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Targeting Mutant NRAS Signaling Pathways in Melanoma

Ha Linh Vu¹ and Andrew E. Aplin^{1,2}

¹Department of Cancer Biology and Sidney Kimmel Cancer Center, Thomas Jefferson University Philadelphia, PA

²Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, PA

Graphical Abstract

Cutaneous melanoma is a devastating form of skin cancer and its incidence is increasing faster than any other preventable cancer in the United States (Jemal et al, 2013). The small GTPase, NRAS, was the first oncogene identified in melanoma and is mutated in 20% of cases (Milagre et al, 2010). Compared to other mutational subtypes of melanoma, patients with mutant NRAS tumors tend to be older and have a history of chronic UV exposure (Curtin et al, 2005, Lee et al, 2011, Jakob et al, 2012, Thumar et al, 2014). Histologically, the mutant NRAS tumors are more aggressive than other subtypes with thicker lesions, elevated mitotic activity, and higher rates of lymph node metastasis (Thumar et al, 2014, Devitt et al, 2011, Ellerhorst et al, 2011). Given the more aggressive disease seen with mutant NRAS patients, it is not surprising that NRAS mutation status is a predictor of poorer outcomes with lower median overall survival compared to non-NRAS mutant melanoma (Jakob et al, 2012, Thumar et al, 2014).

Corresponding Author: Ha Linh Vu, Department of Cancer Biology, Sidney Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107. Phone: 215-503-7296; Fax: 215-923-9248; HaLinh.Vu@Jefferson.edu.

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In normal cells, RAS proteins switch between active GTP-bound forms and inactive GDP-bound forms. The transition between the active and inactive state is mediated by GTPase-activating proteins (GAPs). When examining the types of mutations within NRAS in melanoma patients, approximately 80–90% occur at the Q61 locus and less frequently at the G12 or G13 loci (Colombino et al, 2012). Oncogenic NRAS mutations of Q61 impair the GTP hydrolysis reaction by interfering with the coordination of a catalytic water molecule, which is required for the nucleophilic attack on the gamma-phosphate of GTP (Scheidig et al, 1999, Buhrman et al, 2010). Similarly, oncogenic substitutions in residues G12 and G13 prevent the formation of van der Waals bonds through steric hindrance between NRAS and (GAPs). This perturbs the proper orientation of the catalytic Q61 residue in NRAS, resulting in pronounced attenuation of GTP hydrolysis (Scheffzek et al, 1997). Thus, the outcome of these substitutions is the persistence of the GTP-bound state of NRAS that drives melanoma by promoting cell growth, survival and invasion (Malumbres and Barbacid, 2003). As attempts to target activated RAS directly have not led to clinical therapies, the current focus is to target RAS effector pathways. The major downstream effectors of NRAS relevant in melanoma are RAFs (ARAF, BRAF, and CRAF), phosphatidylinositol 3-kinase (PI3K), and the RAS-like protein (RAL) GEFs (Figure 1) (Downward, 2003). Although 20% of melanomas have activating NRAS mutations, the aggressive nature and complex molecular signaling conferred by this transformation has evaded clinically effective treatment options. This review examines the advances made in targeted therapies that focus on the effector pathways of NRAS.

RAF-MEK Pathway

The RAF-MEK-ERK1/2 pathway is a well-established driver of melanoma. Upon activation, RAS recruits the serine/threonine kinase RAF to the cell membrane, leading to RAF activation. The three RAF isoforms are ARAF, BRAF, and CRAF (also known as RAF1). Activating mutations in BRAF are found in approximately 50% of melanoma patients but in a mutually exclusive manner from NRAS mutations (Davies et al, 2002). RAF kinases activate the dual specificity serine/threonine kinases MEK1 and MEK2 through phosphorylation at the S218 and S222 residues (Hanks and Quinn, 1991). MEK1 and MEK2 share 79% amino acid identity and are equally competent to phosphorylate ERK1 and ERK2 (Dhanasekaran and Premkumar Reddy, 1998), with sequential phosphorylation of ERK1 at T202 and Y204 (T185 and Y187 in ERK2). ERK1/2 signaling impacts diverse cell functions, including regulation of cell proliferation, survival, mitosis, and migration.

PI3K-AKT Pathway

The second best validated class of RAS effectors is class I PI3Ks, which is comprised of a regulatory subunit p85 and a catalytic subunit p110 with α , β , γ and δ isoforms (Mahajan and Mahajan, 2012). RAS binds and activates p110 (Castellano et al, 2013). Upon activation, all type 1 PI3Ks phosphorylate PIP2 (phosphatidylinositol (4,5)-diphosphate), a constitutive membrane component, to create the lipid second messenger PIP3 (phosphatidylinositol (3,4,5)-triphosphate). Point mutations in PIK3CA, which encodes the catalytic subunit p110 α of PI3K, are detected in 2–6% of melanomas (Curtin et al, 2006, Feldman et al, 2005). PTEN (phosphatase and tensin homolog) has D3 phosphoinositide phosphatase

activity and acts as a negative regulator of PI3K by removing the 3' phosphate from its products (Feldman et al, 2005). Loss of function mutations in PTEN occur in 10–30% of melanomas (Stahl et al, 2004).

PIP3 initiates signals through binding to the pleckstrin homology (PH) domain of target proteins, which induces their membrane translocation and/or conformational changes. PIP3 binding to the PH domain of AKT (v-akt murine thymoma viral oncogene homolog) leads to a conformational change that enables mTORC2 (mammalian target of rapamycin 2) to directly phosphorylate AKT S473, which in turn promotes direct phosphorylation of T308 by PDK1 (3-phosphoinositide dependent protein kinase 1). The AKT protein kinase family consists of three members, AKT1-3, and they are involved in cell growth, proliferation, survival and immunity.

RALGEF-RALA/B Pathway

Other RAS effectors are members for the Ral-GEF that activate two highly similar GTPase isoforms, RALA and RALB. Although understudied, the RALA/B-mediated pathways play a key role in mutant NRAS melanomas (Mishra et al, 2010, Zipfel et al, 2010). The best-characterized effector of RAL GTPases is an octameric protein complex termed the exocyst, a large multiprotein complex responsible for the appropriate targeting and tethering of a subset of secretory vesicles to specific dynamic plasma membrane domains during exocytosis (Moskalenko et al, 2002). In addition to its involvement in cell-specific secretory products, the exocyst is also involved in the maintenance of epithelial cell polarity, cell motility and cytokinesis (Grindstaff et al, 1998, Gromley et al, 2005, Spiczka and Yeaman, 2008). In cancer cells, RAL GTPases are chronically activated. The exocyst components SEC5 and EXO84 directly interact with RAL proteins and have tethering-independent functions that support cancer cell survival. RALA regulates anchorage-independent proliferation through an unknown mechanism (Zipfel et al, 2010). RALB activates cell survival and autophagy through its interactions with the SEC5 and EXO84 exocyst components, respectively.

RAF inhibitors

As the RAF-MEK-ERK pathway is a well-established driver in melanoma, several RAF and MEK inhibitors have been evaluated. The ATP-competitive inhibitors of BRAF, vemurafenib (PLX-4032) and dabrafenib (GSK2118436), have received FDA-approval for unresectable or metastatic BRAF V600E melanoma patients. Although BRAF inhibitors could be hypothetically used in mutant NRAS melanoma to target the pathway downstream of NRAS, they are contraindicated due to paradoxical activation of MEK-ERK1/2 signaling in the setting of activated RAS signaling (Hatzivassiliou et al, 2010, Heidorn et al, 2010, Poulikakos et al, 2010, Kaplan et al, 2011). In BRAF wild-type tumors, the addition of selective BRAF inhibitors leads to BRAF recruitment to the plasma membrane in a RAS-dependent manner. Inhibited BRAF dimerizes with CRAF to form a stable complex, leading to CRAF activation and subsequent signaling downstream to MEK-ERK1/2. Therefore, in mutant NRAS tumors, inhibition of BRAF is ineffective due to inhibitor-mediated priming

of signaling through BRAF-CRAF heterodimers (Hatzivassiliou et al, 2010, Heidorn et al, 2010, Poulikakos et al, 2010).

The development of pan-RAF kinase inhibitors (Compound A, TAK-632, and LY3009120) that are active against mutant BRAF as well as wild type BRAF and CRAF do not elicit paradoxical RAF-MEK signaling in RAS-transformed cancer cells (Henry et al, 2015). Compound A has been shown to block the proliferation of NRAS mutant melanomas with dependence on the MAPK pathway *in vitro* (Atefi et al, 2015). Both TAK-632 and LY3009120 inhibit mutant NRAS cell line xenograft *in vivo* as monotherapy (Nakamura et al, 2013, Peng et al, 2015). Based on the promising preclinical data, LY3009120 is currently being evaluated in patients with solid tumors in a phase I study (NCT02014116).

MEK inhibitors

Prior to the advent of pan-RAF kinase inhibitors, the primary focus in mutant NRAS tumors has been to target downstream MEK. The first generation of MEK inhibitors (PD 098059 and U0126) showed promising inhibition of growth in preclinical models of melanoma. However, due to undesirable pharmaceutical properties, the early MEK inhibitors did not progress to clinical trials (Friday and Adjei, 2008). CI-1040 (PD 184352) and its derivative PD-0325901 (PD-901) were the first MEK inhibitors to be utilized in human patients. Despite a good safety profile, the low oral bioavailability and high metabolism of CI-1040 resulted in insufficient plasma drug levels for antitumor activity (Rinehart et al, 2004). PD-901 had some clinical responses, but several adverse effects, including retinal vein occlusion and neuropathy, led to its cessation in phase II studies (LoRusso et al, 2010).

Since CI-1040 and PD-901, newer MEK inhibitors have been developed with better safety profiles and antitumor activity. The two main MEK inhibitors currently utilized in clinical trials are selumetinib (ARRY-142886, AZD6244) and trametinib (GSK1120212). Selumetinib is a selective allosteric inhibitor of MEK1/2 with an IC₅₀ of 14 nM against purified MEK1 (Yeh et al, 2007). Due to the weak interaction between the inhibitor and MEK1 S212, treatment with selumetinib is effective in inhibiting ERK1/2 phosphorylation but leads to increased phospho-MEK (Yeh et al, 2007, Hatzivassiliou et al, 2013). In a phase II study of patients with advanced melanoma treated with either temozolomide (TMZ), an alkylating agent, or selumetinib, no difference in progression-free survival was observed between the two arms (Kirkwood et al, 2012). In a phase II study of the anti-mitotic chemotherapeutic, docetaxel, with or without selumetinib in patients with wild-type BRAF advanced melanoma, there was no difference in overall survival between the two groups (Gupta et al, 2014).

Trametinib (GSK1120212) was identified in a high-throughput screen for antiproliferative compounds that can induce expression of the CDK inhibitor, p15INK4b, as a strong and selective MEK1/2 inhibitor (Yamaguchi et al, 2011). Unlike other MEK inhibitors, trametinib preferentially binds to unphosphorylated MEK and, upon binding, has a low dissociation rate that stabilizes unphosphorylated MEK (Yoshida et al, 2012). Trametinib effectively inhibits phospho-ERK1/2 in all cells but has variable effects in proliferation assays (Gilmartin et al, 2011). A phase I trial of trametinib in patients with advanced

melanoma demonstrated efficacy in mutant BRAF patients but no responses were seen in mutant NRAS patients (Falchook et al, 2012). A phase 3 trial comparing trametinib to the chemotherapeutics dacarbazine or paclitaxel in metastatic BRAF V600E/K melanoma patients showed clinical efficacy (Flaherty et al, 2012b). Trametinib received FDA approval for the treatment of BRAF V600E/K unresectable or metastatic melanoma in May 2013. Additionally, a phase 1 and 2 trial analyzing the efficacy of BRAF inhibition with dabrafenib alone compared to the combination of dabrafenib and trametinib demonstrated efficacy with the combination (Flaherty et al, 2012a). The promising results in early phase trials led to the FDA accelerated approval of the trametinib and dabrafenib combination in unresectable or metastatic melanoma with mutant BRAF V600E/K.

Next-generation MEK inhibitors

Despite the disappointing clinical trials with selumetinib and trametinib in the mutant NRAS melanoma subset (Falchook et al, 2012), newer MEK inhibitors have shown promising pre-clinical and early clinical results in RAS-driven tumors. The compounds CH5126766 (RO5126766), GDC-0623, and G-573 are dominant negative inhibitors of MEK. These dominant negative MEK inhibitors lead to the formation of a stable MEK-RAF complex, where MEK cannot be phosphorylated and released from RAF. The blockade of MEK feedback phosphorylation by RAS-activated RAF makes these inhibitors more likely to be effective in mutant RAS cancers (Hatzivassiliou et al, 2013). A phase 1 study of GDC-0623 in patients with locally advanced or metastatic solid tumors (NCT01106599) was completed but the results have not been published. In phase I studies of CH5126766, there have been responses noted in mutant NRAS melanoma patients (Martinez-Garcia et al, 2012, Zimmer et al, 2014). Of the 8 evaluable mutant NRAS melanoma patients, there were 2 with stable disease and 1 partial response.

Binimetinib (MEK162) and pimasertib (AS703026) are allosteric MEK inhibitors that have shown promise in mutant NRAS melanomas and are currently being investigated in phase 2 and 3 trials selectively in mutant NRAS melanoma. The safety profile of binimetinib and preliminary signs of antitumor activity were reported in a phase 1 trial in patients with advanced solid tumors (Bendell et al, 2011, Finn et al, 2012). In a phase 2 study of binimetinib in advanced mutant NRAS melanoma patients, 6/30 (20%) elicited partial responses and 13/30 (43.3%) showed stable disease (Ascierto et al, 2013). Based on these promising responses, this study was amended to enroll an additional 70 patients with mutant NRAS melanoma (NCT01320085). Additionally, there is an ongoing phase 3 trial in unresectable or metastatic mutant NRAS melanoma patients comparing binimetinib to the chemotherapeutic agent dacarbazine (NCT01763164). In preclinical studies, pimasertib demonstrated strong antiproliferative effects on tumor cell lines with high MEK-ERK1/2 pathway activation but little or no effect in other cells (Goutopoulos et al, 2009). In a phase 1 trial of metastatic melanoma patients, treatment of 17 mutant NRAS patients with pimasertib resulted in 2 partial responses and 2 complete responses (Lebbe et al, 2012). Based on the phase I study, there is an ongoing phase 2 trial of pimasertib compared to dacarbazine in mutant NRAS cutaneous melanoma (NCT01693068). The results from these trials may confirm the efficacy of binimetinib and pimasertib in mutant NRAS melanoma patients.

TAK-733 was discovered as a selective MEK inhibitor using structure-based drug design (Dong et al, 2011). It was found to have favorable pharmacokinetics in several animal models that support once-daily oral dosing in humans (Dong et al, 2011). *In vitro* testing of melanoma cell lines showed sensitivity in 7/12 mutant BRAF, 4/10 mutant NRAS, 3/5 WT/WT, and all 5 uveal cell lines (von Euw et al, 2012). In additional *in vivo* murine models utilizing melanoma patient-derived xenografts (PDX), TAK-733 led to tumor growth inhibition or tumor regression wild-type BRAF PDX (Micel et al, 2015). These studies provide support for the use of TAK-733 in melanomas that were wild-type for BRAF.

Combinatorial Therapies in Mutant NRAS Melanoma

Although the next generation of MEK inhibitors are showing promising clinical efficacy in an activating NRAS background, their use will most likely be optimized when combined with other targeted therapies. The combination of MEK inhibition with RAF, EGFR/PI3K/AKT, and CDK4/6 are currently being evaluated in clinical trials. Preclinical studies support the combination of MEK inhibitors with inhibitors of PLK1, TBK1, and ROCK.

RAF and ERK inhibition

For complete blockade of the MAPK pathway, targeting MEK in combination with its upstream effector, RAF, or with its downstream target, ERK1/2, has been examined. RO5126766 (CH5126766) is a potent and selective dual RAF/MEK inhibitor (Wada et al, 2014). In mutant NRAS melanoma, RO5126766 suppressed the RAF-mediated MEK phosphorylation seen with MEK inhibition alone and suppressed tumor growth *in vivo*. A phase I study of RO5126766 in solid tumors, including melanoma, was recently completed but results remain to be published (NCT00773526). SCH772984 is an ERK1/2 inhibitor with efficacy in melanoma cell lines, regardless of mutational status (Morris et al, 2013). The addition of SCH772984 to MEK inhibition increased apoptosis and delayed resistance in mutant NRAS melanoma cell lines (Rebecca et al, 2014).

EGFR/PI3K/AKT inhibition

EGFR is a receptor tyrosine kinase member of the ERBB receptors family, which signals through the PI3K-AKT pathway to promote survival and maintain. EGFR is frequently expressed in melanoma, and its role in mediating metastasis is well characterized (Rodeck et al, 1991). The compensatory up-regulation of AKT signaling following MEK inhibition is well characterized (Gopal et al, 2010, Deuker et al, 2015). A phospho-proteomic analysis of a MEK-inhibited mutant NRAS melanoma cell line identified up-regulation of EGFR signaling (Fedorenko et al, 2015). A high throughput antibody-based analysis of changes in MEK signal transduction in mutant NRAS melanoma cell lines identified a decrease in MIG6 (Vu et al, 2016). MIG6 is an immediate early response gene that acts as a negative regulator of ERBB receptors (Hackel et al, 2001, Ferby et al, 2006). Functionally, MIG6 was shown to negatively regulate migration and, thus, its down-regulation is likely to promote a pro-migratory phenotype in tumor microenvironments containing high levels of EGF (Vu et al, 2016).

Targeting EGFR signaling in combination with MEK in mutant NRAS melanoma may lead to more durable responses through decreased metastasis. There are few examples of EGFR inhibition in wild-type BRAF melanoma. In an *in vivo* xenograft model of a mutant NRAS melanoma cell line, the EGFR inhibitor, erlotinib, in combination with a VEGF binding antibody, bevacizumab, reduced metastasis to regional lymph nodes and the lungs (Schicher et al, 2009). The EGFR inhibitor gefitinib failed to show significant clinical efficacy as a single-agent therapy for unselected patients with metastatic melanoma (Patel et al, 2011). *In vivo* studies with the combination of MEK and EGFR inhibitors in mutant NRAS melanoma are needed to determine the anti-tumor and anti-metastasis response.

More studies have examined the combined inhibition of MEK and PI3K pathways. MEK and PI3K/mTOR inhibition has been shown to synergistically inhibit the growth of mutant NRAS melanoma cell lines *in vitro* and in xenograft models *in vivo* (Posch et al, 2013). Similarly, in a RAS-driven melanoma genetically-engineered mouse (GEM) model, the combination of selumetinib and the PI3K/mTOR inhibitor BEZ235 exhibited significant antitumor activity (Roberts et al, 2012). There is a phase II clinical study of trametinib in combination with GSK2141795, an AKT inhibitor, in wild-type BRAF melanoma (NCT01941927). Additionally, there are multiple phase I studies examining the combination of binimetinib with PI3K inhibitors in solid tumors, including melanoma (NCT01363232, NCT01337765, NCT01449058).

CDK4/6 inhibition

In an inducible NRAS-driven GEM model of melanoma, monotherapy with MEK inhibitors (selumetinib or trametinib) was not sufficient to drive tumor regression but the combination of MEK with cyclin-dependent kinase 4/6 (CDK4/6) inhibition (PD-0332991/palbociclib) resulted in tumor regression (Kwong et al, 2012). Inhibition of CDK4 and 6, regulators of the G1/S cell cycle checkpoint, arrests the cell cycle in the G1 phase, suppresses DNA synthesis, and inhibits cancer cell growth. There is an ongoing phase 1/2 trial of binimetinib with the CDK4/6 inhibitor ribociclib (LEE011) in mutant NRAS melanoma patients (NCT01781572). Early results from the 21 evaluable patients were very promising, with 7 patients achieving a partial response (33%) and 11 patients with stable disease (52%) (Sosman et al, 2014). There is also an ongoing phase 1/2 trial of trametinib with palbociclib, the CDK4/6 inhibitor, in patients with solid tumors (NCT02065063).

PLK1 inhibition

Polo-like kinase 1 (PLK1) is overexpressed in mutant NRAS melanoma (Jalili et al, 2011). As a regulator of mitosis, PLK1 influences the activation of CDK1/cyclin B, centrosome maturation, spindle formation, kinetochore assembly, regulation of microtubule nucleation, chromosome segregation and cytokinesis (Cholewa et al, 2014). In clinical trials, the PLK inhibitor volasertib was well tolerated but had poor tumor responses (Lin et al, 2014). While the clinical effects of monotherapy with PLK1 inhibitors has been disappointing, the addition of MEK inhibitors induced apoptosis in mutant NRAS melanoma both *in vitro* and *in vivo* (Posch et al, 2015).

TBK1 inhibition

TANK-binding kinase 1 (TBK1), an atypical I κ B kinase family member, is activated downstream of RALB and SEC5, a component of the exocyst (Chien et al, 2006). The RALB/SEC5/TBK1 activation complex supports the host innate immune response triggered by either extracellular double-stranded RNA or exposure to Sendai virus downstream of toll-like receptor (TLR) 3 and 4 (Chien et al, 2006). Additionally, TBK1 is an essential contributor to cancer transformation in RAS-transformed tumors (Barbie et al, 2009) (Chien et al, 2006) and promotes migration in mutant NRAS melanoma (Vu and Aplin, 2014). In mutant NRAS melanoma, targeting TBK1 with small interfering RNA transfection or small molecule inhibitors (BX795 and AZ13102909) cooperates with MEK inhibition to elicit apoptosis in 3D *in vitro* models (Vu and Aplin, 2014).

ROCK inhibition

ROCK1/2 are RHO GTPase-activated serine/threonine kinases that regulate migration, invasion, and metastasis (Amano et al, 2010). In addition to its role in metastasis, ROCK1/2 also has a role in tumor proliferation and survival (Street and Bryan, 2011). In mutant NRAS melanoma, ROCK inhibitors (GSK269962A and fasudil), in combination with trametinib-induced cell death *in vitro* and suppressed the growth of established tumors *in vivo* (Vogel et al, 2015). While there are no clinical trials evaluating fasudil in melanoma, it is a well-tolerated agent that is approved in Japan and China for other clinical indications (Feng et al, 2015).

Conclusion

This is an exciting time for targeted therapies in mutant NRAS melanomas with several clinical trials focusing specifically on this subset. While new MEK inhibitors may prove to be efficacious as monotherapies, their combination with inhibitors of pathways known to cooperate with MEK inhibition will likely maximize antitumor activity. Furthermore, utilization of targeted agents is likely in combination with immune checkpoint inhibitors such as PD-1 antibodies, which alone have shown promising results in mutant NRAS melanoma patients (Johnson et al, 2015). Finally, attempts to directly target forms of active RAS are advancing. For example, a recently identified compound, ARS853, binds to KRAS G12C mutant when in a GDP-bound form and inhibits KRAS G12C activation of the MEK-ERK1/2 and AKT pathways (Ostrem et al, 2013, Lito et al, 2016). These studies highlight the possibility of utilizing structural information to design novel inhibitors to other mutationally activated forms of RAS. Also, recent developments have led to antibodies with some selectivity for Q61R forms of NRAS that may have diagnostic purposes (Dias-Santagata et al, 2016). Improved detection and results from current and future clinical trials will hopefully provide new effective therapeutics options for mutant NRAS melanoma patients.

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Figure 1.
NRAS downstream effector pathways.