Published in final edited form as:

J Immunother. 2012 July ; 35(6): 473–477. doi:10.1097/CJI.0b013e3182618209.

Brief Communication: Immunologic Effects of an Orally Available BRAFV600E Inhibitor in BRAF Wild-Type Murine Models

Gregory S. Vosgianian, M.D.¹, Rinke Bos, PhD.², and Linda A. Sherman, PhD²

¹Division of Hematology/Oncology, Scripps Clinic and Green Hospital, La Jolla, California

²Department of Immunology and Microbial Sciences, The Scripps Research Institute, La Jolla, California

Abstract

Vemurafenib is an orally available small molecule that targets constitutively activated BRAFV600E, an integral part of the MAPK pathway involved in melanomagenesis. We examined the effects of vemurafenib on cytokine production and anti-tumor response in a BRAF WT non-tumor bearing murine model and a BRAF WT murine insulinoma system to determine its effect on immune function during immunotherapy. We demonstrate no significant effect from vemurafenib on CD4+ and CD8+ T cell cytokine production or on a T-cell mediated anti-tumor response. Our data demonstrates that vemurafenib does not significantly impact BRAF WT targets, suggesting that there may be a role for combining vemurafenib treatment with T cell directed immunotherapy.

Keywords

vemurafenib; BRAF; murine model; melanoma; immunotherapy

Introduction

Metastatic melanoma is an aggressive disease for which previous therapies have resulted in low rates of complete response and minimal impacts on overall survival.^{1–3} Treatment strategies for metastatic melanoma have incorporated immunologically directed therapies with high dose IL2 alone or in combination with interferon alpha and cytotoxic chemotherapy suggesting that exogenous immune stimulation decreases tumor burden.^{4,5} These strategies as well as data involving vaccine therapy⁶ and anti-CTLA-4 directed therapy⁷ imply that escape from the immune system may play a key role in melanomagenesis and tumor progression.

Recently, vemurafenib (Genentech, Inc. South San Francisco, CA USA) has received FDA approval for patients with metastatic melanoma bearing the BRAF V600E mutation following the results of a phase III clinical trial demonstrating improved overall survival.¹ As encouraging as these data are, there is evidence that tumors ultimately develop resistance to treatment with vemurafenib.⁸ Any potential off target effect of vemurafenib on the BRAF wild type (WT) host immune system could influence multi-agent therapies.

Corresponding author: Linda A. Sherman, PhD., The Scripps Research Institute, IMM-15, 10550 North Torrey Pines Road, La Jolla, CA 92037, Phone: 858-784-8052; Fax: 858-784-8298, lsherman@scripps.edu.

Financial Disclosure: All authors have declared there are no financial conflicts of interest in regards to this work.

To better understand the effect of vemurafenib on tumor directed T cells, we evaluated its effects on cytokine production in BRAF WT non-tumor bearing mice and on a T-cell mediated anti-tumor response in a previously described murine insulinoma system.⁹

Methods

Preparation of Vemurafenib

Vemurafenib was obtained from Genentech, Inc. Aliquots of 200mg/kg were prepared in an aqueous solution of 2% Klucel LF (Hydroxypropylcellulose, Ashland Inc. Covington, KY, USA) as previously described.¹⁰

Mice

BRAF WT, non-melanoma bearing B10.D2 and B10.D2 rat insulin promoter (RIP)-Tag2-HA mice were used at 10 weeks of age. B10.D2 RIP-Tag2-HA mice develop spontaneous insulinoma at approximately 6 weeks of age.¹¹ B10.D2 Clone 1 TCR transgenic mice, which express a TCR specific for the HA₅₁₈₋₅₂₆ (IYSTVASSL) presented by H-2K^d, and SFE TCR transgenic mice, which express a TCR that recognizes HA₁₁₀₋₁₁₉ (SFERFEIFPK) presented by I-E^d, were bred with the congenic markers Thy1.1 and CD45.1 respectively. Experiments were performed on groups of 3 to 5 mice in the treated and control arms. All mice were bred at our facility and all animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute, La Jolla, CA U.S.A.

Pharmacokinetic Assessment of Serum Vemurafenib concentrations

B10.D2 mice were treated with vemurafenib 200 mg/kg via gavage in a 2% aqueous Klucel LF solution once daily. Blood was collected 2, 4 and 24 hours post dosing in treated mice and at 2 hours in control mice. Serum was separated by centrifugation and vemurafenib extracted in methanol. Drug concentrations were quantified via mass spectroscopy.

Adoptive transfer of naïve transgenic T cells and peptide immunization

Lymph nodes were collected and purified by magnetic cell sorting using CD4⁺/CD8⁺ T-cell enrichment sets (BD Biosciences, San Diego, CA, USA). Purified lymphocytes (0.3×10^6 cells per mouse) were injected intravenously (i.v.) into recipient RIP-Tag2-HA or B10.D2 mice on day 1 of each experiment; mice were concurrently immunized with 10 µg HA₅₁₈₋₅₂₆ peptide, 50 µg HA₁₁₀₋₁₁₉ peptide, and 200 µg poly (inosinic-cytidylic acid) (poly (I:C); EMD Biosciences, San Diego, CA USA) in incomplete Freund's adjuvant (IFA; Difco Laboratories, Sparks, MD USA) via subcutaneous (s.c.) injection.

In vitro cytokine production assay

Following adoptive transfer of naïve transgenic Clone 1 CD8⁺ Thy 1.1 and SFE CD4⁺ CD45.1 T cells on day 1, B10.D2 BRAF WT mice were treated with 200mg/kg vemurafenib in 2% aqueous Klucel LF or vehicle control daily via gavage for 5 days. On day 6, spleens were harvested, morcelized and filtered in ice-cold complete RPMI media. 2×10^6 splenic lymphocytes were incubated with 1 pg/ml HA₅₁₈₋₅₂₆ peptide or 5 pg/ml HA₁₁₀₋₁₁₉ peptide in the presence of 1 µg/ml Golgi Plug. Samples were incubated at 37° C for 5 hours and then prepared for intracellular cytokine staining according to the manufacturer's instructions using the Cytotfix/Cytoperm plus kit (BD Biosciences, BD Biosciences, San Diego, CA USA). CD8⁺ T cells were assayed for IFN-γ, IL-2, and TNF-α production; CD4⁺ T cells were assayed for IFN-γ and IL-2 production by fluorescence-activated cell sorting (FACS).

In vivo assessment of vemurafenib's effect on an immune mediated anti-tumor response

Purified Clone 1 CD8+ Thy 1.1 and SFE CD4+ CD45.1 T cells were injected into B10.D2 RIP-Tag2-HA mice on day 1. Recipient mice were immunized with 10 µg HA₅₁₈₋₅₂₆ peptide, 50 µg HA₁₁₀₋₁₁₉ peptide and 200 µg poly (I: C) in IFA injected s.c. into the right flank. Mice received vemurafenib 200 mg/kg or vehicle control daily via gavage. Serum blood glucose was measured as previously described.⁹

Statistical analysis

Differences between groups were determined by t-test, Mann-Whitney and ANOVA as appropriate. Data are presented as means and means $\pm$ SEM as appropriate.

Results

Vemurafenib Achieves Effective Anti-Tumor Concentrations in B10.D2 Mouse Serum

B10.D2 mice received one dose of vemurafenib 200 mg/kg by gavage and the serum concentration of vemurafenib was measured at 2, 4 and 24 hours post dosing for three representative mice (Fig.1). The results are consistent with previous data describing serum concentrations of vemurafenib that demonstrated anti-tumor activity in a BRAFV600E murine xenograft model.¹⁰ Control mice not receiving vemurafenib had drug levels 1000-fold lower than treated animals (data not shown).

Vemurafenib does not Affect CD4 and CD8 Cytokine Production in B10.D2 BRAF WT Mice

To determine whether vemurafenib affected the antigen specific response of T cells *in vivo*, mice receiving both Thy1.1 Clone 1 cells and CD45.1 SFE cells were immunized with cognate peptides and treated with 200mg/kg of drug for 5 days. Control mice received no drug. The percent of Thy1.1+ CD8+ Clone 1 lymphocytes recovered on day 6 from spleens of treated and non-treated mice showed no significant difference (Fig. 2A, two tailed unpaired *t* test, $p=0.4535$). Similarly, the percent of CD45.1+ SFE CD4+ lymphocytes recovered from the same spleens showed no significant difference between the two groups (Fig. 2B, two tailed unpaired *t* test, $p=0.7535$). Thus, the antigen specific expansion of T cells was unaffected by the drug.

We further tested the activated T cells for cytokine production *in vitro* by intracellular staining on day 6. No significant difference was noted in the percent of Clone 1 CD8+ cells of treated and non-treated mice producing IFN-gamma, IL2, or TNF-alpha (Fig. 3A–C, two tailed unpaired *t* test, $p=1.00$, 0.4155 , and 0.4257 , respectively). Similarly, no significant difference was noted in the percent of SFE CD4+ cells producing IFN gamma and IL2 (Fig. 4A–B, two tailed unpaired *t* test, $p=0.7863$ and 0.5525 , respectively). Collectively, these data demonstrate that treatment with vemurafenib does not affect antigen specific immune responses.

Vemurafenib does not affect the CD4 and CD8 dependent anti-tumor response in Ins-HA tumor bearing mice

To determine whether vemurafenib treatment affects CD4 and CD8 T cell effector function, we used a tumor model in which tumor eradication is dependent on CD4 and CD8 T cells.⁹ RIP-Tag2 HA mice spontaneously develop insulinoma and carry an endogenous T-cell repertoire which is tolerant for this self-antigen.⁹ Previous studies have shown that a combination of SFE CD4+ cells and Clone 1 CD8+ cells, both specific for HA antigens, along with a potent peptide vaccine, can eradicate such tumors. Insulinoma destruction correlates with development of diabetes that is monitored by measurement of blood glucose.⁹ To determine whether vemurafenib has an effect on the T-cell mediated tumor

eradication, BRAF WT insulinoma-bearing RIP-TAG2-HA mice received HA-specific Clone 1 CD8⁺ and SFE CD4⁺ T cells intravenously and were immunized with cognate peptides and Poly (I:C) in IFA via subcutaneous injection. Mice in the treatment group received vemurafenib 200 mg/kg via gavage daily for 30 days. Glucose levels in the blood were measured once a week to assess tumor eradication (Fig. 5).

Administration of vemurafenib had no effect on tumor eradication, replicated as no difference in time to onset of diabetes and peak blood sugar levels. Two-way ANOVA revealed no significant difference between the blood glucose levels of the treated and untreated groups. These data suggest that in a WT BRAF system, vemurafenib does not exert an off-target effect on T-cell mediated anti-tumor response.

Discussion

Vemurafenib is a highly specific inhibitor of mutant BRAF V600E exhibiting a 10 fold greater selectivity for the mutant kinase¹². However, the potential for low-level inhibition of WT BRAF that may affect an immune response *in vivo* could not be excluded. In the present study, we describe the effect of vemurafenib on T-cell function in two BRAF WT murine systems. Activation of CD4⁺ and CD8⁺ T cells *in vivo* was not significantly affected as assessed by cell numbers or cytokine production following T cell activation. Furthermore, using a tumor model in which tumor eradication is dependent on the activation of tumor antigen specific CD4⁺ and CD8⁺ cells and their production of both IFN- γ and IL-2^{11,13} demonstrated that the BRAFV600E inhibitor did not affect tumor eradication. Together, these data suggest that vemurafenib does not exert an off target effect on T-cell mediated anti-tumor responses, consistent with previous reports describing the effect of vemurafenib on immune function in a human *in vitro* system.¹⁴

It would be useful to evaluate whether these effects are reproducible in a murine BRAFV600E mutated system to confirm that there is no down regulation of cytokine production in the presence of melanoma. Furthermore, evaluating the effects of vemurafenib on immune response within the tumor milieu could elucidate targets for combination therapy; future studies will evaluate these issues.

Acknowledgments

We thank Kristi Marquardt for breeding the mice used in these experiments and for her technical assistance.

Conflicts of Interest and Sources of Funding: The authors have no conflicts of interest to report.

This research was supported by a grant from the NIH, Grant Number R01 CA57855.

References

1. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011; 364:2507–2516. [PubMed: 21639808]
2. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010; 363:809–819. [PubMed: 20818844]
3. Middleton MR, Grob JJ, Aaronson N, et al. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *J Clin Oncol*. 2000; 18:158–166. [PubMed: 10623706]
4. Tarhini AA, Kirkwood JM, Gooding WE, et al. Durable complete responses with high-dose bolus interleukin-2 in patients with metastatic melanoma who have experienced progression after biochemotherapy. *J Clin Oncol*. 2007; 25:3802–3807. [PubMed: 17761969]

5. Weber RW, O'Day S, Rose M, et al. Low-dose outpatient chemobiotherapy with temozolomide, granulocyte-macrophage colony stimulating factor, interferon-alpha2b, and recombinant interleukin-2 for the treatment of metastatic melanoma. *J Clin Oncol*. 2005; 23:8992–9000. [PubMed: 16260693]
6. Lopez MN, Pereda C, Segal G, et al. Prolonged survival of dendritic cell-vaccinated melanoma patients correlates with tumor-specific delayed type IV hypersensitivity response and reduction of tumor growth factor beta-expressing T cells. *J Clin Oncol*. 2009; 27:945–952. [PubMed: 19139436]
7. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010; 363:711–723. [PubMed: 20525992]
8. Solit DB, Rosen N. Resistance to BRAF inhibition in melanomas. *N Engl J Med*. 2011; 364:772–774. [PubMed: 21345109]
9. Bos R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. *Cancer Res*. 2010; 70:8368–8377. [PubMed: 20940398]
10. Yang H, Higgins B, Kolinsky K, et al. RG7204 (PLX4032), a selective BRAFV600E inhibitor, displays potent antitumor activity in preclinical melanoma models. *Cancer Res*. 2010; 70:5518–5527. [PubMed: 20551065]
11. Lyman MA, Nugent CT, Marquardt KL, et al. The fate of low affinity tumor-specific CD8+ T cells in tumor-bearing mice. *J Immunol*. 2005; 174:2563–2572. [PubMed: 15728462]
12. Tsai J, Lee JT, Wang W, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci U S A*. 2008; 105:3041–3046. [PubMed: 18287029]
13. Wong SB, Bos R, Sherman LA. Tumor-specific CD4+ T cells render the tumor environment permissive for infiltration by low-avidity CD8+ T cells. *J Immunol*. 2008; 180:3122–3131. [PubMed: 18292535]
14. Comin-Anduix B, Chodon T, Sazegar H, et al. The oncogenic BRAF kinase inhibitor PLX4032/ RG7204 does not affect the viability or function of human lymphocytes across a wide range of concentrations. *Clin Cancer Res*. 2010; 16:6040–6048. [PubMed: 21169256]

Figure 1. Mean Serum Concentration of vemurafenib. Mean serum concentration of vemurafenib following treatment with 200 mg/kg drug PO daily. Drug levels were assayed at 2, 4 and 24 hours post gavage measured in three B10.D2 mice by mass spectroscopy following methanol extraction from plasma.

Figure 2.

Vemurafenib does not significantly effect recovery of *in vivo* activated transgenic T cells. B10.D2 BRAF WT mice were immunized and Thy1.1+ Clone 1 cells and CD45.1+ SFE T cells were injected via the intravenous route on day 1. Mice were treated with 200mg/kg vemurafenib in 2% aqueous Klucel LF or vehicle control daily via gavage for 5 days. Following *in vitro* stimulation with appropriate peptide, splenic lymphocytes were analyzed by FACS on day 6. Mice not receiving *in vivo* stimulation with peptide had significantly fewer T cells recovered on FACS analysis (data not shown). Data are representative of 4 independent experiments with 3–5 mice per group. 2A, no significant difference in % CD8+Thy1.1+ splenic lymphocytes recovered on day 6 in mice treated with vemurafenib or control (two tailed unpaired *t* test, $p=0.4535$). 2B, no significant difference in %CD4+CD45.1+ splenic lymphocytes recovered on day 6 in mice treated with vemurafenib or control (two tailed unpaired *t* test, $p=0.7535$).

Figure 3.

No significant effect of vemurafenib on cytokine production by of *in vivo* activated transgenic CD8+ T cells. B10.D2 BRAF WT mice were immunized and Thy1.1+ Clone 1 cells and CD45.1+ SFE T cells were injected on day 1. Mice were treated with 200mg/kg vemurafenib in 2% aqueous Klucel LF or vehicle control daily via gavage for 5 days. Following *in vitro* stimulation with appropriate peptide, cytokine production by isolated splenic lymphocytes was analyzed by FACS on day 6. Data are representative of 4 independent experiments with 3–5 mice per group. 3A, no significant difference in % of CD8/Thy1.1+ splenic lymphocytes producing IFN- γ in mice treated with vemurafenib or control (two tailed unpaired *t* test, $p=1.00$); 3B, no significant difference in % of CD8/Thy1.1+ splenic lymphocytes producing IL2 in mice treated with vemurafenib or control (two tailed unpaired *t* test, $p=0.4155$); 3C, no significant difference in % of CD8/Thy1.1+ splenic lymphocytes producing TNF- α in mice treated with vemurafenib or control (two tailed unpaired *t* test, $p=0.4257$).

Figure 4.

No significant effect of vemurafenib on cytokine production by of *in vivo* activated transgenic CD4+ T cells. B10.D2 BRAF WT mice were immunized and Thy1.1+ Clone 1 cells and CD45.1+ SFE T cells were injected on day 1. Mice were treated with 200mg/kg vemurafenib in 2% aqueous Klucel LF or vehicle control daily via gavage for 5 days. Following *in vitro* stimulation with appropriate peptide, cytokine production by isolated splenic lymphocytes was analyzed by FACS on day 6. Data are representative of 4 independent experiments with 3–5 mice per group. 4A, no significant difference in % of CD4+/SFE splenic lymphocytes producing IFN- γ in mice treated with vemurafenib or control (two tailed unpaired *t* test, $p=0.7863$); 4B, no significant difference in % of CD4+/SFE splenic lymphocytes producing IL2 in mice treated with vemurafenib or control (two tailed unpaired *t* test, $p=0.5525$).

Figure 5.

No significant effect of vemurafenib on immune mediated anti-tumor response *in vivo*. Thy 1.1+ Clone 1 and SFE+ CD45.1 T cells were injected into B10.D2 RIP-Tag2-HA mice on day 1 concurrently with peptide immunization with HA₅₁₈₋₅₂₆ and HA₁₁₀₋₁₁₉. Mice were treated with vemurafenib 200 mg/kg or vehicle control daily via gavage for 30 days. Serum blood glucose was measured weekly. Development of diabetes correlates with immune mediated destruction of insulinoma. Two-way ANOVA revealed no significant association between serum blood glucose and exposure to vemurafenib ($p=0.2104$). There was no significant difference between peak serum glucose concentrations on day 15 (two tailed unpaired t test, $p=0.3685$). Data are representative of 4 independent experiments with 3–5 mice per group.