Published in final edited form as:

Clin Cancer Res. 2010 July 1; 16(13): 3329–3334. doi:10.1158/1078-0432.CCR-09-3064.

Targeting the mitogen-activated protein kinase pathway: physiological feedback and drug response

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Abstract

MAPK pathway activation is a frequent event in human cancer and is often the result of activating mutations in the BRAF and RAS oncogenes. Targeted inhibitors of BRAF and its downstream effectors are in various stages of preclinical and clinical development. These agents offer the possibility of greater efficacy and less toxicity than current therapies for tumors driven by oncogenic mutations in the MAPK pathway. Early clinical results with the BRAF-selective inhibitor PLX4032 suggest that this strategy will prove successful in a select group of patients whose tumors are driven by ^{V600E}BRAF. Relief of physiologic feedback upon pathway inhibition may, however, attenuate drug response and contribute to the development of acquired resistance. An improved understanding of the adaptive response of cancer cells to MAPK pathway inhibition may thus aid in the identification of those patients most likely to respond to targeted pathway inhibitors and provide a rational basis for tailored combination strategies.

BACKGROUND

One of the central regulators of growth factor-induced cell proliferation and survival in both normal and cancer cells is the RAS protein. Activation of RAS leads to activation of several effector pathways, the best characterized of which are the RAF/MEK/ERK pathway (“the classical MAPK pathway”), the PI3 kinase pathway and the Ral-GEFs (1–6). Constitutive MAPK pathway activation can result from activating mutations in RAS, BRAF and MEK1, loss of the tumor suppressor NF1 (7), or upstream activation mediated by mutations, amplification, or ligand-mediated activation of cell surface receptors (Figure 1). All three RAS family genes (*KRAS*, *NRAS* and *HRAS*) have been shown to be somatically mutated in human cancer, most commonly as a result of single point mutations at codons 12, 13 and 61 (8–11). These mutations impair GTP hydrolysis and thus promote formation of constitutively activated GTP-bound RAS. Somatic point mutations in BRAF occur in approximately 8% of human tumors, most frequently in melanoma, colorectal and thyroid cancers (12,13). BRAF mutations are found, with rare exceptions, in a mutually exclusive pattern with RAS mutations, suggesting that these genetic alterations activate common downstream effectors of transformation.

Activating BRAF mutations are found clustered within the P-loop (exon 11) and activation segment (exon 15) of the kinase domain, and a single point mutation, V600E, accounts for approximately 90% of cases (12,14). Structural analysis of the V600E mutation suggests that it disrupts the interaction between the P-loop and the activation segment, which normally locks the kinase in the inactive conformation (15). “Impaired activity” mutants have also been reported. In contrast to the V600E mutant, these mutations activate the pathway in a RAF1-dependent manner (16).

CLINICAL-TRANSLATIONAL ADVANCES

Several strategies for inhibiting MAPK signaling are now being evaluated as cancer therapies. Clinically effective direct inhibitors of RAS have yet to be identified. Promising therapeutic approaches include targets that function as synthetic lethals in RAS mutant tumors (17) and inhibitors of downstream effectors such as RAF and MEK.

RAF kinase Inhibitors

Sorafenib (Nexavar) was the first RAF kinase inhibitor to enter human clinical testing. Sorafenib is now FDA approved for use in renal cell carcinoma and hepatocellular carcinoma. Although this compound was initially developed as a selective inhibitor of RAF, later studies revealed other biologically relevant targets, including VEGFR2/3, PDGFR, Flt-3, c-kit, and FGFR-1 (18). Sorafenib has virtually no activity as a single-agent in melanoma, the tumor type with highest frequency of BRAF mutations (19). Phase 2 trials combining sorafenib with chemotherapy showed early promise in melanoma but the activity of this combination regimen did not correlate with BRAF mutational status. Furthermore, a phase 3 trial of sorafenib in combination with carboplatin and paclitaxel in patients with advanced melanoma failed to meet its primary endpoint of improvement in overall survival (20). Overall, the data suggest that the primary mechanism of activity of sorafenib in renal cancer is likely anti-angiogenic and that RAF inhibition contributes minimally to its activity in patients with advanced cancer.

The limited activity of sorafenib in tumors with BRAF mutation prompted the development of second-generation RAF inhibitors with greater selectivity for mutant BRAF and greater potency for the target *in vivo*. PLX4032/R7204 (and its close analogue PLX4720, Plexxikon/Roche) (21,22) cause potent pathway inhibition and antiproliferative effects, but in contrast to sorafenib, do so only in cell lines harboring ^{V600E}BRAF (23). In the recent phase 1 clinical trial of PLX4032, high (30–50 μ M) steady state serum levels of the drug were tolerated with modest toxicity. This resulted in profound inhibition of ERK signaling in the tumor. Profound antitumor activity was observed: a 78% response rate by RECIST (Response Evaluation Criteria in Solid Tumors) and tumor shrinkage in almost all patients (24,25). Notably, toxicities included skin rash and the development of squamous cell carcinomas in approximately one third of patients. The average duration of response on the Phase 1 trial was approximately 9 months and a phase 3 trial comparing PLX4032 to dacarbazine as first-line therapy for patients with metastatic melanoma whose tumors harbor ^{V600E}BRAF is currently ongoing.

Testing for BRAF mutations became a prerequisite for study entry early in the trials of PLX4032, and all responses observed were in patients whose tumors harbored ^{V600E}BRAF. Notably, PLX4032 inhibits ERK activation only in cells with BRAF mutations, while in cells with wild-type BRAF, including those with RAS mutations, PLX4032 treatment results in a paradoxical induction of ERK phosphorylation (26–28). The mechanistic explanation for this observation is that binding of PLX4032 to one member of a CRAF homodimer inhibits the bound protomer but results in transactivation of the drug-free protomer (28). The paradoxical activation of ERK by PLX4032 is not observed in cells harboring ^{V600E}BRAF mutations, as in this context, RAS activity is low and dimer formation is not required for activation. Notably, the paradoxical activation of ERK signaling is not unique to PLX4032 but is also observed

with sorafenib and other ATP-competitive inhibitors of RAF (26–28). These results suggest that the clinical activity of PLX4032 will be restricted to tumors harboring activating mutations of BRAF. Furthermore, PLX4032 may enhance the growth of tumors in which the ERK pathway is activated by RAS mutation or upstream receptor kinases. Inhibitors further downstream such as those targeting MEK or ERK may thus be preferable in tumors in which ERK activity is the result of RAS or receptor tyrosine kinase activation. Finally, the toxicities of PLX4032 such as the development of squamous cell carcinomas may be attributable to ERK activation.

Several additional small molecule inhibitors of RAF are also in early clinical testing. XL281 (Exelixis) has shown modest biological activity and modulation of MAPK pathway activity in tumor tissue: clinical benefit (PR or SD) was observed in 43% (13/30) of patients in the dose-escalation phase of a recent Phase 1 study (29). RAF265 (Novartis), which inhibits all three isoforms of RAF, as well as mutant BRAF, and has anti-angiogenic activity through inhibition of VEGFR-2 (30,31), is also being investigated in Phase I clinical trials in malignant melanoma.

MEK inhibitors

CI-1040 (Pfizer Oncology), an allosteric inhibitor of MEK, was the first selective MEK inhibitor to advance into clinical testing (32,33). In contrast to PLX4032, CI-1040 inhibits ERK activity in all cells irrespective of their mutational status. Cells with BRAF mutations are selectively sensitive to CI-1040 but in contrast to PLX4032, a subset of RAS mutant cell lines, and those with wild-type RAS and BRAF also exhibit MEK-dependence and CI-1040 sensitivity (34–36). Modest anti-tumor activity was observed in the phase 1 trial of CI-1040 (37), but clinical activity was disappointing in the phase 2 setting and development of CI-1040 was halted in favor of a second-generation compound PD0325901 (Pfizer Oncology) (38). PD0325901 exhibits 50–100-fold greater potency, improved oral bioavailability, and increased metabolic stability compared to CI-1040 (39,40). RECIST responses were observed in three patients with melanoma on the phase 1 clinical trial of PD0325901, but further clinical development of this agent was not pursued due to concerns over neurological toxicity (41–43). In contrast to the recent trials of PLX4032, trials of CI-1040 and PD0325901 did not restrict eligibility to patients whose tumors harbored mutational activation of the ERK pathway and therefore the activity of these agents in the BRAF mutant class has yet to be clearly defined.

AZD6244 (AstraZeneca) is a third, ATP non-competitive, allosteric inhibitor of MEK1/MEK2. AZD6244 completed phase 2 testing in melanoma, colorectal and lung cancers. In a phase 2 randomization trial of AZD6244 vs. temozolomide in patients with melanoma, antitumor activity with AZD6244 was observed, with partial responses in six patients, five of whom had tumors harboring V600E BRAF mutations (44,45). There was, however, no significant difference between the treatment arms for the primary endpoint of progression free survival. Similar results were observed in phase 2 trials of AZD6244 in non-small cell lung and colon cancer in which the drug was compared to pemetrexed and capecitabine, respectively (46, 47). In summary, the three randomized phase 2 trials of AZD6244 suggested that activity with this agent was comparable to, but not superior to, disease-specific standard chemotherapy. However, these trials did not enrich for patients whose tumors were most likely to respond to MEK inhibition as predicted by preclinical data. Ongoing studies are now testing the efficacy of AZD6244 in trials in which study entry is restricted to only those patients whose tumors harbor activating mutations in BRAF and/or RAS.

Physiological feedback and drug response

Given the diversity of mutations that induce ERK pathway activation, expression of phosphorylated ERK has been postulated as a potential biomarker of ERK pathway activation and thus MEK inhibitor sensitivity. Several reports demonstrate, however, that the expression

of phosphorylated ERK does not correlate with MAP kinase pathway dependence and MEK-inhibitor sensitivity (34,36,48). The explanation for this lack of correlation is that the level of phosphorylated ERK is a poor surrogate for MAPK pathway activity (49). Physiologic activation of RAS/RAF signaling is balanced by inhibitory regulators of the pathway which include the sprouty (SPRY) proteins, the MAP kinase phosphatases (MKPs or DUSPs), KSR-1, and RKIP (2,50–53), and by scaffolding proteins such as 14-3-3 which regulate RAF cellular localization and stability (54,55). Pathway activity is also regulated by cross-talk with parallel signaling pathways, such as by AKT phosphorylation of inhibitory sites on RAF (56) and through PI3K-dependent feedback (57). In non-transformed cells, activation of the ERK pathway is balanced by inhibitory signals, which dampen or limit the duration of its activity. In tumor cells, however, this normal feedback is often disabled, either through mutation or decreased expression of feedback regulators (58–62). This disruption of normal pathway feedback allows for unhindered ERK pathway activation and is likely a prerequisite for ERK-dependent transformation.

The ERK pathway is thus a classical feedback loop in which negative regulators of the pathway are transcriptionally controlled by ERK. Specifically, ERK activation leads to increased expression of DUSPs and SPRYs that in turn downregulate pathway activity in normal cells. In BRAF mutant tumors, the upstream feedback at the level of the RAF is disrupted, at least in part by the inability of Sprouty family proteins to bind to and inhibit mutant BRAF (63, 64). Feedback at the level of ERK, mediated by the MAPK phosphatases, remains intact and therefore, in BRAF mutant cells, steady state levels of phosphorylated ERK are not dramatically elevated, despite high levels of MEK phosphorylation and high levels of ERK pathway output (49). These findings provide a mechanistic basis for the lack of correlation between phosphorylated ERK expression and ERK pathway output. They also suggest assays of phosphorylated MEK expression and PCR based methods to detect elevated expression of the transcriptional output of ERK may be useful surrogates for high MAPK pathway activity.

The mechanisms whereby MAP kinase pathway feedback is disrupted in BRAF mutant cells may also explain in part the exquisite dependence of BRAF mutant tumors on MEK-ERK signaling and the selectivity of PLX4032 for tumors cells harboring ^{V600E}BRAF. As discussed above, PLX4032 and other ATP-competitive inhibitors of RAF induce a paradoxical activation of ERK in tumors with RAS mutation and in those with wild-type BRAF and RAS (28). Activation of ERK by PLX4032 requires direct binding of the compound to the ATP-site of RAF and results from transactivation of the unbound protomer within RAF dimers (28). As RAS activation promotes RAF dimer formation, pathway activation is observed prominently in cells expressing mutant RAS. In contrast to the paradoxical activation of ERK observed in RAS mutant cells, PLX4032 does not induce ERK activation in cells with ^{V600E}BRAF. One possible explanation for this result is that RAS activity is low in cells harboring ^{V600E}BRAF due to the high levels of sprouty and other negative feedback regulators induced by the oncogene. Ectopic expression of mutant RAS in ^{V600E}BRAF cells can, however, induce resistance to PLX4032 (28), suggesting that the pattern of feedback dysregulation observed in ^{V600E}BRAF mutant cells may be critical in determining response to selective inhibitors of RAF.

Relief of upstream feedback within the MAPK pathway may also attenuate the response to selective inhibitors of RAF and MEK and contribute to drug resistance. In ^{V600E}BRAF tumor cells, MEK phosphorylation is high and not further induced by treatment with MEK inhibitors (49). This contrasts with the response of most tumor and normal cells, including those driven by receptor tyrosine kinase activation and RAS mutations, in which MEK inhibition induces a rapid and profound stimulation of MEK phosphorylation, presumably due to relief of feedback inhibition of MEK (49,65,66). This relief of physiological feedback inhibition upstream of MEK following treatment with MEK inhibitors may attenuate drug response in

one of several ways. First, activation of RAF, RAS or upstream receptor tyrosine kinases upon downregulation of Sprouty and other feedback elements may lead to activation of parallel signaling pathways previously suppressed by the high levels of SPRYs and DUSPs. As many of these parallel pathways harbor the potential to redundantly regulate the same common downstream effectors of transformation regulated by ERK, such as D cyclins, their activation may attenuate drug response. Alternatively, the hyperphosphorylation of MEK following treatment with an inhibitor such as PD0325901 may promote a state of hyperactivation during times in which the drug is not present at sufficient concentrations to inhibit the enzyme. Such a scenario would occur if continuous drug exposure were not possible as a result of toxicity limitations.

Finally, feedback regulation of the ERK pathway is mediated not only by transcriptional events, but also via direct phosphorylation of CRAF by ERK. CRAF contains six inhibitory sites, the phosphorylation of which require ERK activation (67). CRAF mediated phosphorylation of MEK is thus observed following treatment with MEK inhibitors (49,65,66), in the setting of impaired ERK activation by dominant negative kinase suppressor of RAS (KSR) (68), and in cells overexpressing IMP (69). One translational implication of these findings is that the clinical activity of selective inhibitors of BRAF may be attenuated by relief of feedback inhibition of CRAF. Consistent with this possibility, overexpression of CRAF has been shown to be a mechanism of acquired resistance to the selective RAF inhibitor AZ628 (70).

Conclusion

Activation of the MAP kinase pathway is a frequent event in human cancer and pathway activity is often the result of activating mutations in RAS and BRAF. Agents that target RAF and its primary downstream effector MEK are in early stage clinical development, and in the case of the RAF inhibitor PLX4032 have demonstrated sufficient clinical activity to warrant progression to definitive Phase 3 trials. As the activity of these agents correlates with the mechanism responsible for pathway activation (BRAF mutation, RAS mutation or receptor tyrosine kinase activation), prospective genotyping of patients to enrich for those most likely to respond will be critical in the future development of drugs targeting this pathway. Relief of physiological feedback may attenuate the response of cells to selective inhibitors of RAF and MEK and may contribute to drug resistance. A further understanding of the role of feedback elements in promoting transformation and attenuating drug response may thus inform the development of combination strategies that maximize tumor response.

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Figure 1. The RAS-RAF-MEK-ERK signaling pathway

The classical MAP kinase pathway is activated in human tumors by several mechanisms including the binding of ligand to receptor tyrosine kinases (RTKs), mutational activation of an RTK, by loss of the tumor suppressor NF1, or by mutations in RAS, BRAF and MEK1. Phosphorylation and thus activation of ERK regulates transcription of target genes which promote cell cycle progression and tumor survival. The ERK pathway contains a classical feedback loop in which the expression of feedback elements such as SPRY and DUSP family proteins are regulated by the level of ERK activity. Loss of expression of SPRY and DUSP family members due to promoter methylation or deletion is thus permissive for persistently elevated pathway output. In the case of tumors with ^{V600E}BRAF expression, pathway output is enhanced by impaired upstream feedback regulation.