup> and BRAF<sup>V600E</sup> patients (P = 0.2; Fig. 2C).

Among BRAF<sup>WT</sup> patients, NRAS mutation status was assessed for 3 patients with PRs: 2 were NRAS<sup>WT</sup> at the commonly mutated sites (exon 1, codons 12 and 13; exon 2, codon 61); 1 was NRAS<sup>WT</sup> at exon 2, codon 61, but exon 1 could not be sequenced (data not shown).

**Tumor biopsies**

Three patients were taken off study drugs by D23. For these, biopsies were obtained only on D1 and D2. Tumor biopsies were available pretreatment for all patients with PRs, but on D23 only for one (Table 1).

**Changes in phosphoproteins in melanoma metastases**

To determine the effects of temsirolimus alone (at 24 hours) and long-term effects of combination treatment (at D23) on mTOR signaling and parallel pathways, tumor biopsies were analyzed by Western blot analysis and RPPA. Changes by RPPA were modeled, with adjustments for multiple comparisons (Supplementary Table S2, online only). After mTOR inhibition (24 hours), there were significant decreases in phospho-S6Kinase (pS6K)S235/236, S240/244, and ratio of pS6K S240/244 to total S6, and these changes persisted to day 23 (P < 0.001, Fig. 4A and Supplementary Table S2). These changes in pS6K were also confirmed by Western blot analysis, Fig. 4B). Possible treatment-related changes in 11 other phosphoproteins were suggested from the RPPA data, if using a less stringent cutoff for multiple comparisons (P < 0.025). These include decreases in YB1, Bcl-2, p70S6K (T389), Xiap, ratio of S235/236 to total S6, and ratio of Chk1/total; and increases in Stathmin, Tau, Kras, EGFR, Cox-2 (Supplementary Table S2). Changes in phosphoERK (pT202) and pAKT (pT308) and (pS473) were assessed in the RPPA (Supplementary Fig. S3 and Supplementary Table S2). There were no consistent changes in phosphoERK or clear associations with clinical response (pT202, Supplementary Fig. S3A). Similarly, there was no consistent change in ratios of pAKT to total AKT, nor were there clear associations with clinical response (Supplementary Fig. S3C and S3D). There was a very small increase in phosphoAKT-pT308 from day 1 to 2, but it did not persist thereafter. The P value for that 1-day increase in pT308 was 0.02, which was not significant overall in our modeling for multiple comparisons (Supplementary Table S2). The RPPA also showed no clear association between PTEN protein level and clinical response (Supplementary Fig. S3B).

**Immunohistochemical analysis of tumor biopsies**

Ki67<sup>+</sup> melanoma cells in tumor biopsies decreased significantly by day 23 (P = 0.007, F test), most notably in clinical responders (Fig. 4C). No obvious changes were evident in immune cell infiltrates (CD45), or in caspase3, T-bet, CD1a, CD31, CD83, DC-Lamp, GATA3, and VEGFR2 (data not shown).
Systemic immune function in patients treated with temsirolimus and bevacizumab

Effects of therapy on immune function were assessed in a set of exploratory analyses. To determine the effect of the combination of temsirolimus and bevacizumab on immune cell subsets and function, we assayed blood for patients with blood samples available through day 23 (n = 11). There were minor decreases in circulating lymphocytes (not shown). We also tested PBMC for their function in response to mitogens [phytohemagglutinin and phorbol 12-myristate 13-acetate (PMA)/ionomycin, Fig. 5A] and to a panel of MHC-restricted viral recall antigens from cytomegalovirus, Epstein–Barr virus, and influenza (CEF panel, Fig. 5B). Overall, these functional activities were maintained through day 23 without significant change. NK cell lytic activity against the human NK target cells, K562, was also preserved over time, as assessed in chromium-release assays (not shown). However, treatment-related decreases were observed in T-cell proliferation to alloantigens in mixed lymphocyte reactions (MLR, Fig. 5C), and there was also a significant decrease in circulating FoxP3+ CD25hi CD127lo CD4+ T cells (putative regulatory T cells, Fig. 5D).

Discussion

This study provides evidence for clinical activity of combination temsirolimus and bevacizumab in patients with advanced metastatic melanoma, particularly in BRAFWT patients. The study design criterion for clinical activity was met, with 3 objective responses; this favors a clinical response rate of 25%, versus the null hypothesis rate of 5%. A prior study found moderate activity and tolerability with another mTOR inhibitor, everolimus, and bevacizumab, in metastatic melanoma (RR = 12%, 7/57; ref. 31); however, that study did not assess tumor BRAF mutation status and lacked molecular or immunologic correlates. In the present study, we found that the patients with objective responses had BRAFWT tumors (RR = 30%, 3/10), and their tumors were NRASWT at the most commonly mutated sites, suggesting that this regimen may offer a therapeutic opportunity for patients with BRAFWT NRASWT melanomas, for whom current options are...
limited. It is possible that mTOR and VEGF pathways contribute more to the malignant phenotype when driver mutations in BRAF and NRAS are absent.

The clinical activity of this combination regimen could be due to either agent or to synergistic effects of the combination. In a prior study of temsirolimus alone, the overall response rate was only 3% (1/32; ref. 9). On the other hand, 2 studies of bevacizumab alone for advanced melanoma provide conflicting results: in one, there were no objective responses (0/16; ref. 10); in the other, the objective clinical response rate was 17% (6/35) but without association of clinical response and BRAF status (11). Thus, the clinical benefit observed in the present study, with combination temsirolimus and bevacizumab, may be due in part to bevacizumab alone; however, the association of response with mutational status raises the possibility that there could be different mechanisms when given in combination with temsirolimus: VEGF blockade with bevacizumab creates an hypoxic environment, and mTOR is critical for managing cell functions during stress (32, 33). mTOR has a critical role in modulating VEGF expression, and its blockade can normalize VEGF (34, 35). Thus, there is rationale for synergy of these agents.

We hypothesized that signaling downstream of mTOR would be decreased early after temsirolimus (by D2) and compensatory signaling would be evident later (after D2). Marked downregulation of S6K was observed early, with robust statistical analysis adjusting for multiple comparisons. Changes with less stringent P values should be viewed with caution, but further supported effects downstream of mTOR: decreases in p70S6K ($P = 0.009$) and in pS6K ($pS235/236$):total S6 ratio ($P = 0.007$, Supplementary Table S2). Significant changes in mitogen-activated protein kinase (MAPK) signaling were not evident. However, other changes of borderline significance are of potential interest: downregulation of XIAP has increased ER stress–induced apoptosis (36); and early decreases in the cell-cycle arrest protein Chk1 may contribute to the decrease in melanoma cell proliferation (Ki67) and to clinical activity. On the other hand, Bcl-2 has antiapoptotic function—decreases may reflect

Figure 5. Immunologic correlates of treatment with temsirolimus plus bevacizumab. PBMC were obtained day 1 (0 hour), before the administration of study drug(s) and at each of the following: day 1 (6, 12, 24 hours), and for day 22 (0, 6, 12, 24 hours); D1 + 24 hours = D2; D22 + 24 hours = D23. IFN-γ-secreting cells were enumerated by ELISpot assay after mitogen induction (PMA/ionomycin, A) or viral recall antigen stimulation (CEF peptide pool, B). Graphs of both individual patients and mean/medians are shown. C, proliferation after alloantigen exposure (mixed lymphocyte reaction) was determined after 5 days ($n = 11$); D, circulating regulatory T cells were estimated by determining the proportion of CD4+ cells that were CD25hi FoxP3+ and CD127lo at each time-point ($n = 7$). For C and D, data are presented with box plots showing median values (dot), 25th and 75th percentiles (box), maximum and minimum values (whiskers); *, $P < 0.02$; **, $P < 0.001$. 

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mechanisms of tumor escape signaling and increases in Stathmin (38). KRas, EGFR, and Cox-2 may represent other escape pathways after mTOR and VEGF blockade. Analysis of tumor biopsies can be affected by tumor necrosis, heterogeneity, and sampling error. Each of the tumor biopsy specimens was divided, so that a portion was formalin-fixed for histologic and immunohistologic evaluation, and other portions were evaluated for protein expression and other studies. Thus, each was evaluated and confirmed to contain melanoma. The RPPA (GAPDH and β-actin) and Western (GAPDH) analyses included housekeeping proteins as controls, and their analyses were controlled in that way. It is possible that small variations in a subset of samples could be missed in these studies, as a function of heterogeneity. However, across the whole study population, sample variability should be random. Decreases in pS6K and in Ki67 staining with treatment were consistently observed; and thus seem to be treatment-related changes.

Together, this study provides evidence of clinical activity and acceptable toxicity with combination temsirolimus and bevacizumab in metastatic melanoma. Correlative studies support possible combinations with immune therapy. These data also raise the possibility clinical activity may be better for BRAFVT tumors. A possible explanation for better responses in BRAFVT patients is that those tumors may depend more on AKT/mTOR signaling than those with constitutive BRAF-driven activation of the MAPK pathway. Blocking VEGF may increase hypoxic stress, thus increasing the need for mTOR; so blocking both in these tumors may contribute to tumor control. The same hypothesis may apply for NRAS-driven activation. However, the regimen of mTOR and VEGF blockade should be studied in larger cohorts of BRAFVT patients, with and without NRAS mutations, to obtain a more precise estimate of the clinical response rate in those groups. It would be valuable also to test this regimen for tumors with PTEN inactivation, as this is associated with activation of mTOR/PI3K signaling, and these tumors might be predicted to respond to regimens incorporating mTOR blockade. If the sensitivity of BRAFVT tumors is confirmed in a larger study, we recommend further study of other molecular driver pathways. Truncating or missense mutations in tuberous sclerosis protein 1 (TSC1) have been implicated in the response of human bladder cancer to the mTOR inhibitor everolimus (39); so this regimen also may have relevance in patients with TSC1 mutations. We have also reported that VEGFR2 is expressed on melanoma cells in a minority of patients (8); though we did not observe it in tumors evaluated in this trial (data not shown), patients whose melanoma cells express VEGFR2 may also respond to combination mTOR and VEGF blockade (7). Further studies may also include evaluation of newer mTOR and VEGF inhibitors, and studies of mTOR and/or VEGF blockade in combination with other active therapies, including immune therapies.

Disclosure of Potential Conflicts of Interest
C.R. Weiss has commercial research support from Bristol–Myers Squibb. J.E. Gershenwald is a consultant/advisory board member of Navidea and GSK. No potential conflicts of interest were disclosed by the other authors.

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