Editorial

Combined BRAF and MEK inhibition in BRAF^{V600E} mutant melanoma: a synergistic and potentially safe combination partner with immunotherapy

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Submitted Sep 16, 2015. Accepted for publication Sep 25, 2015.
doi: 10.3978/j.issn.2305-5839.2015.10.17
View this article at: http://dx.doi.org/10.3978/j.issn.2305-5839.2015.10.17

The combination of immune checkpoint blockade using PD-1 and or CTLA-4 inhibition with BRAF inhibition for BRAF^{V600} mutant advanced melanoma is a potentially attractive option given the substantial anti-tumor activity of these two treatment modalities. However, initial clinical trials indicated that combined inhibition of CTLA-4 and BRAF may be associated with intolerable toxicity. MEK inhibition, when added to BRAF inhibition, enhances the inhibitory effect on MAPK pathway signaling in BRAF mutant cells while limiting paradoxical activation of the MAPK pathway in BRAF wild type cells, leading to enhanced clinical efficacy and possibly toxicity of combined BRAF/MEK inhibition compared to BRAF inhibition alone. This provides a rationale for the addition of MEK inhibition in a combined BRAF-directed immunotherapy approach. However, MEK inhibition had been associated with decreased T cell function *in vitro*. Expanding on previous studies in an adoptive transfer model with TCR transgenic T cells (pmel) recognizing the melanoma differentiation antigen (MDA) gp100 endogenously expressed on syngeneic BRAF^{V600E} mutant SM1 melanoma, Hu-Liewskovan perform systematic analyses of T cell frequencies and functionality, immune cell subtypes in the tumor microenvironment, and gene expression signatures. The studies show that the addition of MEK inhibition to combined BRAF inhibition and two different immunotherapy modalities [adoptive T cell transfer (ACT) and PD-1 inhibition] does not impede T cell function *in vivo*, has favorable effects on immune suppressive cells, and provides superior tumor control. These data support the investigation of combined BRAF/MEK inhibition with immunotherapy in patients with advanced melanoma.

Two distinct treatment approaches, developed in parallel over the last decade, have revolutionized the therapy for advanced melanoma: (I) immunotherapy with monoclonal antibodies directed at the inhibitory molecules CTLA-4 and PD-1 expressed on T cells has shown improved overall survival and objective tumor response rates of 40-60%—with many of the responses durable, lasting for many years (1-3); (II) targeted therapy with small molecules directed at BRAF and MEK induces high rates of rapid-onset tumor responses and improved overall survival in the approximately 50% of advanced melanoma patients with BRAF^{V600} mutant tumors (4-7). The convergence of these two novel and fundamentally different approaches to cancer therapy have rendered melanoma a prime example of the opportunities and complexities of combined targeted and immunotherapy.

Activation of signaling pathways in tumor cells have long been implicated in promoting suppressive immune networks in the tumor environment (8,9). A link between the MAPK pathway and the anti-tumor immune response in melanoma has been established in preclinical models and advanced melanoma patients. MAPK pathway inhibition affects the melanoma microenvironment on multiple levels with both beneficial and also potentially adverse impacts on T cells, dendritic cells (DC), tumor cells, stromal cells, and soluble factors (10,11). These effects include:

(I) Increased expression of tumor antigens. BRAF and MEK inhibition leads to overexpression of the MDA gp100, MART-1, and tyrosinase mediated through upregulation of microphthalmia-associated

transcription factor (MITF) and other pathways (12). The increased expression of tumor antigens is associated with improved antigen-recognition by T cells. Notably, in metastatic tumors of advanced melanoma patients treated with BRAF inhibition, MDA expression was down-regulated and CD8 cell numbers were decreased at the time of progression and restored upon initiation of combined BRAF/MEK inhibition (13). BRAF/MEK inhibition can also conceivably result in increased tumor antigen expression through cross presentation of tumor antigens from apoptotic melanoma cells.

(II) Migration of T effector cells into the tumor or expansion/proliferation of tumor infiltrating lymphocytes (TILs) in the tumor. In tumors of patients treated with BRAF inhibition, increased numbers of CD8 TIL, which returned to baseline levels at the time of disease progression and rose again with combined BRAF and MEK inhibition, were found. An increase in T cell clonality as determined by sequencing of the rearranged TCR beta chain was found in TIL upon BRAF inhibition suggesting that the invasion of T cells into the tumor is a (tumor) antigen driven event (14).

(III) Increased functionality of TIL. In an ACT mouse model using the murine BRAF^{V600E} mutant melanoma SM1 (syngeneic to immunocompetent C57BL/6 mice) and transgenic T cells recognizing gp100 the authors of the current article had previously shown that ACT given in combination with vemurafenib induced superior anti-SM1 tumor immune responses compared to either of the therapies alone (15). In contrast to some of the clinical observations and other mouse models, no increase in TIL frequencies was observed in this model, whereas the adoptively transferred T cells secreted more IFN-γ when stimulated with gp100 and exhibited higher cytotoxicity in mice treated with ACT and vemurafenib.

Conversely, an increase in the T cell exhaustion markers PD1, PD-L1, and TIM-3 was seen in melanoma biopsies of patients treated with BRAF inhibition; furthermore, PD-L1 expression was up-regulated in melanoma cell lines resistant to BRAF inhibition compared to BRAF sensitive parental lines (13,16). Assuming that an immune response plays a role in the treatment effect of MAPK inhibition, upregulation of these inhibitory immune pathways may indicate a potential resistance mechanism to BRAF inhibition. Notably, PD-L1 overexpression in melanoma cell lines could be overcome by MEK inhibition (16).

Based on the enhanced anti-tumor activity and improved overall survival of combined MEK/BRAF inhibition compared to BRAF monotherapy reported in phase 2 and 3 studies, combination therapy was approved by the FDA and has become the standard therapy for advanced BRAF^{V600} mutant melanoma over BRAF inhibition alone. Notably, combined BRAF/MEK inhibition is associated with lower toxicity compared to BRAF inhibition alone, most likely because of the MEK inhibitor mediated suppression of a paradoxically up-regulated MAPK pathway in BRAF wild type cells.

Both MEK and BRAF inhibition in melanoma cells lead to increased T cell effector and DC function *in vitro* in co-culture experiments (where immune cells are not directly exposed to BRAF or MEK inhibition) (12,17,18). In contrast, T cell and DC function appear to be unaffected or even improved by direct BRAF inhibition, whereas direct MEK inhibition leads to dampening of both T cell and DC function *in vitro* (12,18), raising the concern that the addition of MEK inhibition to combined BRAF inhibition and immunotherapy may be detrimental to the immune response. Conversely, some of the above *in vivo* observations (e.g., restoration of down-regulated MDA expression, increased TIL infiltration upon initiation of combined BRAF/MEK inhibition at the time of tumor progression on BRAF inhibition) suggest that the "net effect" of combined MEK/BRAF inhibition on the immune response is favorable.

Given the enhanced anti-tumor activity of BRAF/MEK inhibition over BRAF and MEK inhibition alone in advanced melanoma patients and the resulting interest and rationale for "triple therapy" with immunotherapy and combined BRAF/MEK inhibition, Hu-Lieskovan *et al*. set out to test the hypothesis that MEK inhibition can enhance the immune-stimulating effect of BRAF inhibition when combined with immunotherapy. The investigators expand on their previous work assessing the impact of the BRAF inhibitor vemurafenib on the immune response in a murine melanoma ACT model. In this model, T cells transgenic for a TCR specific for the melanosomal antigen gp100 are adoptively transferred into myelodepleted C57/BL/6 mice bearing BRAF^{V600E} mutant SM1 melanoma (which expresses gp100 endogenously).

As expected, BRAF inhibition with dabrafenib and MEK inhibition with trametinib either as monotherapy or in combination lead to suppression of phosphorylated

ERK (pERK) in SM1 melanoma cells. In contrast, pERK was "paradoxically" up-regulated in the gp100 specific transgenic T cells with dabrafenib alone, whereas both trametinib or combined dabrafenib and trametinib resulted in pERK inhibition. The viability of the transgenic T cells was unaffected with either of the therapies given as monotherapy or in combination.

Treatment with ACT plus dabrafenib and trametinib (triple combination) was more effective against SM1 tumors than combined dabrafenib and trametinib without ACT, ACT alone and ACT given with either dabrafenib or trametinib monotherapy. ACT plus MEK inhibition was more effective than ACT plus BRAF inhibition in this model. Of note, ACT by itself had only modest, if any, anti-tumor activity.

To determine whether BRAF and MEK inhibition differentially affect the homing of T effector cells, adoptively transferred pmel-effector cells and both endogenous and adoptively transferred CD8 cells were quantified by flow cytometry in tumors and spleen. BRAF and MEK inhibition lead to an increase in TILs when given as monotherapy and in combination. Notably, BRAF inhibition alone resulted in the numerically highest increase in $CD3^+CD8^+$ T cells as compared to MEK inhibition alone or combined MEK/BRAF inhibition. When adoptively transferred pmel effector cells were quantified by bioluminescence imaging, TIL numbers were elevated with BRAF and MEK inhibition alone and in combination compared to ACT alone and peaked at day 5. No differences between the MAPK inhibition modalities (BRAF inhibition alone, MEK inhibition alone, combination BRAF/MEK inhibition) were seen.

To address the question of compromised T cell functionality mediated by MEK inhibition raised from *in vitro* assays (confirmed in the current study) in their adoptive transfer model, Hu-Lieskovan *et al*. measured the activation state of pmel effector cells *in vivo*. No statistical differences in IFN-γ secretion upon restimulation by gp100 peptide were found in pmel effector cells harvested from SM1 tumors and spleens from animals treated with BRAF and MEK inhibition alone or in combination, although BRAF inhibition alone appeared to be associated with numerically higher IFN-γ–secretion (not statistically significant). To assess T cell functionality independently from the effect of BRAF and MEK inhibition on the melanoma, the cytotoxicity of adoptively transferred pmel effector cells pulsed *in vivo* with gp100 was assessed by measuring the proliferation of CFSE-labeled BRAF wild type target splenocytes. Again, cytotoxicity showed no statistically significant differences between the BRAF and MEK monotherapy and combined therapy, although it was numerically increased with dabrafenib. The authors' conclusion that MEK inhibition does not compromise the immune-sensitizing effects of BRAF inhibition *in vivo* is supported by this series of experiments.

In a subsequent set of experiments, frequencies of additional immune cell populations including myeloid-derived suppressor cells (MDSC), tumor associated macrophages (TAM), and T regulatory cells (T-regs) were assessed by FACS analysis. A striking shift towards increased MDSC of the monocyte subtype and decreased MDSC of the granulocyte subtype was observed with BRAF inhibition, MEK inhibition as monotherapy and combined BRAF/MEK inhibition (with no differences between the three modalities). BRAF and combined BRAF/MEK inhibition was associated with increased numbers of TAM, whereas BRAF inhibition lead to increased T-reg numbers.

Analysis of the tumor microenvironment using gene expression profiling of SM1 tumors after treatment showed clusters of genes that were up- or down-regulated with BRAF and/or MEK inhibition independent of additional ACT, suggesting a direct effect of the targeted agents, and genes that were up-regulated with BRAF and/or MEK inhibition only when given in combination with ACT, suggesting that the effect was dependent on the presence of tumor specific effector T cells. Interestingly, expression of MHC and MDA, both found to be critical for sensitization of tumor cells by MAPK inhibition, appears differentially mediated according to these categories—MDA was up-regulated independently of ACT, whereas MHC expression was increased only with concurrent ACT.

Gene expression profiling also revealed upregulation of IFN-γ, granzyme B, and PD-L1 in SM-1 tumors after BRAF and MEK inhibition, suggesting increased infiltration of tumors with T effector cells. Consistent with these findings, PD-L1 measured by FACS was found to be up-regulated after BRAF and/or MEK inhibition in tumors, while no differences were seen in spleens. Overall in line with the ACT model, PD-1 inhibition combined with BRAF and MEK inhibition showed more effective tumor control compared to PD-1 inhibition with either BRAF or MEK inhibition alone. Notably, PD-1 inhibition itself had no anti-tumor activity in this model.

Taken together, these elegant studies show that both BRAF and MEK inhibition have mostly beneficial effects on multiple components of the tumor immune

microenvironment, including increased frequencies and enhanced activation status of tumor infiltrating T cells, inhibition of suppressive immune cells types (TAM, MDSC, T-regs), and chemokines. No detrimental effect of MEK inhibition on effector T cell function was seen *in vivo* (the concern from previous *in vitro* experiments) and therefore MEK inhibition should not compromise the efficacy of T cell directed immunotherapy when used in a triple regimen in humans.

Despite the numerous effects on molecules seen in the gene expression profiles it remains elusive whether there is a principle mechanism for the immune-sensitization seen with BRAF and MEK inhibition. As Hu-Lieskovan *et al*. show, PD-L1 expression seems an important driver of these events, a link that could be more firmly established for example by using siRNA mediated knockdown of the PD-1/PD-L1 axis. Pertubation of individual components contributing to the immune response could identify potential other key drivers. On the T cell level, using technologies such as shRNA libraries or CRISPR/CAS9, it is now possible to directly modulate genes in primary T cells (19,20).

Hu-Lievskovan *et al*.'s experiments clearly demonstrate that combined BRAF/MEK inhibition leads to superior tumor control compared to BRAF or MEK inhibition alone when added to two distinct immunotherapy modalities (ACT and PD-1 inhibition). It is possible that the enhanced tumor control of the triple therapy versus "double therapy" (immunotherapy plus BRAF or MEK inhibition) is mediated predominantly by the superior anti-tumor activity of combined BRAF/MEK inhibition, independent of immunotherapy.

Since in patients (I) combined BRAF/MEK inhibition has emerged as a superior strategy for BRAF^{V600} mutant melanoma compared to BRAF inhibition alone and (II) BRAF inhibition in combination with CTLA-4 blockade was associated with unacceptable toxicity, the observation that MEK inhibition does not compromise T cell effector function *in vivo*, coupled with its ability to counteract paradoxical MAPK pathway inhibition in BRAF wild type cells (thereby potentially mitigating toxicity) is highly encouraging with respect to combined strategies with immunotherapy.

Nevertheless, whether combined MEK/BRAF inhibition provides enhanced immuno-sensitization compared to BRAF inhibition alone (i.e., whether combined BRAF/MEK inhibition should be the preferred combinatorial partner versus BRAF inhibition alone from a pure immunotherapy/targeted therapy synergy perspective) remains to be fully elucidated. The observations that BRAF inhibition leads to an increase in T-regs (which can be partially overcome by MEK inhibition) and the effect of combined BRAF/MEK on chemokines contrast with the findings of a seemingly more pronounced increase of tumor infiltrating CD8CD3 T cells, PD-L1 expression, and IFN-γ secretion, as well as numerically higher T cell cytotoxicity in a series of experiments where the effect on cytotoxicity was uncoupled from the direct effect on the tumor. The relatively modest efficacy of both immunotherapy modalities (ACT and PD-1 inhibition) when used as monotherapy as well as the lower anti-tumor activity of BRAF *vs*. MEK inhibition are not fully in line with the effects of these treatments in humans. Ultimately, ongoing clinical trials will hopefully answer this important question.

Acknowledgements

The author thanks Rizwan Haq and Sameer Chopra for their input and thoughtful discussions.

Footnote

Provenance: This is a Guest Editorial commissioned by Executive Editor Bing Gu, MD (Department of Laboratory Medicine, the Affiliated Hospital of Xuzhou Medical University, China).
Conflict of Interest: The author has no conflicts of interest to declare.

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Cite this article as: Ott PA. Combined BRAF and MEK inhibition in $BRAF^{V600E}$ mutant melanoma: a synergistic and potentially safe combination partner with immunotherapy. Ann Transl Med 2015;3(20):313. doi: 10.3978/j.issn.2305-5839.2015.10.17

