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Introduction: Targeted therapy and immunotherapies are the novel pharmacologic treatment strategies for metastatic melanoma. BRAF and MEK inhibitors effectively block the hyperactivation of the MAPK pathway in BRAF mutant melanomas and also have several other effects on melanoma cells and on the immune response. The aim of this work is to discuss the rationale, evidence and perspectives of approaches combining target and immunotherapy against melanoma.

Areas covered: We first review the effects of BRAF and MEK inhibitors on melanoma cells and on the different components of the immune system. Afterwards, we summarize the results of the preclinical and clinical studies that have combined targeted therapy and immunotherapy for the treatment of melanoma.

Expert opinion: Clinical applications of immunotherapy strategies have recently changed the therapeutic mainstay for metastatic melanoma. Biologic and initial preclinical data support their integration with innovative molecular targeted therapies, opening enormous perspectives for researchers in the effort of finding a definitive cure. Main open challenges are the definition of reliable research models, assessment of effective schedules, safety issues and designing of personalized approaches.

Keywords: BRAF inhibitors, immunotherapy, melanoma, targeted therapy

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1. Introduction

The novel pharmacologic treatment strategies for metastatic melanoma are divided into two categories: i) targeted therapies that specifically block signaling pathways and ii) immunotherapies that boost the immune systems' response to tumor cells.

Approximately half of melanomas harbor a somatic point mutation of BRAF [1,2], which determines a constitutive activation of the MAPK pathway. This leads to tumor progression, evasion of senescence and apoptosis, unchecked replicative potential, angiogenesis, tissue invasion and metastasis as well as evasion of immune response [3].

The BRAF inhibitors (BRAFi) vemurafenib and dabrafenib and the MEK inhibitor (MEKi) trametinib are approved for the treatment of BRAF V600 mutant metastatic melanoma [4-6].

The different mechanisms of action and effects of target inhibitors and immunotherapies offer the rationale of combining these two approaches. Targeted therapies lead to quick cell death and release of tumor antigens that sensitize the immune system and can induce a stronger response [7]. The rapid tumor regression following targeted therapy decreases the tumor-associated immunosuppression typical of the tumors' microenvironment and offers a favorable window for immunotherapy. Moreover, targeted therapy can directly modulate the immune system components to boost the immune-mediated tumor destruction.

The aim of this work is to discuss the rationale, evidence and perspectives of approaches combining targeted and immunotherapy against melanoma. We first analyze the effects of the BRAFi and MEKi on both melanoma and immune system cells (Figure 1). We then evaluate preclinical and clinical data of combined treatments (Table 1).

2. Effects of BRAFi and MEKi on melanoma cells

BRAFi and MEKi specifically block the MAPK pathway and cause tumor growth arrest [8-10]. This direct effect on the signaling pathway leads to tumor cell death and rapid release of tumor antigens that could be picked up by antigen-presenting cells (APC), presented to T cells and could potentiate the immune system response. However, there are still no experimental data to support this hypothesis [7].

Beyond the direct effect of BRAFi and MEKi on the MAPK pathway, other effects were described on melanoma cells. In this paragraph, we review how BRAFi and MEKi could indirectly modulate the immune response through their effect on melanoma cells.

The expression of some surface molecules appears to change during target inhibitor treatment and this could affect the melanoma-specific immune response on the level of antigen-specific T-lymphocyte response.

Preclinical studies and patient specimen analysis showed an increased expression of melanocyte differentiation antigens following BRAFi and MEKi treatment [11-13]. It was hypothesized that target inhibitors could reverse the suppression of microphthalmia-associated transcription factor, a transcription factor essential for melanocyte differentiation, induced by the presence of the oncogenic BRAF mutation [14].

The expression of MHC class I and II in melanoma cells also increases during BRAFi treatment [15].

BRAF and MEK inhibition also resulted in reduced expression of CD200 mRNA in melanoma cells [16]. CD200, a type I glycoprotein, a member of the immunoglobulin superfamily, is highly expressed on melanoma cells. CD200 is regulated by ERK activation [17] and mediates an inhibitory signal interacting with its receptor on macrophages and dendritic cells (DCs) [16,18]. Both BRAFi and MEKi reduce CD200 levels in melanoma cells, prevent the inhibitory effect on DCs and therefore restore the ability of DCs to activate T cells [16].

The effect of BRAFi on the expression of programmed cell death ligand (PD-L)1 and PD-L2 is a matter of debate. PDL1 and PD-L2 are ligands for programmed cell death protein 1 (PD1), a co-stimulatory molecule that plays an inhibitory role in regulating T-cell activation in the periphery [19]. First preclinical and in vivo studies suggest an increase in PD-L1 tumor expression following treatment with BRAFi alone

and in combination with MEKi [13,20]. A more recent study described an initial transient in vitro inhibition of PD-L1 by BRAFi followed by steadily increased level of PD-L1 [21]. In cell lines made resistant to BRAFi, an increased expression of PD-L1 was described [20,21]. A possible relation between mechanisms of resistance and targeted therapy was also postulated: a resistance to BRAFi due to the activation of alternative signaling pathways was accompanied by the induction of PDL1 expression, whereas the resistance due to the reactivation of the MAPK pathway had no effect on PD-L1 expression [22]. The analysis of sequential biopsies taken prior to treatment, early during treatment and at time of progression from patients treated with BRAFi or combinations of BRAF and MEKi showed no difference in PD-L1 positivity rates. A different trend was noticed when patients were stratified on the basis of pre-treatment PD-L1 positivity. Patients' tumors that were PD-L1 positive at baseline showed a significant decrease in PD-L1 expression at progression, whereas patients' tumors that were PD-L1 negative at baseline showed a significant increase in PD-L1 expression at progression irrespective of treatment with BRAF or combination of BRAFi and MEKi [23].

Targeted therapy can also affect the tumor microenvironment and the immune response by modulating the tumor cell cytokine secretion. BRAF and MEK inhibition were reported to decrease the production of VEGF, IL-10 and IL-6 [24]. VEGF is an endothelial cell-specific growth factor and the principal regulator of angiogenesis under normal and pathological conditions in most organs [25]. Its downregulation during BRAFi treatment was confirmed in vitro and in melanoma patient biopsies before and during the treatment [26]. BRAFi inhibit VEGF production by reducing the binding of c-Myc to the VEGF promoter. This down-regulation of VEGF has a beneficial effect because the blockage of the interaction of VEGF with its receptor upregulates endothelial adhesion molecules in tumor vessels, which can in turn increase the number of leukocytes infiltrating the tumor [27]. Moreover, it can contribute to normalize tumor vessels and create a homogeneous distribution of perfused vessels throughout the tumor. This was shown to reprogram the tumor microenvironment away from immunosuppression toward potentiation of immunotherapy strategies [28].

3. Effects of BRAFi and MEKi on host immunity cells

In this section, we focus on the direct effect of BRAFi and MEKi on the different components of the immune system.

3.1 Tumor infiltrating lymphocytes

The number of tumor-infiltrating lymphocytes (TILs) within and around patients' metastases increases during BRAFi treatment [29]. CD8+ TILs were consistently increased during BRAFi treatment, while the increase in CD4+ TILs could not be confirmed in all studies [13,30]. The increase of CD8+ TILs during BRAFi treatment was correlated with decrease in tumor size and a correlation with patients' response was hypothesized: CD8+ amount increases after the beginning of BRAFi therapy and decreases at tumor progression [13,30]. However, a more recent study demonstrated that TILs increase in both responders and non-responders to BRAFi treatment. The difference between these two groups appeared to be related to the presence of a pre-existing population of tumor-infiltrating T-cell clones rather than to the infiltration of neoplastic lesions by new T-cell clones. In fact, 80% of the T-cells clones detected after initiation of BRAFi treatment are new clones, but only the pre-existing clones could predict a response to the treatment. Patients who had a higher proportion of pre-existing clones responded better to therapy than patients who had a low proportion of such pre-existing clones [31].

BRAFi specifically target the mutant form of BRAF harbored by melanoma cells. The mechanism of action of BRAFi appears to be different in cells that are wild type for BRAF. Studies performed in RAF wild-type tumors proposed that under physiological conditions BRAFwt maintains itself in an inactive conformation through its own kinase activity. BRAFi, interfering with BRAF activity, allow BRAF to escape from this auto-inhibited state and to be recruited to the plasma membrane by RAS where it forms a stable complex with CRAF. In these BRAF-CRAF dimers, BRAF seems to act as a scaffold whose function is to enhance CRAF activation that leads to hyperactivation of the MAPK pathway. This CRAF-mediated paradoxical activation is RASdependent [32-34]. The effect of BRAFi on non-neoplastic BRAFWT cells, as T cells, is matter of debate. Different studies reported no impact on the function of T cells during BRAFi treatment [26,35,36]. However, a more recent work proposed that BRAFi potentiate T-cell activation in vitro and in vivo in a dose-dependent manner. This activation requires a T-cell activating stimulus, such as engagement of the T-cell receptor (TCR), which triggers ERK signaling through RAS. The same effect was observed also using a pan-RAF inhibitor. This suggests that the CRAF-mediated hyperactivation cannot entirely explain the paradoxical activation of the MAPK pathway [37].

The effect of MEKi on T cells is also not clear. In vitro MEKi had an inhibitory effect on T lymphocytes [12], but no difference was observed in the absolute number of CD8+ TILs comparing patients receiving BRAFi alone or a BRAFi plus a MEKi [13,23]. It has been hypothesized that CD8+ TILs may be insensitive to MEK inhibition since they consist mostly of antigen-experienced memory cells and MEKi may have a less pronounced effect on memory T cells than on their naı¨ve counterparts [38].

BRAFi appear to affect not only the amount of TILs, but also their phenotype. In a mouse model, BRAFi promoted the expression of CD40L and IFN-g on intratumoral CD4+ TILs and reduced the accumulation of regulatory T cells (Tregs). These changes lead to the development of a more immune stimulatory microenvironment [39]. The number of PD1+ TILs increased early during BRAFi treatment compared with pre-treatment patients' biopsies, while this was not observed in patients treated with a combination of BRAFi and MEKi. This could be due to a defect in T-cell differentiation when the patient is receiving both BRAFi and MEKi, but could also be due to the lack of statistical power of the study [23].

3.2 Peripheral blood cells

A drug-specific effect rather than a class-specific effect was postulated for BRAFi on peripheral blood cells. No changes in the absolute number of T-cells (both CD4+ and CD8+), B-cells, natural killer (NK) cells, DCs, monocytes and Tregs or the ex vivo functionality of T cells were found following dabrafenib treatment [35,40]. Meanwhile, a decline in CD4+ T cells and an increase in circulating NK cells were observed in patients treated with vemurafenib [41]. The MEKi trametinib alone or in combination with dabrafenib suppressed T-lymphocyte proliferation, cytokine production and antigen-specific expansion [40].

3.3 Dendritic cells

DCs are a sentinel component of the innate immune system and can function as APCs to induce adaptive responses by processing and presenting antigens to naive T-lymphocytes in lymphoid organs [42]. Melanoma cells have an inhibitory effect on DCs function. Lysates of primary melanoma cells and cell lines suppress the IL-12 secretion of autologous patients' and healthy donor DCs, subsequently limiting the ability of DCs to initiate a Th1 response [43]. This inhibitory effect can be reversed by both BRAFi and MEKi [24,43]. BRAFi showed no direct effect on DCs function [44] and their numbers in peripheral blood [35]. The direct effect of MEKi on DCs is controversial.

Some studies described an enhanced DC maturation [40,45,46], others reported no or minimal impact on DC function [47,48]. One study found an inhibition of DCs maturation [44] during MEKi treatment. The discrepant results could be due to the different maturation stimuli used and investigated in each of the above-mentioned studies. An enhanced maturation of DCs would implicate a loss in DCs ability to endocytose, capture and internalize immune complexes resulting in reduced cross-presentation of tumor antigens [40].

3.4 Natural killer cells

NK cells play critical roles in immunity against cancer. NK cells recognize the tumor cells via stress or danger signals. Activated NK cells directly kill target tumor cells and act as regulatory cells when interacting with DCs, improving their antigen uptake and presentation and facilitating the generation of an antigen-specific T-cell response [49]. A preclinical study found that NK cells are also crucial for the therapeutic effect of BRAFi. BRAFi increase the proliferation of mouse and human NK cells in vitro (with increased levels of p-ERK and CD69) and enhance the frequencies of NK cells in lung metastases of a mouse model. In the same model, depletion of NK cells significantly impaired the antimetastatic effects of BRAFi treatment [50].

3.5 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC) suppress T- and NK-cell function by increasing the expression of inflammatory mediators [51]. Patients with metastatic melanoma had a higher number of circulating MDSC compared with healthy donors and patients with localized disease [52]. The amount of MDSC in the blood of melanoma patients declined during BRAFi treatment in individuals achieving clinical responses. This seemed to rely on the decreased release of soluble factors that induce MDSC, such as IL-6 from melanoma cells, more than on the direct effect of BRAFi on MDSC. In fact, the conditioned medium from untreated melanoma cells induced MDSC that exerted robust immunosuppressive effects on T cells. In contrast, the conditioned medium from BRAFitreated melanoma cells did not [53].

4. Preclinical and clinical data on target and immunotherapy combinatory regimens

In this section, we will review the preclinical and clinical data already available on treatment regimens which combined BRAFi and/or MEKi with an immunotherapy strategy

(Table 1).

4.1 BRAFi combined with IFN-a-2b

The overexpression of MHC class I and II molecules on melanoma cells during BRAFi treatment, which leads to a more favorable melanoma-specific immune response, is mediated by IFN [15]. Therefore, the combination of BRAFi and IFN was first investigated in preclinical studies. In a mouse model, it was able to prolong rodent survival [54]. This led to the design of clinical trails, which test the safety and efficacy of such combinations (NCT01959633, NCT01943422). The trials are currently ongoing and preliminary data are at present not available.

However, a possible concern could raise from the recent evidence of the crucial role of NK cells for the antimetastatic activity of BRAFi [50]. In fact, the MHC class I overexpression in tumor cells induced by IFN-a-2b can trigger an inhibitory signal in NK cells.

4.2 BRAFi combined with IL-2

IL-2 was the first immunotherapy approved for the treatment of metastatic melanoma. High-dose (HD) IL-2 demonstrated a complete response rate of 6% and partial response rate of 10% in patients with advanced melanoma, with some longlasting responses [55]. However, due to its significant acute toxicity (severe hypotension, pulmonary edema, systemic edema with significant weight gain and renal insufficiency, rash and fatigue), this regimen requires hospitalization and it is reserved for patients in a good performance status and without involvement of the CNS [56,57].

The rationale of combining IL-2 with BRAFi comes from the observation that BRAFi increase the expression of tumor-specific antigens leading to an enhanced T-cell recognition [11-13], which could be sustained by IL-2 that plays a central role in the activation and stimulation of T-lymphocytes. Moreover, IL-2 could boost the NK response, which was shown to be crucial for the antimetastatic activity of BRAFi [50]. However, HD IL-2 was shown to enhance the expansion of highly suppressive Tregs more than anyother lymphocyte subsets, and this increased level of Treg in blood following the first cycle of HD IL-2 was also correlated with worse outcomes [58].

Clinical trails investigating the combination of a BRAFi with IL-2 are currently ongoing (NCT01683188, NCT01603212).

4.3 BRAFi and/or MEKi combined with adoptive T-cells transfer

Adoptive T-cells transfer (ACT) involves the direct administration of autologous ex vivo expanded tumor reactive T-lymphocytes to preconditioned recipients. Several of the aforementioned effects of targeted therapy lead to the hypothesis of their effective combination with ACT. The tumor antigens released from dead melanoma cells during targeted therapy can provide an 'endogenous' vaccine-like stimulus, which could enhance the ACT effectiveness. In fact, in a mouse model ACT was enhanced by vaccination. The up-regulation of melanoma antigens during both BRAFi and MEKi treatment [11,12] can also improve tumor cell recognition by ACT. The increased T-cell infiltrate, which was observed during targeted therapy [13,23,30], and the observed inhibition of MDSC [53] could also favor the outcome of an ACT therapy.

In a mouse model, BRAFi treatment was shown to increase tumor infiltration of adoptively transferred gp100-specific T cells and to improve antitumor responses [26]. In another mouse model of syngeneic BRAF(V600E)-driven melanoma, SM1, ACT based on both T cells with a TCR recognizing chicken ovalbumin expressed by the tumor or on pmel-1 TCR transgenic lymphocytes recognizing gp100 endogenously expressed by the tumor, resulted in superior antitumor responses when combined with BRAFi treatment [59]. In the same mouse model, SM1, the combination of BRAFi and MEKi with pmel-1 ACT showed complete tumor regression, increased T-cell infiltration into tumors and improved in vivo cytotoxicity [60]. Clinical trials investigating the combination of ACT and targeted therapy are currently ongoing (NCT01585415, NCT02354690, NCT01659151) [61].

4.4 BRAFi combined with immune-checkpoint modulators (Ab anti-CTLA-4 and anti-PD1)

Ipilimumab is an inhibitory monoclonal antibody directed against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). The resulting blockade of CTLA-4 signaling prolongs T-cell activation, restores T-cell

proliferation and thus amplifies T-cell-mediated immunity, which enhances the patient's capacity to mount an antitumor immune response [62]. The observation that BRAFi promote the development of a more immune stimulatory microenvironment by increasing intratumoral cytotoxic T cells suggests a possible synergistic effect with ipilimumab [13,30,31].

The first clinical trial which explored the concurrent administration of ipilimumab and vemurafenib was interrupted because of hepatotoxicity [63], but the combination of the other BRAFi, dabrafenib, with ipilimumab showed a more favorable safety profile [64] and is currently under investigation in patients (NCT01767454, NCT01940809, NCT02200562) [61].

Sequential treatment has also been evaluated. Ipilimumab does not appear to negatively influence response to BRAFi, but ipilimumab therapy following BRAFi treatment was associated with low responses and poor survival [65,66]. These results could be due to the fact that patients who discontinue the BRAFi usually have rapid progression and therefore have insufficient lifespan to benefit from ipilimumab, which typically requires weeks or months to show a response. The low response to ipilimumab after BRAFi resistance can also be due to the changes in tumor antigen expression and tumor microenvironment over time. The favorable tumor microenvironment characterized by increased tumor melanocytic antigen expression and CD8+ T cells, typical of the beginning of BRAFi treatment, is no longer evident in tumor samples collected at time-of-progression [13,30].

PD1 and its ligands, PD-L1 and PD-L2 play an important role in regulating immune response through various mechanisms [19]. In a BRAF(V600E)/Pten(-/-) syngeneic tumor graft immunocompetent mouse model, the administration of anti-PD1 or anti-PD-L1 with a BRAFi led to an enhanced response, a prolonged survival and slower tumor growth. Moreover, the combinatory regimen increased the number and activity of TILs [67].

In another mouse model of syngeneic BRAF(V600E)- driven melanoma, SM1, the combination of BRAFi, MEKi and anti PD1 therapy showed a superior antitumor effect compared with anti-PD1 plus either therapy alone or isotope control with both BRAFi and MEKi [60].

In clinical trials, anti-PD1 antibodies showed a higher efficacy in patients with PD-L1-positive tumors [68,69]. The increased expression of PD-L1 in tumors treated with a BRAFi can explain the benefit of a combination with anti-PD1 or PD-L1 antibodies. Such combinatory regimens are currently under investigation (NCT02130466, NCT02027961, NCT02357732, NCT02224781, NCT01656642) [61].

5. Expert opinion

In this review, we discuss the rationale supporting potential synergisms between various immunotherapy and targeted therapy approaches for melanoma. We discuss goals, perspectives, critical aspects and limitations in research models that need to be faced by scientists in the field. In the previous paragraphs, we emphasized the possible positive correlation between target therapies and antitumor immune response. However, we also need to consider and explore potential downsides of these treatments, such as bystander effects of targeted therapy on tumor microenvironment and 'negative regulators' of immune response.

The development of new therapies without the consideration of the intrinsic biological variability of tumors and patients is a methodological weakness. For example, tumor immune infiltration is highly variable among patients. The amount and quality of tumor immune infiltration is a prerequisite for the

effectiveness of immuno-modulatory therapies [70]. This could also influence the outcomes of possible combination therapies and should be taken into account in the upfront selection of patients and in the data interpretation. Similarly, the tumor microenvironment can also affect the response to treatment. The predictive role and the potential susceptibility to treatments of negative regulatory elements such as MDSC and Treg is an important end point to evaluate.

The first goal in order to rationally elucidate the basis for experimental treatments that involve immunotherapy is the investigation of the tumor 'neo-antigen landscape'. In fact, the presence of relevant and immunogenic antigens is mandatory for the ultimate efficacy of adaptive immune responses. The genetic instability and the mutational load of tumors might positively correlate with the generation of immunogenic neo-epitopes [71,72]. From a practical point of view, the incorporation of such analyses in future studies requires the introduction of translational end points and a tight collaboration of clinical and preclinical researchers. In order to achieve this, deep sequencing data and prediction of immunogenic molecules should be done on tumor samples; whenever possible, longitudinal tumor biopsies should be scheduled to collect tumor samples representative of relapse or chemo-resistance conditions.

Another ambitious goal for new experimental treatments is to identify and eradicate cancer stem cells (CSC), which are considered responsible for disease relapse and chemo-resistance. Some preclinical evidence supports the possibility that immunotherapy may be effective against CSC in solid tumors including melanoma [73-75] and potential synergisms with targeted therapy in this direction are an intriguing field to explore.

Another important factor for the future preclinical research is the definition and availability of appropriate in vivo models to investigate synergisms of immunotherapy and molecular targeted approaches. Murine immune-competent models may be indicative but not entirely representative of the complex and patient-specific interactions between immune system and tumors. Tumor xenografts in immunodeficient animals, on the other hand, are very useful to assess the activity of targeted therapy but miss the potential to explore functions and modulations of the immune response.

Researchers are currently exploring the possibility to generate 'humanized mice', where immunodeficient animals are engrafted with human hematopoietic stem cells (HSC) capable of durable immune reconstitution [76,77]. This system would allow exploring the effect of molecular targeted drugs on immune effectors. However, it still holds important limitations given by the HLA-mismatch between the engrafted immune system and the implanted tumor. In theory, it would be ideal to reconstitute mice with autologous HSC collected from cancer patients, but this is obviously not practicable outside exceptional situations.

These limitations may be partially overcome for studies exploring adoptive cell therapy (ACT) approaches. In this case, it is feasible to collect adoptive immune effectors (e.g., TIL, NK, LAK, CIK cells) and infuse them into immunodeficient mice engrafted with autologous tumors.

However, these models would still be incomplete. They would not represent potential negative modulations operated by the tumor-conditioned immune system, as seen in patients, but could enable a transitory platform to investigate synergism with molecular targeted approaches.

While the association of targeted therapy with ACT is intriguing and supported by the reported biologic evidences, the optimal schedule is still to be defined.

We speculate that a sequential treatment may benefit from the immunogenic effect of targeted therapy on tumor cells followed by an augmented antigen presentation, while a concomitant administration might exploit the reported activation exerted by RAFi on circulating lymphocytes. Even when animal models should be available, unpredictable safety issues should always be considered, and 'first-in-human' studies should consider progressive dose-escalation schedules and accurate monitoring for undesired effects.

It has to be considered that combinatorial approaches of molecular targeted drugs with immune checkpoint modulators have logistic advantages over ACT. The ACT therapies require dedicated facilities and procedures compliant with rigorous good manufacturing procedures requirements, currently limiting its application to a few specialized centers.

An intriguing evolution of ACT includes the possibility to genetically redirect T lymphocytes against specific tumor antigens. Redirected specificity may be conferred by transferring genes encoding for either a tumor-antigen-specific TCR or an antibody-based chimeric receptor. Initial promising clinical results against melanoma have been reported with the infusion of TCR-engineered T cells against MART-1, GP-100 and NY-ESO antigens [78-80]. Clinical data of synergism between engineered ACT and molecular targeted therapies have not been reported yet. However, positive premises are in place and it is conceivable to expect interesting studies to come in the next future.

Overall, we are experiencing exciting times in melanoma research, which will hopefully lead the way to biologic knowledge and innovative treatments exportable to other tumor models. The central point of this process is immunotherapy and its possible integration with innovative molecular targeted approaches. From a 'clinical practice' point of view, the melanoma treatment is moving towards a 'personalized' approach based on biologic basis. The field will have to find a balance between the ever-increasing treatment possibilities generated by translational research and the desire of the clinician for easily understandable and manageable approaches with the ultimate goal of finding the best cost--effectiveness in curing melanoma.

Declaration of interests

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Bibliography

Papers of special note have been highlighted as either of interest (□) or of considerable interest

(□□) to readers.

1. Hodis E, Watson IR, Kryukov GV, et al. A landscape of driver mutations in melanoma. Cell 2012;150:251-63

2. Fedorenko IV, Gibney GT, Sondak VK, Smalley KSM. Beyond BRAF: where next for melanoma therapy? Br J Cancer 2015;112:217-26

3. Maurer G, Tarkowski B, Baccarini M. Raf kinases in cancer--roles and therapeutic opportunities. Oncogene 2011;30:3477-88

4. U.S. Food and Drug Administration. Approved Drugs - Trametinib and Dabrafenib [Internet]. Available from: http://www.fda.gov/Drugs/ InformationOnDrugs/ApprovedDrugs/ ucm381451.htm [Cited 25 April 2015]

5. Food and Drug Administration. Approved Drugs - Dabrafenib [Internet]. Available from: http://www.fda.gov/ Drugs/InformationOnDrugs/ ApprovedDrugs/ucm354477.htm [Cited 25 April 2015]

6. U.S. Food and Drug Administration. Approved Drugs - Trametinib [Internet]. Available from: http://www.fda.gov/ Drugs/InformationOnDrugs/ ApprovedDrugs/ucm354478.htm [cited 25 April 2015]

7. Hu-Lieskovan S, Robert L, Homet Moreno B, Ribas Al. Combining targeted therapy with immunotherapy in BRAF-mutant melanoma: promise and challenges. J Clin Oncol Off J Am Soc Clin Oncol 2014;32:2248-54

8. Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature 2010;467:596-9

9. 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;70:5518-27

10. Gilmartin AG, Bleam MR, Groy A, et al. GSK1120212 (JTP-74057) Is an Inhibitor of MEK Activity and Activation with Favorable Pharmacokinetic Properties for Sustained In Vivo Pathway Inhibition. Clin Cancer Res 2011;17:989-1000

11. Kono M, Dunn IS, Durda PJ, et al. Role of the mitogen-activated protein kinase signaling pathway in the regulation of human melanocytic antigen expression. Mol Cancer Res MCR 2006;4:779-92 .. First preclinical evidence that MAPK inhibition may assist targeting of melanomas for immunotherapy.

12. Boni A, Cogdill AP, Dang P, et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. Cancer Res 2010;70:5213-19 .. Immune evasion of melanomas mediated by oncogenic BRAF may be reversed by targeted BRAF inhibition without compromising T-cell function.

13. Frederick DT, Piris A, Cogdill AP, et al. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. Clin Cancer Res Off J Am Assoc Cancer Res 2013;19:1225-31 .. First data about the effects of MAPK inhibition on the tumor microenvironment in melanoma patients.

14. Wellbrock C, Rana S, Paterson H, et al. Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. PLoS One 2008;3

15. Sapkota B, Hill CE, Pollack BP. Vemurafenib enhances MHC induction in BRAF(V600E) homozygous melanoma cells. Oncoimmunology 2013;2:e22890

16. Petermann KB, Rozenberg GI, Zedek D, et al. CD200 is induced by ERK and is a potential therapeutic target in melanoma. J Clin Invest 2007;117:3922-9

17. Shields JM, Thomas NE, Cregger M, et al. Lack of extracellular signalregulated kinase mitogen-activated protein kinase signaling shows a new type of melanoma. Cancer Res 2007;67:1502-12

18. Hoek RM, Ruuls SR, Murphy CA, et al. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science 2000;290:1768-71

19. Nguyen LT, Ohashi PS. Clinical blockade of PD1 and LAG3 ---- potential mechanisms of action. Nat Rev Immunol 2015;15:45-56

20. Jiang X, Zhou J, Giobbie-Hurder A, et al. The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. Clin Cancer Res Off J Am Assoc Cancer Res 2013;19:598-609

21. Liu L, Mayes PA, Eastman S, et al. The BRAF and MEK inhibitors dabrafenib and trametinib: effects on immune function and in combination with immunomodulatory antibodies targeting PD-1, PD-L1, and CTLA-4. Clin Cancer Res Off J Am Assoc Cancer Res 2015;21(7):1639-51

22. Atefi M, Avramis E, Lassen A, et al. Effects of MAPK and PI3K pathways on PD-L1 expression in melanoma. Clin Cancer Res Off J Am Assoc Cancer Res 2014;20:3446-57

23. Kakavand H, Wilmott JS, Menzies AM, et al. PD-L1 expression and tumorinfiltrating lymphocytes define different subsets of MAPK inhibitor treated melanoma patients. Clin Cancer Res Off J Am Assoc Cancer Res 2015. [Epub ahead of print]

24. Sumimoto H, Imabayashi F, Iwata T, Kawakami Y. The BRAF-MAPK signaling pathway is essential for cancerimmune evasion in human melanoma cells. J Exp Med 2006;203:1651-6

25. Streit M, Detmar M. Angiogenesis, lymphangiogenesis, and melanoma metastasis. Oncogene 2003;22:3172-9

26. Liu C, Peng W, Xu C, et al. BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. Clin Cancer Res 2013;19:393-403

27. Dirkx AEM, oude Egbrink MGA, Castermans K, et al. Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyteendothelium interactions and infiltration in tumors. FASEB J Off Publ Fed Am Soc Exp Biol 2006;20:621-30

28. Huang Y, Yuan J, Righi E, et al. Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. Proc Natl Acad Sci USA 2012;109:17561-6

29. Long GV, Wilmott JS, Haydu LE, et al. Effects of BRAF inhibitors on human melanoma tissue before treatment, early during treatment, and on progression. Pigment Cell Melanoma Res 2013;26:499-508

30. Wilmott JS, Long GV, Howle JR, et al. Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. Clin Cancer Res Off J Am Assoc Cancer Res 2012;18:1386-94 31. Cooper ZA, Frederick DT, Juneja VR, et al. BRAF inhibition is associated with increased clonality in tumor-infiltrating lymphocytes. Oncoimmunology 2013;2:e26615

32. Poulikakos PI, Zhang C, Bollag G, et al. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wildtype BRAF. Nature 2010;464:427-30

33. Heidorn SJ, Milagre C, Whittaker S, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 2010;140:209-21

34. Hatzivassiliou G, Song K, Yen I, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 2010;464:431-5

35. Hong DS, Vence L, Falchook G, et al. BRAF(V600) inhibitor GSK2118436 targeted inhibition of mutant BRAF in cancer patients does not impair overall immune competency. Clin Cancer Res Off J Am Assoc Cancer Res 2012;18:2326-35

36. 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 Off J Am Assoc Cancer Res 2010;16:6040-8

37. Callahan MK, Masters G, Pratilas CA, et al. Paradoxical activation of T cells via augmented ERK signaling mediated by a RAF inhibitor. Cancer Immunol Res 2014;2:70-9

38. Shindo T, Kim TK, Benjamin CL, et al. MEK inhibitors selectively suppress alloreactivity and graft-versus-host disease in a memory stage-dependent manner. Blood 2013;121:4617-26

39. Ho P-C, Meeth KM, Tsui Y-C, et al. Immune-based antitumor effects of BRAF inhibitors rely on signaling by CD40L and IFNg. Cancer Res 2014;74:3205-17

40. Vella LJ, Pasam A, Dimopoulos N, et al. MEK inhibition, alone or in combination with BRAF inhibition, affects multiple functions of isolated normal human lymphocytes and dendritic cells. Cancer Immunol Res 2014;2(4):351-60

41. Schilling B, Sondermann W, Zhao F, et al. Differential influence of vemurafenib and dabrafenib on patients' lymphocytes despite similar clinical efficacy in melanoma. Ann Oncol Off J Eur Soc Med Oncol ESMO 2014;25:747-53

42. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer 2012;12:265-77 43. Jackson AM, Mulcahy LA, Zhu XW, et al. Tumour-mediated disruption of dendritic cell function: Inhibiting the MEK1/2-p44/42 axis restores IL-12 production and Th1-generation. Int J Cancer 2008;123:623-32

44. Ott PA, Henry T, Baranda SJ, et al. Inhibition of both BRAF and MEK in BRAF(V600E) mutant melanoma restores compromised dendritic cell (DC) function while having differential direct effects on DC properties. Cancer Immunol Immunother CII 2013;62:811-22

45. Puig-Kr€oger A, Relloso M, Ferna´ndez-Capetillo O, et al. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. Blood 2001;98:2175-82

46. Aguilera-Montilla N, Chamorro S, Nieto C, et al. Aryl hydrocarbon receptor contributes to the MEK/ERK-dependent maintenance of the immature state of human dendritic cells. Blood 2013;121:e108-17

47. Ardeshna KM, Pizzey AR, Devereux S, Khwaja A. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. Blood 2000;96:1039-46

48. Arrighi JF, Rebsamen M, Rousset F, et al. A critical role for p38 mitogenactivated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. J Immunol Baltim 166:3837-45

49. Cheng M, Chen Y, Xiao W, et al. NK cell-based immunotherapy for malignant diseases. Cell Mol Immunol 2013;10:230-52 50. Ferrari de Andrade L, Ngiow SF, Stannard K, et al. Natural Killer Cells Are Essential for the Ability of BRAF Inhibitors to Control BRAFV600EMutant etastatic Melanoma. Cancer Res 2014;74:7298-308

.. First preclinical evidence that NK cells are crucial for the therapeutic effect of BRAF inhibitors.

51. Lindau D, Gielen P, Kroesen M, et al. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology 2013;138:105-15

52. Schilling B, Sucker A, Griewank K, et al. Vemurafenib reverses immunosuppression by myeloid derived suppressor cells. Int J Cancer J Int Cancer 2013;133:1653-63

53. Schilling B, Paschen A. Immunological consequences of selective BRAF inhibitors in malignant melanoma: Neutralization of myeloid-derived suppressor cells. Oncoimmunology 2013;2:e25218

54. Ascierto PA, Flaherty K, Queirolo P, et al. Phase I-II study of the combination vemurafenib plus peg-interferon in advanced melanoma patients harboring the V600BRAF mutation. [Internet]. J Clin Oncol 2014.32:5s. Available from: http://meetinglibrary.asco.org/content/ 129060-144 [Cited 8 February 2015]

55. West WH, Tauer KW, Yannelli JR, et al. Constant-infusion recombinant interleukin-2 in adoptive immunotherapy of advanced cancer. N Engl J Med 1987;316:898-905

56. Kaufman HL, Kirkwood JM, Hodi FS, et al. The Society for Immunotherapy of Cancer consensus statement on tumour immunotherapy for the treatment of cutaneous melanoma. Nat Rev Clin Oncol 2013;10(10):588-98

57. Sanlorenzo M, Vujic I, Posch C, et al. Melanoma immunotherapy. Cancer Biol Ther 2014;15:665-74 58. Sim GC, Martin-Orozco N, Jin L, et al. IL-2 therapy promotes suppressive ICOS + Treg expansion in melanoma patients. J Clin Invest 2014;124:99-110

59. Koya RC, Mok S, Otte N, et al. BRAF inhibitor vemurafenib improves the antitumor activity of adoptive cell immunotherapy. Cancer Res 2012;72:3928-37

60. Hu-Lieskovan S, Mok S, Homet Moreno B, et al. Improved antitumor activity of immunotherapy with BRAF and MEK inhibitors in BRAFV600E melanoma. Sci Transl Med 2015;7:279ra41

61. U.S. National Institutes of Health. ClinicalTrials.gov [Internet]. Available from: http://clinicaltrials.gov/ [cited 27 August 2014]

62. Tarhini A, Lo E, Minor DR. Releasing the Brake on the Immune System: Ipilimumab in Melanoma and Other Tumors. Cancer Biother Radiopharm 2010;25:601-13

63. Ribas A, Hodi FS, Callahan M, et al. Hepatotoxicity with combination of vemurafenib and ipilimumab. N Engl J Med 2013;368:1365-6

64. Puzanov I, Callahan MK, Linette GP, et al. Phase 1 study of the BRAF inhibitor dabrafenib (D) with or without the MEK inhibitor trametinib (T) in combination with ipilimumab (Ipi) for V600E/K mutation--positive unresectable or metastatic melanoma (MM). [Internet]. J Clin Oncol 2014.32:5s. Available from: http:// meetinglibrary.asco.org/content/129765- 144 [Cited 29 January 2015]

65. Ackerman A, Klein O, McDermott DF, et al. Outcomes of patients with metastatic melanoma treated with immunotherapy prior to or after BRAF inhibitors. Cancer 2014;120:1695-701

66. Ascierto PA, Simeone E, Sileni VC, et al. Sequential treatment with ipilimumab and BRAF inhibitors in patients with metastatic melanoma: data from the Italian ipilimumab expanded access programme (EAP). J Immunother Cancer 2013;1:P69

67. Cooper ZA, Juneja VR, Sage PT, et al. Response to BRAF inhibition in melanoma is enhanced when combined with immune checkpoint blockade. Cancer Immunol Res 2014;2(7):643-54

68. Brahmer JR, Tykodi SS, Chow LQM, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 2012;366:2455-65

69. Long GV, Atkinson V, Ascierto PA, et al. Nivolumab improved survival vs dacarbazine in patients with untreated advanced melanoma. J Transl Med 2015;13:O6

70. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515:568-71

71. Snyder A, Makarov V, Merghoub T, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 2014;371:2189-99

72. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 2015;348:124-8

73. Gammaitoni L, Giraudo L, Leuci V, et al. Effective activity of cytokineinduced killer cells against autologous metastatic melanoma including cells with stemness features. Clin Cancer Res Off J Am Assoc Cancer Res 2013;19:4347-58

74. Gammaitoni L, Leuci V, Mesiano G, et al. Immunotherapy of cancer stem cells in solid tumors: initial findings and future prospective. Expert Opin Biol Ther 2014;14:1259-70

75. Sangiolo D, Mesiano G, Gammaitoni L, et al. Cytokine-induced killer cells eradicate bone and soft-tissue sarcomas. Cancer Res 2014;74:119-29

76. Ishikawa F, Yasukawa M, Lyons B, et al. Development of functional human blood and immune systems in NOD/SCID/ IL2 receptor {gamma} chain(null) mice. Blood 2005;106:1565-73

77. McDermott SP, Eppert K, Lechman ER, et al. Comparison of human cord blood engraftment between immunocompromised mouse strains. Blood 2010;116:193-200

78. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 2006;314:126-9

79. Johnson LA, Morgan RA, Dudley ME, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 2009;114:535-46

80. Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol Off J Am Soc Clin Oncol 2011;29:917-24

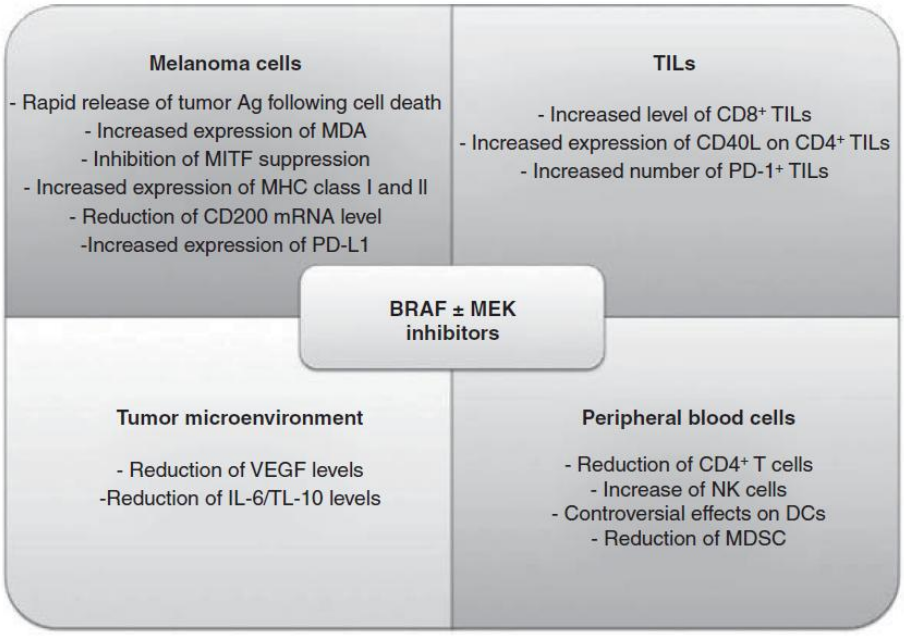

Figure 1. Main effects of BRAF and MEK inhibitors in melanoma and immune system cells.

DCs: Dendritic cells; MDA: Melanocyte differentiation antigens; MDSC: Myeloid-derived suppressor cells; MITF: Microphthalmia-associated transcription factor;

NK: Natural killer; TILs: Tumor-infiltrating lymphocytes.

Table 1. Clinical trials in melanoma exploring BRAF inhibitors (with/without MEK inhibitors) in combination with cancer immunotherapies (June 2015).

Clinical trial number	Phase	Intervention – Schedule	Status of the trial
NCT01959633	I/II	Vemurafenib + Peg-IFN IFN treatment should start after 15 days of vemurafenib	Recruiting
NCT01943422	I/II	Vemurafenib + high-dose IFN-α-2b IFN-α is administered intravenously for 5 consecutive days every week for 4 weeks	Recruiting
NCT01683188	IV	Vemurafenib + high-dose IL-2	Terminated
NCT01603212	I/II	Vemurafenib + IFN-α-2b + IL-2 Vemurafenib (21days cycle) + IL-2 (days 2 – 5) + IFN-α (days 1 – 5)	Active, not recruiting
NCT01585415	I	Vemurafenib + white blood cell therapy After 2 weeks of vemurafenib and a lymphocyte depleting preparative regimen (cyclophosphamide and fludarabine), young TILs are infused and followed by IL-2	Active, not recruiting
NCT02354690	I/II	Vemurafenib + TIL therapy After 2 weeks of vemurafenib, the tumor is harvested; TILs are isolated and expanded in the lab. After lymphodepleting chemotherapy the patient receives TILs and IL-2	Recruiting
NCT01659151	II	Vemurafenib + lymphodepletion + adoptive cell transfer & high-dose IL-2 After 2 weeks of vemurafenib, the tumor is harvested; TILs are isolated and expanded in the lab. After lymphodepleting chemotherapy the patient receives ACTs and IL-2	Recruiting
NCT01767454	I	Dabrafenib + ipilimumab ± trametinib	Recruiting
NCT01940809	I	Ipilimumab ± dabrafenib, ± trametinib	Recruiting
NCT02200562	I/II	Ipilimumab + dabrafenib	Recruiting
NCT02130466	I/II	Pembrolizumab ± trametinib ± dabrafenib	Recruiting
NCT02027961	I/II	MEDI4736 (anti PD-L1) + trametinib ± dabrafenib	Recruiting
NCT02357732	I	Nivolumab + dabrafenib ± trametinib	Active, not recruiting
NCT02224781	III	Dabrafenib + trametinib followed by ipilimumab + nivolumab versus ipilimumab + nivolumab followed by dabrafenib + trametinib	Active, not recruiting
NCT01656642	I	MPDL3280A (anti PD-L1) + vemurafenib ± cobimetinib	Recruiting

ACT: Adoptive T-cells transfer; PD-L1: Programmed cell death ligand 1; Peg-IFN: Pegylated-IFN; TIL: Tumor-infiltrating lymphocyte.