Selective BRAF**V**600E Inhibition Enhances T-Cell Recognition of Melanoma without Affecting Lymphocyte Function

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Abstract

Targeted therapy against the BRAF/mitogen-activated protein kinase (MAPK) pathway is a promising new therapeutic approach for the treatment of melanoma. Treatment with selective BRAF inhibitors results in a high initial response rate but limited duration of response. To counter this, investigators propose combining this therapy with other targeted agents, addressing the issue of redundancy and signaling through different oncogenic pathways. An alternative approach is combining BRAF/MAPK-targeted agents with immunotherapy. Preliminary evidence suggests that oncogenic BRAF (BRAF**V**600E) contributes to immune escape and that blocking its activity via MAPK pathway inhibition leads to increased expression of melanocyte differentiation antigens (MDA). Recognition of MDAs is a critical component of the immunologic response to melanoma, and several forms of immunotherapy capitalize on this recognition. Among the various approaches to inhibiting BRAF/MAPK, broad MAPK pathway inhibition may have deleterious effects on T lymphocyte function. Here, we corroborate the role of oncogenic BRAF in immune evasion by melanoma cells through suppression of MDAs. We show that inhibition of the MAPK pathway with MAPK/extracellular signal-regulated kinase kinase (MEK) inhibitors or a specific inhibitor of BRAF**V**600E in melanoma cell lines and tumor digests results in increased levels of MDAs, which is associated with improved recognition by antigen-specific T lymphocytes. However, treatment with MEK inhibitors impairs T lymphocyte function, whereas T-cell function is preserved after treatment with a specific inhibitor of BRAF**V**600E. These findings suggest that immune evasion of melanomas mediated by oncogenic BRAF may be reversed by targeted BRAF inhibition without compromising T-cell function. These findings have important implications for combined kinase-targeted therapy plus immunotherapy for melanoma.

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Introduction

Melanoma is a major world health problem with an incidence that is increasing at a rate faster than any other solid malignancy. The overall survival for patients with metastatic melanoma is poor (1, 2), and novel forms of treatment are needed.

Key genetic alterations have been identified in melanomas, resulting in increased oncogenic potential (3). A better understanding of these oncogenic signaling pathways has stimulated interest in therapeutic strategies targeting molecular mechanisms. Somatic mutations in the BRAF oncogene occur in most melanomas, with >60% of melanomas harboring an activating point mutation in a specific codon of the BRAF gene (V600E; ref. 4). This leads to constitutive activation of the mitogen-activated protein kinase (MAPK) signaling pathway and increased oncogenic potential through a variety of mechanisms, including reduced apoptosis, increased invasiveness, and increased metastatic behavior (3).

Various inhibitors of BRAF and the MAPK signaling pathway have been tested in clinical trials. Treatment with a selective MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor yielded modest response rates (12%) among patients with a known BRAF mutation in a phase II trial involving melanoma (5). Preliminary results of a phase I/II trial with a selective BRAF inhibitor suggest that objective responses are seen in the majority of patients whose tumors harbor BRAF**V**600E (6). However, even the most encouraging of these agents yielded a median duration of response of only 8 months.

Functional redundancy and compensatory activity through alternate signaling pathways might explain the low response rates to MEK inhibitors and the emergence of resistance seen in patients treated with selective BRAF inhibitors (7, 8). To address these issues, combination of BRAF/MAPK-targeted therapy with other signal transduction inhibitors or with conventional chemotherapy has been proposed (9).

Another potential approach involves combining BRAF/MAPK-targeted therapy with immunotherapy. To date,
one of the most successful forms of immunotherapy incorporates adoptive transfer of autologous T lymphocytes after a lymphodepleting preconditioning regimen (10). However, this type of therapy is limited by the ability to generate tumor-reactive T cells and by the technical and financial resources required (11). An important variant involves the genetic modification of patient’s peripheral blood lymphocytes (PBL) to express T-cell receptors (TCR) targeting melanocyte differentiation antigens (MDA) for use in adoptive transfer. This strategy has shown promise in generating antitumor responses in clinical trials of patients with melanoma (12) but requires antigen expression by the tumor cells. Other immunemediated approaches such as vaccines, monoclonal antibodies, and cytokines are also available but are associated with lower response rates (13, 14).

The rationale for combination targeted therapy–immunotherapy is based on evidence that MAPK pathway inhibition leads to increased expression of MDAs (15). Recognition of antigens (including MDAs) is central to melanoma immunotherapy. This is supported by findings that loss of antigen expression can lead to relapse after immunotherapy (16).

We sought to test the hypothesis that treatment of melanoma cell lines with MEK inhibitors or a specific BRAF inhibitor would increase the expression of MDAs, resulting in improved recognition by MART-1–specific and gp100-specific T lymphocytes. We analyzed the effect of several different MAPK pathway inhibitors on T-cell function to provide a basis for rational combinations of systemic targeted therapy and immunotherapy for melanoma.

**Materials and Methods**

**Cell lines and tumor digests**

Melanoma cell lines 888 MEL, 624 MEL, and 1300 MEL were provided by the Surgery Branch, National Cancer Institute (NCI). Melanoma cell lines SKMEL28, UACC903, and Mel-Juso were provided by Dr. Hensin Tsao (Massachusetts General Hospital, Boston, MA). Fresh tumor digests were isolated from patients with metastatic melanoma enrolled on a tissue procurement protocol. All cell lines/digests were genotyped for BRAF mutation. Melanoma lines/digests were cultured in RPMI 1640 containing 10% fetal bovine serum at 37°C in 5% CO₂.

**Chemicals**

MEK inhibitors U0126 and PD0325901 were obtained from Cell Signaling Technology. The BRAF-V600E inhibitor PLX4720 was obtained from Symansis.

**Quantitative PCR**

Differential expression of MDAs in melanoma lines was validated using Taqman Gene Expression Assays (Applied Biosystems) with glyceraldehyde-3-phosphate dehydrogenase as control. Total RNA (250 ng) was used as template, and SuperScript III Platinum kit (Invitrogen) was used for one-step quantitative reverse transcription-PCR in a 25 μL reaction.

**TCR transduction of PBLs**

Retroviral vectors with TCR specific for the MART-1 and gp100 antigens were provided by the Surgery Branch, NCI. A green fluorescent protein (GFP)–encoding vector served as negative control. PBLs were obtained from melanoma patients on protocol and stimulated *in vitro* and transduced with TCR-encoding retroviral vectors as previously described (12).

**Flow cytometric analysis**

Melanoma cell lines were stained with intracellular stains for MART-1 using a phycoerythrin-conjugated antibody (Santa Cruz Biotechnology) and for surface staining of HLA class I expression using β2-microglobulin. Transduced PBLs were stained for MART-1 and gp100 tetramer, CD3, CD4, and CD8. Intracellular staining was also performed for IFN-γ. Cells were analyzed on a FACScalibur instrument (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

**Immunofluorescence**

Melanoma cell lines were cultured on glass slides and treated as described. Cells were fixed and stained with a monoclonal antibody targeting MART-1 and a FITC-conjugated secondary antibody (Abcam). Images were acquired using a Nikon Eclipse-80i fluorescence microscope.

**Cytokine release assay**

IFN-γ release was measured using commercially available ELISA kits (IFN-γ; Endogen).

**Cell viability assay for T cells treated with MEK or BRAF inhibitors**

PBLs were treated for 3 days with either U0126 or PLX4720. Each cell sample was then collected and assayed for viability using CellTiter-Glo Luminescent Cell Viability Assay from Promega.

**Results**

**MAPK pathway inhibition increases expression of MDAs in melanoma cell lines and fresh tumor digests**

Given the critical role of the MAPK signaling pathway in melanoma, we sought to investigate the role of oncogenic BRAF in modulating expression of MDAs. We expanded on prior literature by increasing the number of antigens assayed and also by testing the effects of treatment with a specific inhibitor of BRAF<sup>V600E</sup>. Melanoma cell lines were treated with two specific MEK inhibitors (U0126 and PD0325901), and mRNA levels of gp100, MART-1, Typrp-1, and Typrp-2 were analyzed. We observed a significant increase in mRNA levels for all four genes after treatment with MEK inhibitors, regardless of BRAF mutational status. In contrast, treatment of melanoma cell lines with a selective inhibitor of BRAF<sup>V600E</sup> (PLX4720; ref. 17) increased antigen expression only in BRAF-mutant lines (Fig. 1A). Fresh tumor digests were also treated with these agents, showing similar results (Fig. 1B). Western blot analysis showed that suppression of phospho-ERK...
Figure 1. MAPK pathway inhibition increases mRNA expression of MDAs. mRNA levels of gp100, MART-1, Tyrp-1, and Tyrp-2 in melanoma cell lines (A) and fresh tumor digest (B) treated with MEK inhibitors U0126 (10 μmol/L) and PD0325901 (0.2 μmol/L) or with the selective BRAFV600E inhibitor PLX4720 (1 μmol/L). Expression levels are shown as fold increase over the untreated control (X axis). Experiments were repeated at least three times with similar results.
correlated with upregulation of MDAs (Supplementary Fig. S1). There was no change in HLA class I expression (Supplementary Fig. S2). Expression of MDAs was proportionally upregulated in each cell line, although the level of induction varied significantly between different melanoma cell lines (e.g., 2-fold induction in 888 MEL versus 100-fold induction in UACC903), a point that will be discussed further later.

Increased expression of MDAs is associated with increased antigen-specific T-cell recognition

Next, we performed studies to test the ability of MAPK pathway inhibition to induce protein expression of MDAs and to improve antigen-specific recognition by T cells targeting these antigens. Melanoma cell lines were treated with MEK inhibitors (U0126 and PD0325901) or a selective BRAFV600E inhibitor (PLX4720), and MART-1 protein expression was assessed by flow cytometry. Treatment with MEK inhibitors increased MART-1 expression regardless of BRAF mutational status, whereas treatment with a specific BRAFV600E inhibitor increased MART-1 expression only in BRAF-mutant lines (Fig. 2A). This increase in protein expression was verified using immunofluorescence (Fig. 2B). Treated and untreated melanoma cell lines were cultured with MART-1–specific or gp100-specific T lymphocytes versus control (GFP-transduced) lymphocytes. Transduction efficiency exceeded 60% in every experiment (Supplementary Figure 2).
Increased MDA expression is associated with enhanced antigen-specific T-cell recognition. A, HLA-A2+ UACC903 melanoma cells were treated as above with a MEK (U0126) or BRAF (PLX4720) inhibitor and cultured with CTL specific for MART-1 or gp100 versus control lymphocytes (GFP-transduced) at various E:T ratios. IFN-γ release was measured by ELISA. Experiments were repeated at least three times with similar results. B, intracellular staining for IFN-γ was performed after overnight coculture of MART-1–specific or gp100–specific HLA-A2–restricted lymphocytes with treated (PD0325901 or PLX4720) and untreated (NT) melanoma cell lines UACC903 (HLA-A2+) or 888 MEL (HLA-A2−). The number in the upper-right quadrant represents the percentage of IFN-γ–positive cells in the tetramer-positive subset.
Fig. S3). IFN-γ release was used to assess T-cell recognition of tumor cells. Significant increases in IFN-γ were observed when MART-1–specific and gp100-specific T cells were cultured with melanoma cell lines treated with the MEK inhibitor or the selective BRAFV600E inhibitor PLX4720 (Fig. 3A) but not with control lymphocytes (P < 0.05). These findings were verified with intracellular staining for IFN-γ (Fig. 3B) and cytotoxicity assay (Supplementary Fig. S4).

These data suggest that MAPK pathway inhibition triggers upregulation of melanocyte antigen expression, which confers improved recognition by antigen-specific T cells.

**MEK inhibition, but not BRAFV600E inhibition, impairs T lymphocyte function**

One critical question in planning combined targeted molecular therapy and immunotherapy is the effect of systemic drug exposure on CTLs. We evaluated the effects of MEK inhibitors and a selective inhibitor of BRAFV600E on T lymphocyte function as assayed by T-cell expansion and antigen-specific recognition. T lymphocytes were stimulated with interleukin-2 and OKT3 and treated on days 2 to 8 with MEK inhibitors or a specific inhibitor of BRAFV600E at the same drug doses that induced MDA expression. T cells cultured in the presence of U0126 showed a decrease in proliferative response. In contrast, cells treated with PLX4720 showed no change in proliferation compared with untreated lymphocytes (Fig. 4A). Viability measurements revealed the same patterns of susceptibility by T lymphocytes to MEK inhibitor treatment (Fig. 4B). To assess the effects on T-cell recognition of tumors, treated and untreated T lymphocytes were cultured with treated and untreated HLA-A2+ UACC903 melanoma line. T-cell release of IFN-γ decreased significantly when lymphocytes were treated with MEK inhibitors (P < 0.05). Treatment with a specific inhibitor of BRAFV600E had no measurable effect on T-cell secretion of IFN-γ as assessed using both gp100-specific and MART-1–specific T cells (Fig. 4C).

**Discussion**

The development of selective BRAF inhibitors for treatment of BRAF-mutant malignancies represents the first successful application of targeted therapy in melanoma. Whereas the percentage of patients who show tumor regression early in the course of therapy is high, the proportion of patients who maintain a response for more than 12 months is small (6). The mechanism of cell death following treatment with BRAF inhibitors in humans is unknown, but in animal models apoptosis seems to predominate over necrosis (18). It is hypothesized that induction of apoptotic cell death with either targeted therapy or chemotherapy would result in the release of tumor antigens and effective vaccination of
the host. This might set the stage for more effective treatment with active immunotherapy, such as cytokines or immune checkpoint inhibitors.

Cytokine-based immunotherapy is associated with durable complete responses in a subset of patients with metastatic melanoma. Monoclonal antibodies blocking CTLA-4 are associated with an objective response rate 10% to 15% of patients, and the majority of these responses are durable (19). Thus, the longevity of clinical response is well established for this approach, whereas the percentage of responding patients remains low.

In the present study, we provide preclinical evidence supporting the use of combination BRAF-targeted therapy and immunotherapy for melanoma. We show that MAPK pathway inhibition using a selective inhibitor of BRAF(V600E) leads to increased expression of MDAs, conferring enhanced antigen-specific recognition by CTL without compromising lymphocyte function. In contrast, MEK inhibition has a similar effect on MDAs but deleterious effects on T cells. Clinical in vivo validation of these findings in patients being treated with these agents is a critical next step, and these studies are currently under way.

Further investigation is also required to uncover the mechanism underlying the effect of oncogenic BRAF on antigen expression in melanoma. Preliminary data suggest that oncogenic BRAF suppresses MDA expression through microphthalmia-associated transcription factor, as suggested by Wellbrock and colleagues (Supplementary Fig. S5; ref. 20). However, additional signaling pathways are important to melanoma pathophysiology and may account for the variability in MDA induction we observed across different melano- noma cell lines. In clinical trials, it will be critical to assay genetic alterations and status of pathway activation in the network of pathways beyond MAPK and correlate this with expression of MDAs, as significant heterogeneity may exist even among BRAF-mutant tumors. Variability in MDA expression changes with BRAF-targeted therapy will likely identify those patients for whom the addition of immunotherapy will be most effective. In those for whom MDA expression is minimally altered, additional signaling inhibitors might be used to achieve maximal antigen expression. Our data provide a clear rationale for the combination of targeted therapy and immunotherapy for melanoma and provide candidate pharmacodynamic markers to investigate in clinical trials.

Disclosure of Potential Conflicts of Interest

K.T. Flaherty: advisory board, Roche Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

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