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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Running title: Activity and safety of CCI-779 plus bevacizumab for melanoma

Keywords: melanoma, mTOR, VEGF, targeted therapy, biomarkers, BRAF, clinical outcome

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Conflicts of interest: None

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Statement of Translational Relevance

This manuscript 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 appeared 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 down-modulation 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.
Abstract

Purpose: A CTEP-sponsored phase II trial was performed 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 25mg weekly and bevacizumab 10mg 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 two had grade 4 lymphopenia and one developed reversible grade 2 leukoencephalopathy. Best clinical response was partial response (PR) in three patients (17.7%, 90%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
BRAF<sup>wt</sup> melanoma. Mixed effects on immunologic function also support combination with immune therapies.
Introduction

Targeted therapies directed at commonly overexpressed pathways in melanoma have induced clinical responses. The BRAF inhibitor vemurafenib has been approved by the FDA for BRAF-mutant metastatic melanomas [1]. However, the response duration is short, and patients with wild-type BRAF (BRAF WT) do not benefit. Other single-agent regimens have failed to provide lasting control of metastatic melanoma, perhaps because of multiple parallel cell survival signaling pathways [2]. Thus, there is a need to target multiple pathways.

The 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 IGF-1, bFGF, HGF and vascular endothelial growth factor (VEGF), which serve both autocrine and paracrine functions for cell proliferation and migration [5]. VEGF blockade has anti-angiogenic 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,) induced clinical responses in only 3% of melanoma patients (1/32) [9]. Single agent therapy with bevacizumab has had variable results, with response rates of 0% (0/16) and 17% (6/35) [10, 11].

Our laboratory identified synergistic anti-tumor activity in vitro with a combination of mTOR inhibition (rapamycin) and VEGF blockade (bevacizumab) in VEGFR2 + melanomas [7]. Additional anti-tumor synergy was expected in vivo by blocking VEGF-mediated angiogenesis. Thus, we performed a Cancer Therapy Evaluation Program (CTEP)-sponsored phase II clinical trial of combination therapy with temsirolimus and bevacizumab in patients with advanced melanoma (NCI protocol # 7190,
NCT00397982). This combination had the potential to impact systemic immune function: temsirolimus is converted in vivo to sirolimus in vivo, and sirolimus has known immunosuppressive effects [12-16], but also can improve CD8+ T cell memory [17-19]. Also VEGF blockade can improve T cell immunity and dendritic cell function [20-23]. Combinations of immune and targeted therapies can be considered if the targeted therapies preserve or potentiate immune function.

The primary aim of the study was to estimate the objective response rate (ORR) with the combination therapy. Other 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.
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 Supplemental 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 three 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 US Food and Drug Administration 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 IV weekly) and bevacizumab (10 mg IV every 2 weeks). Secondary objectives included: to describe the adverse event profile, and to obtain preliminary assessments of pre- and post-treatment measurements of biomarkers and vascular and immune system parameters in these participants. Tumor biopsies were obtained pretreatment (Cycle 1, Day 1, C1D1, 0h),
24h after temsirolimus only (C1D2, at 24h), and 24h after treatment with both agents (C2D8, D23) (Schema, Figure 1). Treatment lasted up to 26 cycles (1 year).

The study was designed to differentiate between objective response rates (ORR) of 5% and 25%, with a two-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 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 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 (Supplemental Text). All patients were observed for progression-free and overall survival.

**Toxicity Assessment and Stopping Rules**

Adverse events were recorded using NCI CTCAE v3.0, and serious adverse events were reported to CTEP using Adverse Event Expedited Reporting System (AdEERS). Comprehensive Adverse Events and Potential Risks Lists (CAEPRs) 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 Supplemental Text (online only).

Collection of PBMCs and Tissue Samples

Research peripheral blood (100 ml) was collected prior to administering study drug(s), and 80 ml was collected at 6h, 12h, and 24h for both C1D1 and C2D8 (D23, Figure 1). Of each sample, serum was obtained from 20 ml, and viable peripheral blood mononuclear cells (PBMC) were isolated from the remainder by Ficoll gradient centrifugation. Normal skin (two 3 mm punch biopsies) and tumor tissue (about 0.3 mm³; core or excisional biopsies) were collected D1, D2, and D23. Details are in Supplemental Text and Figure 1.

BRAF and NRAS testing

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

Western Blot and Reverse-phase Protein Array (RPPA) 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 performed using 4-12% NuPage gradient gels (Life Technologies, Grand Island, NY) with transfer to PVDF membranes (Millipore, Billerica, MA). Blocking, subsequent antibody incubations, washing, visualization, and analysis were performed using protocols for the Odyssey near-infrared scanner (Li-Cor, Lincoln, NE) per manufacturer’s instructions. Antibodies were used to: AKT, phospho-AKT, B-actin, e-NOS, phospho-e-NOS, GAP-DH, 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 RPPA analysis [24-27]; analysis for significance included adjustments for multiple comparisons. GAPDH and beta-actin were used as controls. (Details in Supplemental Text, online only).

Immunologic Function Assays

Proliferation of PBMC in response to mitogen or alloantigen was measured by [\(^{3}\)H] thymidine incorporation in a 3d or 5d assay, respectively, reporting a stimulation index (SI) as fold increase over the mean background response, as described [28], with details in Supplemental 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].

IFNγ ELISpot assays were performed on PBMCs directly ex vivo after cryopreservation (direct ELISpot), as reported [30]. Spots were enumerated on the Bioreader 4000 automated plate reader.

NK cell activity was determined in vitro using a 4h ⁵¹Cr release assay.

Details are provided in Supplemental 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 Supplemental 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 5/8/2007 to 2/8/2011. 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; five (29%) had elevated serum LDH. Four had prior adjuvant systemic therapy (high-dose interferon (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), non-compliance (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 two had grade 4 lymphopenia. The two 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 ([Supplemental Figure S1, online only](#)). Changes in other blood cell counts during treatment are shown in [Supplemental Figure 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 ([Supplemental 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: non-compliance (1) and consent withdrawal (1).

Clinical outcomes

Patient and response data are shown in Table 1. Of the sixteen patients evaluable for clinical response, three had objective partial responses (PR), two 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, Figure 2D). PRs for the other two patients were durable for 3 and 35 months (patients 7 and 16). Nine patients had stable disease (SD) at 8 weeks, and 4 had progressive disease (PD) as best response (Table 1, Figure 2A). Thus, ORR was 17.7% (3/17, 90%CI 5.0-39.6%), with SD in 53%. Images are shown in Figure 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). 6 patients had BRAF$^{V600E}$ mutated tumors, with no clinical responses. However, the 10 evaluable patients with BRAF$^{WT}$ tumors included all three RECIST responders (30%, 90%CI 8.7-60.7%). Five additional BRAF$^{WT}$ 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 (Figure 2A) and for BRAF\textsuperscript{WT} patients (Figure 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\textsuperscript{WT} and BRAF\textsuperscript{V600E} patients (p = 0.2; Figure 2C).

Among BRAF\textsuperscript{WT} patients, NRAS mutation status was assessed for the 3 patients with PRs: 2 were NRAS\textsuperscript{WT} at the commonly mutated sites (exon 1, codons 12 and 13; exon 2, codon 61); 1 was NRAS\textsuperscript{WT} 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 pre-treatment for all patients with partial responses (PR), but on D23 only for one (Table 1).

**Changes in phosphoproteins in melanoma metastases**

To determine effects of temsirolimus alone (at 24h) and long-term effects of combination treatment (at day 23) on mTOR signaling and parallel pathways, tumor biopsies were analyzed by western blot and reverse-phase protein array (RPPA). Changes by RPPA were modeled, with adjustments for multiple comparisons (Supplemental Table S2, online only). After mTOR inhibition (24h), 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, Figure 4A and Supplemental Table S2). These changes in pS6K were also confirmed by
Western analysis, Figure 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 (Supplemental Table S2). Changes in phosphoERK (pT202); pAKT(pT308) and (pS473) were assessed in the RPPA (Supplemental Figure S3; Supplemental Table S2). There were no consistent changes in phosphoERK or clear associations with clinical response (pT202, Supplemental Figure S3A). Similarly, there was no consistent change in ratios of pAKT to total AKT, nor were there clear associations with clinical response (Supplemental Figure S3C,D). 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 (Supplemental Table S2). The RPPA also showed no clear association between PTEN protein level and clinical response (Supplemental Figure S3B).

Immunohistochemical analysis of tumor biopsies

Ki67+ melanoma cells in tumor biopsies decreased significantly by day 23 (p = 0.007, F-test), most notably in clinical responders (Figure 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 (PHA and PMA/ionomycin, Figure 5A) and to a panel of MHC-restricted viral recall antigens from cytomegalovirus, Epstein-Barr virus and influenza (CEF panel, Figure 5B). Overall, these functional activities were maintained through day 23 without significant change. Natural killer (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, Figure 5C), and there was also a significant decrease in circulating FoxP3\textsuperscript{+} CD25\textsuperscript{hi} CD127\textsuperscript{lo} CD4\textsuperscript{+} T cells (putative regulatory T cells, Figure 5D).

**Discussion**

This study provides evidence for clinical activity of combination temsirolimus and bevacizumab in patients with advanced metastatic melanoma, particularly in BRAF\textsuperscript{WT} 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) \textsuperscript{[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 BRAF$^{WT}$ tumors ($RR = 30\%, 3/10$), and their tumors were NRAS$^{WT}$ at the most commonly mutated sites, suggesting that this regimen may offer a therapeutic opportunity for patients with BRAF$^{WT}$ NRAS$^{WT}$ 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) [9]. On the other hand, two studies of bevacizumab alone for advanced melanoma provide conflicting results: in one, there were no objective responses (0/16) [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 ($p = 0.007$, Supplemental Table S2). Significant changes in MAPK signaling were not evident. However, other changes of borderline significance are of potential interest: downregulation of YB1 has been associated with decreased melanoma cell proliferation, invasion, and survival [36]; blockade of XIAP has increased ER-stress-induced apoptosis [37]; 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 anti-apoptotic function – decreases may reflect 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 beta-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 appear 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 BRAF$^{WT}$ tumors. A possible explanation for
better responses in BRAF\textsuperscript{WT} patients is that those tumors may depend more on AKT/mTOR signaling than those with constitutive BRAF-driven activation of the MAP kinase 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 BRAF\textsuperscript{WT} 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 BRAF\textsuperscript{WT} 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\cite{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\cite{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\cite{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.
**Figure Legends**

**Figure 1. Clinical trial schematic.** Drug treatment (top row) and tissue and blood collection (bottom row) are indicated. DLT, dose-limiting toxicity.

**Figure 2. Waterfall plots of best overall response (BOR) after treatment.** Percent change in tumor diameters by RECIST criteria. (A) All 16 evaluable patients; patients with BRAF<sup>WT</sup> tumors are in blue and those with BRAF<sup>V600E</sup> tumors are in red. RR, response rate. DCR, disease control rate; (B) BRAF<sup>WT</sup> patients only; C) Kaplan-Meier estimates for overall survival of patients by BRAF status (blue dotted line, WT; red solid line, V600E); (D) Percent reduction in tumor diameters (RECIST) over time, compared to pre-treatment measurements, for the 3 patients with PR. Patient 7 came off treatment after cycle 4 due to toxicity, with progression at 35 months. Patient 10 had 30% decrease in tumor diameters at cycle 8 (17.5 weeks), 29% decrease at confirmation 8 weeks later, and subsequent lack of progression for 9 months. Patient 16 came off study due to a new lesion (*) at cycle 12. One cycle = 2 weeks.

**Figure 3. Durable partial response, Patient 7.** Extensive cutaneous melanoma metastases in the right groin are shown pretreatment (A) and after 17 months (B) for the patient with the best response to therapy. Sites of disease that were biopsied for the study are shown with dotted lines in (A), and the scars are evident in (B). CT scan images are shown for inguinal, iliac, and periaortic adenopathy pretreatment (C) and after 21-30 months (D). Sites of disease are marked with red circles.
Figure 4. Changes in phosphoproteins and Ki67 expression by melanoma cells in metastases with treatment. (A) Normalized protein expression of phosphorylated S6K (P-Ser235/236 and P-Ser240/244) in tumor samples detected by Reverse phase protein array (RPPA), before treatment (D1), after 24h temsirolimus alone (D2), and after combination treatment (D23). Values are shown for all patients with tissue available for analysis. Protein expression was normalized for protein loading and transformed to linear values. (B) Western blot of total S6K protein (Total S6) and phosphorylated S6 [P-Ser235/236] from tumor biopsies obtained pre-treatment (D1), 24h post-temsiirolimus alone (D2), and post-combination treatment, day 23 (D23). Samples from 3 patients (Patient 1, 8, and 7) are shown. Patient 7 did not have tumor biopsy on day 23. GAPDH is blotted as a loading control. (C) Ki67+ melanoma cells were enumerated in tumor biopsies by immunohistochemistry, and values per high powered field (HPF) are shown for BRAFWT and BRAFV600E melanomas. Wide black lines are two patients with PRs. Dashed lines are in patients with PD.

Figure 5. Immunologic correlates of treatment with temsirolimus plus bevacizumab. PBMC were obtained Day 1 (0h), prior to the administration of study drug(s) and at each of the following: Day 1 (6, 12, 24h), and for Day 22 (0, 6 12, 24h); D1 + 24h = D2; D22 + 24h = D23. Interferon gamma (IFNγ) 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 CD127$^{lo}$ at each time point (n=7). For (C) and (D), data are presented with box plots showing median values (dot), 25$^{th}$ and 75$^{th}$ percentiles (box), maximum and minimum values (whiskers); * p < 0.02; ** p<0.001.
### 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 (yrs)</th>
<th>Sex</th>
<th>Primary site</th>
<th>Stage</th>
<th>M stage</th>
<th>Metastatic sites</th>
<th>LDH(^a)</th>
<th>Prior therapy(^b)</th>
<th>BRAF mutation status</th>
<th>Best response</th>
<th>% change in RECIST measures</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</td>
<td>IIIB</td>
<td>--</td>
<td>Intransit skin</td>
<td>.</td>
<td>None</td>
<td>V600E</td>
<td>SD</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</td>
<td>IV</td>
<td>M1c</td>
<td>Liver</td>
<td>1.6</td>
<td>None</td>
<td>V600E</td>
<td>PD</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>L flank</td>
<td>IV</td>
<td>M1b</td>
<td>L flank skin, lung</td>
<td>.</td>
<td>HD IL2</td>
<td>V600E</td>
<td>SD</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>L back</td>
<td>IV</td>
<td>M1b</td>
<td>Lung</td>
<td>.</td>
<td>HD IL2</td>
<td>WT</td>
<td>SD</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</td>
<td>IV</td>
<td>M1c</td>
<td>Bone, Nodes</td>
<td>.</td>
<td>Vaccine trial (adjuvant); TMZ</td>
<td>V600E</td>
<td>SD</td>
<td>-23.4%</td>
<td>9.4</td>
<td>1, 2, 23</td>
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<td>6</td>
<td>0</td>
<td>55</td>
<td>M</td>
<td>Other: Supraclavicular</td>
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<td>M1c</td>
<td>Small bowel, nodes</td>
<td>.</td>
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<td>V600E</td>
<td>SD</td>
<td>-3.1%</td>
<td>3.9</td>
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<td>7</td>
<td>1</td>
<td>67</td>
<td>F</td>
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<td>IV</td>
<td>M1a</td>
<td>Soft tissue/skin, nodes</td>
<td>.</td>
<td>None</td>
<td>WT</td>
<td>PR</td>
<td>-51.7%</td>
<td>32.7</td>
<td>1, 2</td>
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<td>M</td>
<td>Ocular</td>
<td>IV</td>
<td>M1c</td>
<td>Skin, lung, liver, bone, brain, heart</td>
<td>.</td>
<td>DTIC, gamma knife</td>
<td>WT</td>
<td>PD</td>
<td>+4.3%</td>
<td>1.1</td>
<td>1, 2, 23</td>
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<tr>
<td>9</td>
<td>1</td>
<td>34</td>
<td>F</td>
<td>Scapular skin</td>
<td>IV</td>
<td>M1c</td>
<td>Soft tissue, Lung, liver, bone</td>
<td>5.3</td>
<td>HD IL2</td>
<td>V600E</td>
<td>PD</td>
<td>+37.1%</td>
<td>1.7</td>
<td>1, 2, 23</td>
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<tr>
<td>10</td>
<td>0</td>
<td>72</td>
<td>M</td>
<td>Head</td>
<td>IV</td>
<td>M1b</td>
<td>Skin, Lung LN</td>
<td>.</td>
<td>HD IFN (adjuvant); HD IL2</td>
<td>WT</td>
<td>unconf PR</td>
<td>-30.3%</td>
<td>12.5</td>
<td>1, 2, 23</td>
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<tr>
<td>11</td>
<td>1</td>
<td>69</td>
<td>M</td>
<td>Scalp</td>
<td>IV</td>
<td>M1c</td>
<td>Lymph nodes</td>
<td>1.03</td>
<td>None</td>
<td>WT</td>
<td>NA</td>
<td>0</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</td>
<td>IV</td>
<td>M1a</td>
<td>Skin, LN</td>
<td>.</td>
<td>HD IFN (adjuvant)</td>
<td>WT</td>
<td>SD</td>
<td>-12.9%</td>
<td>5.2</td>
<td>1, 2</td>
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<tr>
<td>13</td>
<td>0</td>
<td>63</td>
<td>F</td>
<td>Other: vulvar</td>
<td>IV</td>
<td>M1c</td>
<td>Adrenal, Soft tissue</td>
<td>.</td>
<td>Vaccine trial (adjuvant)</td>
<td>WT</td>
<td>SD</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</td>
<td>IV</td>
<td>M1a</td>
<td>Nodes</td>
<td>.</td>
<td>HD IL2</td>
<td>WT</td>
<td>PD</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</td>
<td>IV</td>
<td>M1c</td>
<td>Liver, pancreas, soft tissue, LN, spleen, bone</td>
<td>1.7</td>
<td>HD IL2</td>
<td>WT</td>
<td>SD</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</td>
<td>IV</td>
<td>M1b</td>
<td>Lung</td>
<td>.</td>
<td>TMZ</td>
<td>WT</td>
<td>PR</td>
<td>-33.3%</td>
<td>5.6</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
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<td>0</td>
<td>81</td>
<td>M</td>
<td>Upper extremity</td>
<td>IV</td>
<td>M1c</td>
<td>Lung, LN, soft tissue, bone</td>
<td>1.1</td>
<td>None</td>
<td>WT</td>
<td>SD</td>
<td>-2.7%</td>
<td>3.6</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

\(^a\) LDH reported as ratio of upper limit of normal (\(=\) within normal limits)

\(^b\) HD IL2 = high dose interleukin-2; HD IFN = high dose interferon alpha; TMZ = temozolomide; DTIC = dacarbazine; gamma knife = gamma knife radiosurgery for brain metastasis; Vaccine trial = NCT00118274 (Mel44); adjuvant = therapy administered in the adjuvant setting prior to advanced melanoma.

\(^c\) These patients were required to have tumor tissue available from a previous biopsy to satisfy the requirement of pre-treatment tissue.


CD4 T Cell Cultures Is Not Due to the Selective Expansion of Naturally Occurring Regulatory T Cells but to the Induction of Regulatory Functions in Conventional CD4 T Cells. JI 2006;177:944-9.


[21] Ziogas AC, Gavalas NG, Tsiatas M, Tsitsilonis O, Politi E, Terpos E, et al. VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2. Int J Cancer 2012;130:857-64.


Figure 1

### 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>
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<tr>
<td>T</td>
<td>TB</td>
<td>T</td>
<td>TB</td>
<td>T</td>
<td>TB</td>
<td>T</td>
<td>TB</td>
<td>T</td>
<td>→</td>
</tr>
</tbody>
</table>

#### Treatment

- **T** – Temsirolimus/CCI-779
- **B** – Bevacizumab

#### PBMC

<table>
<thead>
<tr>
<th></th>
<th>PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>++++</td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
</tr>
</tbody>
</table>

#### Tumor biopsy

<table>
<thead>
<tr>
<th></th>
<th>Tumor biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>+</td>
</tr>
<tr>
<td>Day 2</td>
<td>+</td>
</tr>
<tr>
<td>Day 23</td>
<td>+</td>
</tr>
</tbody>
</table>

T – Temsirolimus/CCI-779  
Tumor/skin biopsies were obtained days 1, 2, 23.

B – Bevacizumab  
Blood drawn 0, 6, 12, 24 hours after doses days 1, 22.
Figure 2

A

All patients
N = 16

3 PR, 9 SD, 4 PD
RR = 19%
DCR = 75%

B

BRAF-wt patients
N = 10

3 PR, 5 SD, 2 PD
RR = 30%
DCR = 80%

C

WT

V600E

Survival probability (%)

0 10 20 30 40 50 60 70 80 90 100

Time (months)

D

% Reduction in
Pre-treatment RECIST

0 10 20 30 40 50 60

Cycle #

Patient 7
Patient 10
Patient 16
Figure 3
Figure 5

A. PMA/lo

B. CEF

C. MLR

D. CD25^hi FoxP3^+ CD127^lo
Clinical Cancer Research

Clinical activity and safety of combination therapy with temsirolimus and bevacizumab for advanced melanoma: A Phase II trial (CTEP 7190/ Mel47)


Clin Cancer Res  Published OnlineFirst April 25, 2013.

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http://clincancerres.aacrjournals.org/content/suppl/2016/09/21/1078-0432.CCR-12-3919.DC2

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