Molecular Pathways: The Basis for Rational Combination Using MEK Inhibitors in KRAS-Mutant Cancers

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Abstract

Mutations in \textit{RAS} oncogenes are frequently observed in human cancers, and the mutations result in activation of the RAS-RAF-MEK-ERK pathway, leading to cell proliferation and survival. The pathway is, therefore, a potent therapeutic target in the \textit{RAS} mutant cancers. MEK inhibitors can specifically block the pathway and are one of the key drugs for the treatment of the \textit{RAS} mutant cancers. As \textit{RAS} proteins activate other downstream signaling proteins in addition to the RAS-RAF-MEK-ERK pathway, combination therapeutic approaches with MEK inhibitors are also being evaluated. Moreover, MEK inhibitors can arrest cancer cells in G1 phase and repress pro-survival Bcl-2 family proteins such as MCL-1 and BCL-2/BCL-XL, and increase expression of Bim, a pro-apoptotic BH3-only family protein. This mechanism may explain the efficacy of the combination of MEK inhibitors with cytotoxic agents or other targeted inhibitors. A better understanding of the pathway will help us with development of rational combinations for the treatment of the \textit{RAS} mutant cancers.

Background

Recent advances in the development of targeted therapies have improved a survival in patients with a variety of cancers. For instance, in lung cancer, some of the most compelling clinical examples include the treatment of epidermal growth factor receptor (EGFR) mutant or anaplastic lymphoma kinase (ALK) rearranged non-small cell lung cancers (NSCLC) with either EGFR or ALK kinase inhibitors, respectively (1-5). Oncogenic mutations in \textit{RAS} gene, most commonly in \textit{KRAS}, are
detected in approximately 30% of human cancers (6). Despite RAS mutations having been identified more than 30 years ago, successful therapies directly targeting the mutant RAS protein have not been developed. Mutant RAS activates several downstream signaling pathways that are involved in cell proliferation, differentiation, and survival (Figure 1A) (7). Among them, one of the most studied and characterized pathway is the RAS-RAF-mitogen-activated extracellular signal-regulated kinase (MEK)-extracellular signal-related kinase (ERK) pathway (RAS-ERK pathway). Efforts to directly inhibit mutant RAS, or its membrane association using farnesyl transferase inhibitors, failed to show clinical benefit in RAS mutant cancers (8), with the result that the focus of recent efforts has shifted to the development of inhibitors of downstream kinases, including MEK inhibitors.

The RAS genes are located on the short arm of chromosome 12 and encode 21 kd, membrane-localized small GTPases, which function as switches between the active GTP-bound RAS and the inactive GDP-bound one (Figure 1A). There are four highly homologous RAS GTPases; HRAS, NRAS, and KRAS (KRAS4A and KRAS4B). Oncogenic KRAS mutations occur primarily in lung, colorectal, and pancreatic cancers, whereas HRAS mutations are detected in thyroid, kidney, and bladder cancers, and NRAS in hepatocellular cancer, melanoma, and hematological malignancies (7). In normal epithelial cells, activation of RAS signaling is triggered in response to cell–membrane receptors, including receptor tyrosine kinases (RTKs), cytokine receptors, and G protein-coupled receptors. The activation of RAS is regulated by two proteins; guanine nucleotide exchange factors
(GEFs) stimulate GDP for GTP exchange for RAS, whereas GTPase-activating proteins (GAPs) accelerate GTP hydrolysis (9). Upon the activation of the cell membrane receptors, an adaptor protein Grb2 recruits guanine nucleotide exchange factors (GEFs) to cell membrane where RAS proteins exist through prenylation, leading to increasing activated GTP–RAS proteins. Subsequently, the activated RAS interacts with RAF (A-RAF, B-RAF, C-RAF/RAF-1), a serine/threonine kinase, and the RAF is activated through a complex events including dimerization. Subsequently, RAF proteins directly phosphorylate and activate MEK1 and MEK2, tyrosine and serine/threonine dual-specificity kinases, which in turn activate ERK1 and ERK2 proteins that trigger activation of nuclear and cytoplasmic targets associated with transcription, cell proliferation, differentiation, and metabolism (Figure 1A) (9).

**KRAS mutations in human cancer**

Mutations in *KRAS* lead to constitutive activation of the RAS-ERK pathway. As intrinsic GTPase activity of RAS is low, GTP hydrolysis of RAS, that is, inactivation of RAS relies heavily on the GAP function. *KRAS* mutations are frequently found in sites associated with binding of GAPs to RAS; codon 12 and 13 of exon 1, and codon 61 of exon 2. Amino acids in codon 12 and 13 are involved in van der Waals bonds between the RAS and GAPs, and mutations in the codon 12 and 13 prevent the formation of the bonds (9). Similarly, mutations in the codon 61 also impair GTP hydrolysis, which in turn induce permanent activation of RAS proteins.
In NSCLC, \textit{KRAS} is the most frequently mutated oncogene. \textit{KRAS} mutations account for over 90\% of \textit{RAS} mutations and are found mainly in lung adenocarcinoma subtype (~30\%), but rarely in squamous cell carcinoma of the lung or in small cell lung cancer (SCLC) (10, 11). Among the amino acid substitutions resulting from \textit{KRAS} mutations, Gly12Cys (G12C) is the most frequent, followed by Gly12Val (G12V) and Gly12Asp (G12D) (12, 13). Unlike NSCLC, in colorectal cancer (CRC), G12V is the most frequent, followed by G12D and G13D (14). In NSCLC, types of \textit{KRAS} mutations seem to be related to smoking, and G12C \textit{KRAS} mutations are found most commonly in former or current smokers whereas G12D mutations are the dominant \textit{KRAS} mutation in never smokers (15). The phenotypic properties of mutant RAS proteins might differ between the different mutant amino acid substitutions. For example, a murine study demonstrated that mice with \textit{Kras}-G12V tumors had more aggressive phenotype compared to those with \textit{Kras}-G12D tumors (16). Moreover, the \textit{KRAS}-G12V protein interacts with Raf-1 and signals through the RAS-ERK pathway, whereas the \textit{KRAS}-G12D protein don’t activate the RAS-ERK pathway and instead transduce signals through the PI3K-AKT, JNK, p38 and FAK pathways (16). Ihle et al. also reported that in NSCLC cell lines, \textit{KRAS}-G12C and \textit{KRAS}-G12V resulted in activated RAL signaling pathway, a downstream of RAS, and decreased growth factor-dependent AKT activation. In contrast, \textit{KRAS}-G12D resulted in AKT but not RAL activation (17).

\textbf{Mechanisms of MEK inhibitor-induced apoptosis}
The constitutive activation of the RAS-ERK pathway as a result of \textit{KRAS} mutations, has led to the development of MEK inhibitors as a potential therapeutic strategy for \textit{KRAS} mutant cancers. \textit{In vitro} studies demonstrated a tendency toward sensitivity to MEK inhibitor in cell lines harboring \textit{KRAS} mutations compared to those without \textit{KRAS} mutations (18, 19), and in part inspired the development of clinical trials of MEK inhibitors for patients with \textit{KRAS} mutant cancers. There are several MEK inhibitors in clinical development and one, trametinib, that is currently approved by the food and drug administration (FDA), although for \textit{BRAF} mutant melanoma. As MEK1/2 doesn’t have known targets other than ERK1/2, inhibition of MEK should lead to inhibition of all ERK1/2 target proteins. Moreover, most MEK inhibitors have the advantage of being non-competitive inhibitors of ATP binding to MEK, thus conferring high specificity (20).

Unlike MEK proteins, ERK1/2 proteins have several nuclear and cytoplasmic targets. For instance, the ERK protein plays a critical role in cell cycle. ERK1/2 can promote phosphorylation of Retinoblastoma protein (Rb) through activation of Cyclin D1 and repression of p27\textsuperscript{kip1} (21, 22). Since phosphorylation of Rb results in G1/S cell-cycle progression, deregulation of the RAS-ERK pathway leads to the sustained cell cycle progression. MEK inhibitors have been shown to reduce cyclin D1 levels and induce the p27\textsuperscript{kip1} expression as well as cause dephosphorylation of Rb, arresting human cancer cells in G1 phase (23, 24).

A second mechanism of anti-tumor activity of MEK inhibitors is a result of altered
expression of the Bcl-2 family proteins, consisting of the pro-survival proteins (Bcl-2, Mcl-1, Bcl-XL), the pro-apoptotic Bcl-2 homology 3-only (BH3-only) proteins, and the pro-apoptotic Bax/Bak subgroup. The balance between the pro-survival and pro-apoptotic proteins is crucial to apoptosis caused by anti-cancer drugs, and when the pro-apoptotic members are dominant, downstream Bax and Bak proteins can stimulate release of apoptosis-inducing factor and cytochrome c from mitochondria, resulting in apoptosis (Figure 1B). As ERK1/2 represses the pro-apoptotic proteins and induces expression of the pro-survival members, MEK inhibitors can tilt the balance towards apoptosis. In human lung cancer cells, exposure to the MEK inhibitor selumetinib (AZD6244), resulted in an increase of the pro-apoptotic proteins, Bim, PUMA, and NOXA (25), and in pancreatic cancer cells, the MEK inhibitor PD98059 caused a down-regulation of the expression levels of Bcl-2, Bcl-XL, and MCL-1 (24). However, despite these effects, the efficacy of MEK inhibitors alone might be insufficient as treatment for KRAS mutant cancers (26, 27). Indeed, an in vitro study demonstrated the limited efficacy of MEK inhibitor alone in KRAS mutant NSCLC (28), suggesting that combination therapies are likely to yield more robust clinical benefits.

Clinical-Translational Advances

KRAS mutations as prognostic factors

KRAS mutations have both prognostic and predictive roles in human cancer. In CRC,
mutations are associated with a worse prognosis (29), and also with a lack of clinical benefit from treatment with anti-EGFR monoclonal antibody therapies (30). Recent analysis also demonstrated that the amount of KRAS mutations detected using a sensitive PCR based assay may correlate with the efficacy of anti-EGFR therapy in patients with human CRC (31). Similarly, in pancreatic cancer, KRAS mutations (codon 12 or 13) are associated with worse prognosis (32) and in NSCLC, patients with advanced KRAS mutant cancer have a shorter survival compared to those with EGFR mutant or EGFR/KRAS wild type cancers (33, 34). In addition, KRAS mutations predict for resistance to treatment with EGFR tyrosine kinase inhibitors (17, 35, 36). Ihle et al. reported that NSCLC patients whose tumors had either KRAS-G12C or KRAS-G12V mutations, had a worse PFS compared with patients whose tumors had other KRAS mutant tumors or lacked KRAS mutations (17).

**MEK inhibitors in patients with KRAS mutant cancer**

MEK inhibitors have been evaluated as both single agents or in combination with chemotherapy in KRAS mutant cancers. A number of reports demonstrated the preclinical efficacy both in vitro and in vivo of MEK inhibitors in KRAS mutant tumors, but efficacy data obtained from clinical studies is, currently, limited to lung cancer. In CRC, a study suggested that inhibition of MAPK pathway using MEK inhibitors exhibits favorable efficacy in patients with KRAS or BRAF mutant tumors (26), but this has not yet been demonstrated in a clinical study. There is limited clinical evidence of the efficacy of MEK inhibitors in patients with KRAS mutant pancreatic cancer.
In lung cancer, a randomized phase II study evaluated selumetinib or pemetrexed in previously treated NSCLC and demonstrated that the PFS in the selumetinib group was equivalent to that in the pemetrexed group although this was a study of unselected NSCLC patients (37). In contrast, a small study also examined the efficacy of selumetinib specifically in \textit{KRAS} mutant NSCLC but demonstrated no responses (38). Trametinib has also been evaluated in \textit{KRAS} mutant lung cancer. In a phase II study, patients with \textit{KRAS} mutant NSCLC were randomly assigned to either trametinib (GSK1120212) or docetaxel as second-line chemotherapy. The primary endpoint was PFS and although there was no statistically significant difference in PFS between the treatment groups; however patients treated with trametinib had a response rate of 12% (39).

In a combination chemotherapy trial, the randomized clinical phase II study comparing selumetinib and docetaxel versus docetaxel alone as 2\textsuperscript{nd}-line therapy demonstrated encouraging efficacy of the combination in patients with \textit{KRAS} mutant NSCLC (40). In this study, median PFS was prolonged in the combination group compared with the placebo group (5.3 vs. 2.1 months) and the combination had a significantly higher response rate (37\% vs. 0\%) than docetaxel alone. The median overall survival was not statistically significant though, but the preferable tendency toward survival in the combination has shed light on the treatment for the \textit{KRAS} mutant NSCLC. On the basis of these results, a phase III study of docetaxel plus selumetinib or placebo in patients with \textit{KRAS} mutant NSCLC (SELECT-1) has been initiated (NCT 01933932). A single arm phase I/Ib
study evaluating the efficacy of the trametinib and docetaxel in patients with \textit{KRAS} mutant and wild-type NSCLC has also been performed (41). In the preliminary analysis, the response rate was 28\% in patients with \textit{KRAS} mutant NSCLC, and there appeared to be more anti-tumor activity in \textit{KRAS}-G12C tumors compared to non-G12C tumors (41, 42). One challenge with the docetaxel/MEK inhibitor combinations is toxicity. The most dramatic of these is hematologic toxicity. Febrile neutropenia was significantly increased in the docetaxel/selumetinib combination compared to docetaxel alone (18\% vs. 0\%). Future studies will incorporate the use of primary prophylaxis with granulocyte colony stimulating factor (G-SCF) in an effort to reduce this side effect.

Although the mechanism(s) of efficacy of the combination with docetaxel and a MEK inhibitor remain unclear, results from preclinical studies provide some intriguing observations (43, 44). In a murine study of \textit{Kras} G12D NSCLC, the combination of docetaxel/selumetinib produced response rates of 92\% compared with the rates of 30\% in docetaxel (tumor volume difference was 43.7\%, \textit{P} = 0.03273), and the combination led to a prolonged PFS compared to docetaxel alone (12 weeks vs. 6 weeks, \textit{P} = 0.0003) in the murine study (43). However, the efficacy of the combination was significantly blunted in mice whose tumors harbored both a \textit{Kras} mutation and loss of \textit{Lkb1} (43). \textit{LKB1} is a tumor suppressor gene and encodes a serine/threonine protein kinase (45). Germ-line mutations of \textit{LKB1} cause Peutz-Jeghers syndrome, leading to an increased risk of cancer development (46). \textit{LKB1} mutations are frequently accompanied by \textit{KRAS} mutations and observed in...
smokers with NSCLC (47, 48). It will be important to determine in future clinical trials if KRAS mutant NSCLC patients whose tumors also harbor a concurrent loss of LKB1 similarly do not derive benefit from the combination of docetaxel and MEK inhibitors. Other *in vitro* studies have further demonstrated a mechanistic explanation for the clinical efficacy of docetaxel and MEK inhibitors. Microtubule inhibitors, such as taxane or vinorelbine, can lead to activation of the RAS-ERK pathway, and combining MEK inhibitors with these agents suppressed the RAS mediated signaling and increased the efficacy of the drugs in cancers (49, 50). Furthermore, Kawabata et al. suggested, in a preclinical study, an interesting possibility that efficacy of the combination with microtubule inhibitors and MEK inhibitors is largely schedule dependent (44). The study demonstrated that when the microtubule inhibitor was administered first followed by a MEK inhibitor cell death was observed but not when the sequence was reversed (44). This observation may be related to the different phases of the cell cycle where MEK inhibitors and chemotherapy act. MEK inhibitors can arrest most of cells in G1 phase, whereas microtubule inhibitors like docetaxel can affect cells in M phase (Figure 1B). However, treatment with microtubule inhibitors can sometimes cause mitotic slippage, in which the cells enter the following G1 phase without undergoing cell division (51). Thus sequential treatment with MEK inhibitors could also lead to clinical efficacy. Analogously, combining gemcitabine or pemetrexed, which affect cells mainly in S phase and inhibit DNA synthesis, with MEK inhibitors might be also schedule dependent. For instance, both concurrent
combination with a MEK inhibitor AZD6244 and gemcitabine and sequential combination with AZD6244 followed by gemcitabine had limited efficacy, while gemcitabine followed by AZD6244 had efficacy in biliary cancer cells (52). These results may be, in part, due to G1 arrest caused by AZD6244, reducing gemcitabine–induced inhibition of DNA synthesis in S phase. This observation may also be true for pemetrexed. An in vitro study reported that pretreatment with a MEK inhibitor before pemetrexed reduced S–phase arrest and apoptosis induced by pemetrexed in A549 KRAS mutant lung cancer cells (53). Given these findings, the schedule of administration of chemotherapy and MEK inhibitors should be further evaluated in future clinical trials, and such studies may help with development of rational drug combinations with MEK inhibitors in other histological cancers with KRAS mutations.

In lung cancer, there are a number of other ongoing clinical trials of combinations with MEK inhibitors and other targeted agents. Among them, the well–studied targets are proteins in the PI3K-AKT-mTOR signaling cascade (Figure 1A). Data from preclinical studies in a Kras mutant murine lung cancer model demonstrated that activation of the PI3K-AKT-mTOR pathway contributes resistance to AZD6244, and dual inhibition of the PI3K-AKT-mTOR and RAS-ERK pathway had synergistic effects (54-56). Several clinical trials have been initiated combining MEK inhibitors with agents targeting one or more components of PI3K signaling pathway. Preliminary data from one such phase Ib study of the combination with a MEK inhibitor GDC-0973 and a PI3K
inhibitor GDC-0941 demonstrated that in advanced solid tumors, among 30 patients enrolled, 6 of 15 patients had a FDG-PET partial metabolic response and 5 patients had tumor reduction in RECIST–measurable targets (57). A major challenge has been toxicity of the treatment combination. It is possible and even likely that inhibition of both PI3K and MEK signaling sufficiently to fully inhibit these signaling pathways, albeit effective in preclinical models, is not tolerable in humans and alternative schedules and/or treatment approaches will need to be developed.

The other intriguing targets for the combination are anti-apoptotic Bcl-2 family proteins (Figure 1B). A preclinical experiment using shRNA–drug screen library have indicated that a Bcl-XL inhibitor ABT-263 (navitoclax) in combination with a MEK inhibitor led to dramatic apoptosis in KRas mutant xenografts and cell lines (58). An another preclinical study by Tan et al. also reported the efficacy of the combination with ABT-263 and a MEK inhibitor, and further demonstrated that addition of a PI3K inhibitor GDC-0941 to the combination led to increased apoptosis in KRAS mutant cancer cells (59). So far, navitoclax has been studied mainly in small-cell lung cancers though (60, 61), as MEK inhibitors can activate the pro-apoptotic proteins and suppress the pro-survival proteins, inhibition of Bcl-2 pro-survival family proteins in combination with a MEK inhibitor may be expected to be synergistic and thus could represent a potent therapeutic strategy in KRAS mutant cancers.

**Future Studies of MEK Inhibitors in NSCLC**
To date the most compelling clinical data exists for MEK inhibitors combined with docetaxel. Although this combination is now in phase III clinical development for KRAS mutant NSCLC, several important questions and challenges remain. As highlighted above, hematologic toxicity is a potential limitation of the current chemotherapy combinations and likely to be also associated with any other future chemotherapy combinations. Thus alternative scheduling of MEK inhibitors and chemotherapy may need to be considered. This toxicity may also impact on the choice of MEK inhibitors to combine with chemotherapy as MEK inhibitors with a short half life may be more amenable to combine with chemotherapy compared to ones with a long half life.

The development of chemotherapy/MEK inhibitors has so far focused on KRAS mutant NSCLC but it is not clear that this combination is specific for KRAS mutant cancers. It is possible, that MEK inhibitors merely enhance the efficacy of chemotherapy, independent of KRAS mutations, and hence would also be effective in KRAS wild type (WT) cancers. In fact, the phase I trial of docetaxel/trametinib demonstrated a response RR of 21% in KRAS WT patients (41). Further studies will be necessary to determine the benefits of chemotherapy/MEK inhibitors specifically in KRAS WT tumors and whether the magnitude of benefit is similar or smaller than in KRAS mutant cancers.

Until now all KRAS mutant tumors have been lumped together. However, it is more than likely that not all KRAS mutant tumors will derive the same degree of benefit from MEK inhibitors or MEK inhibitor combinations. Data is already emerging on the outcome differences based on
different subtypes of \textit{KRAS} mutations. In addition, the genomic context of \textit{KRAS} mutations is likely to impact therapeutic efficacy of MEK inhibitor combinations. Moving forward, it will be critical to comprehensively characterize \textit{KRAS} mutant cancers and correlate these with clinical efficacy.

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of MEK inhibitor AZD6244 combined with gemcitabine for the treatment of biliary cancer.


Figure 1. Roles of RAS-RAF-MEK-ERK pathway. A. RAS downstream signaling pathways. Under physiological conditions, RAS transduces signals from cell surface receptors to intracellular proteins. Growth factors stimulate the receptors on plasma membranes, and the activated receptors increase the active RAS–GTP form, with the help of GEFs. Activated RAS triggers a multistep phosphorylation cascades such as RAS-RAF-MEK-ERK, PI3K/AKT/mTOR pathways, leading to cell survival and growth (62). B. The RAS-RAF-MEK-ERK pathway regulates expression levels of BCL-2 family proteins. Activated ERK1/2 proteins inhibit Bim and Bad, whereas increase expression of MCL-1, BCL-2, and BCL-XL, leading to cell survival and anti-apoptosis.
Figure 1:

(A) PI3K/AKT/mTOR inhibitors

- PI3K
  - AKT
  - TORC1/2
  - RHEB
  - RSK
  - mTOR

- Survival, protein synthesis, cell-cycle progression, cell migration

(B) MEK inhibitors

- MEK inhibitors
  - MEK
  - ERK
  - PUMA
  - BAD
  - tBID
  - BIM
  - MCL-1
  - BCL-2
  - BCL-XL
  - NOXA
  - ABT-263 (navitoclax)

- Apoptosis
  - BAX, BAK
  - BAD
  - p90RSK

- Calcium signaling
  - Vesicle trafficking

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