Targeting the Mitogen-Activated Protein Kinase Pathway in the Treatment of Malignant Melanoma
David J. Panka, Michael B. Atkins, and James W. Mier

Abstract
The mitogen-activated protein kinase (MAPK; i.e., Ras → Raf → Erk) pathway is an attractive target for therapeutic intervention in melanoma due to its integral role in the regulation of proliferation, invasiveness, and survival and the recent availability of pharmaceutical agents that inhibit the various kinases and GTPases that comprise the pathway. Genetic studies have identified activating mutations in either B-raf or N-ras in most cutaneous melanomas. Other studies have delineated the contribution of autocrine growth factors (e.g., hepatocyte growth factor and fibroblast growth factor) to MAPK activation in melanoma. Still, others have emphasized the consequences of the down-modulation of endogenous ras inhibitors, such as Sprouty family members (e.g., SPRY2) and ras-1 kinase inhibitory protein, in the regulation of the pathway. The diversity of molecular mechanisms used by melanoma cells to ensure the activity of the MAPK pathway attests to its importance in the evolution of the disease and the likelihood that inhibitors of the pathway may prove to be highly effective in melanoma treatment. MAPK inhibition has been shown to result in the dephosphorylation of the proapoptotic Bcl-2 family members Bad and Bim. This process in turn leads to caspase activation and, ultimately, the demise of melanoma cells through the induction of apoptosis. Several recent studies have identified non–mitogen-activated protein/extracellular signal-regulated kinase kinase–binding partners of ras and suggested that the prosurvival effects of ras and the lethality of ras inhibition are mediated through these alternative targets, independent of the MAPK pathway. Other studies have suggested that endothelial cells are the primary targets of ras inhibitors in vivo and that the antitumor effect of these agents are largely attributable to angiogenesis inhibition. This article reviews the genetic and biochemical factors contributing to MAPK activation in melanoma, the mechanisms by which inhibition of the pathway might prove deleterious to tumor cells, and the potential of MAPK inhibitors in the treatment of the disease.

Activation of the Mitogen-Activated Protein Kinase Pathway in Melanoma

The mitogen-activated protein kinase (MAPK) pathway is activated in virtually all melanomas (1). Several factors contribute to this phenomenon, including mutations in the genes of key kinases or G proteins and the absence of endogenous inhibitors that would otherwise constrain the activity of this signaling pathway. For example, ~60% of cutaneous melanomas have an activating mutation in the catalytic domain of the serine-threonine kinase B-raf (2, 3). A T1796A transversion that results in the substitution of a glutamate for valine at position 600 (V600E) is by far the most common such mutation. This single base pair change renders the kinase constitutively active, resulting in the phosphorylation of MEK and targets further downstream (Fig. 1). This particular genetic alteration is not one predicted to result from the failure to repair UV light–induced pyrimidine dimers, and its connection with sun exposure is therefore unclear (2). Although these B-raf mutations play an essential role in the ontogeny of melanoma, they are found in 80% of benign nevi (4) and are therefore by definition insufficient to cause the disease in the absence of other factors.

A few melanomas, primarily those lacking B-raf mutations, have activating mutations in N-ras, a small G protein previously identified as a contributing oncogene in neuroblastomas (5). When GTP-bound, this GTPase binds and activates various ras isoforms, resulting in a signaling cascade qualitatively similar to that activated by the aforementioned B-raf mutation. Nonmutated ras and ras also contribute to MAPK activation in melanoma. Ras is GTP-loaded and c-ras is phosphorylated in melanoma (6), presumably through the action of constitutively produced growth factors, such as hepatocyte growth factor and fibroblast growth factor. The receptor for hepatocyte growth factor, c-met, is frequently overexpressed on melanomas (7, 8),

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and neutralizing antibodies against hepatocyte growth factor and fibroblast growth factor substantially reduce the level of ERK phosphorylation in cultured melanoma cells (6). These data suggest that ERK phosphorylation in these cells is driven in part through an autocrine loop involving the interaction of secreted growth factors with their respective membrane tyrosine kinase receptors.

The acquisition of mutations in B-raf or N-ras is not the only strategy used by melanoma cells to ensure that the MAPK pathway is constitutively active. In addition to these genetic alterations, melanomas frequently fail to express genes that encode proteins that negatively regulate ERK phosphorylation. For example, raf-1 kinase inhibitory protein is conspicuously down-regulated in melanomas (9). The restored expression of this inhibitor reduces ERK phosphorylation in melanoma cells, indicating that the loss of this protein contributes to MAPK activation in these cells. Members of the Sprouty (SPRY) family are thought to play a similar role. Originally described as critical participants in the tracheal bifurcation that occurs during Drosophila development, at least two members of the family (SPRY2 and SPRY4) have been shown to bind raf-1 and wild-type B-raf, but not B-raf^{V600E}, and inhibit their kinase activity (10–12). A recent microarray study comparing the gene expression profiles of melanomas harboring the B-raf^{V600E} oncogene with those possessing only the wild-type allele specifically identified SPRY2 as a gene down-modulated in melanomas lacking a mutated B-raf (13). This finding suggests that absence of SPRY2 may contribute to the activity of the MAPK pathway in cells lacking a B-raf oncogene. Collectively, these observations attest to the need of melanoma cells to maintain the activity of the MAPK pathway and the consequent diversity of strategies used by these cells to achieve this objective.

**Mechanism of Apoptosis Induced by MAPK Inhibition**

The MAPK pathway regulates numerous cellular activities in melanoma, including survival. Effective disruption of this pathway, either through pharmacologic inhibition or the deletion of one or more key pathway components, results in cell death. Studies with small interfering RNAs targeting c-raf and B-raf have affirmed the critical role played by the B-raf^{V600E} oncogene in the maintenance of melanoma cell viability (14). Other studies have shown the lethality of MEK inhibitors in melanoma (15–17), suggesting that MAPK inhibition may be an important component of the mechanism by which the B-raf^{V600E} knockdown induces apoptosis. One such study implicated the dephosphorylation of the proapoptotic BH3-only Bcl-2 family member Bad as the key event leading to the death of melanoma cells from MAPK inhibition (15). In this model, the ERK substrate ribosomal S6 kinase (p90^rsk) phosphorylates Bad on Ser^{75} (Fig. 1). This phosphoserine lies within a consensus sequence that binds to the cytoplasmic protein 14-3-3, sequestering Bad as an inert complex in the cytosol. In the presence of a MEK inhibitor, ERK and p90^rsk are inactive and Bad is dephosphorylated. Bad then dissociates from 14-3-3 and translocates to the mitochondria, where it binds the antiapoptotic proteins Bcl-2 and Bcl-X_L and undermines their prosurvival function (15, 18, 19). Other studies suggest that the phosphorylation of another BH3-only Bcl-2...
family member Bim accounts for the prosurvival effect of MAPK activation. When active, ERK1 and ERK2 phosphorylate Bim, which targets the phosphoprotein to the proteasome where it is degraded (20, 21). Inhibition of the MAPK pathway results in the accumulation of Bim and its association with the proapoptotic Bcl-2 family member Bax, resulting in the death of the cell.

Although these models are consistent with the toxicity of MEK inhibitors, they do not provide an adequate explanation for the lethality of raf inhibitors such as sorafenib for melanoma cells. Studies by Panka et al. (22), for example, have shown that the knockdown of Bad offers no protection against sorafenib-induced apoptosis in most melanoma cell lines examined, suggesting that the lethal effects of raf inhibition may not be entirely due to the inactivation of the MAPK pathway and the dephosphorylation and mitochondrial translocation of Bad. This notion is supported by the recent identification of several raf-binding proteins unrelated to MEK (23–25) and numerous studies suggesting that raf can function independently of MEK (26, 27). For example, in developing murine cardiac muscle cells, the prosurvival effect of c-raf is attributable to its ability to bind and suppress apoptosis signal-regulating kinase-1 (ASK1), a kinase upstream of c-Jun-NH2-kinase (23), rather than its ability to activate MEK. The proapoptotic consequences of a selective cardiac-targeted c-raf deletion can be prevented by the concurrent deletion of ASK1, clearly implicating this stress-associated kinase in the myocyte death observed in these genetically modified mice. Mammalian sterile 20-like kinase 2 (MST2), another kinase whose activity is negatively regulated by raf, induces apoptosis through an elaborate signaling cascade involving the recently characterized tumor suppressor Lats1 (28). The enhanced susceptibility of raf-1−/− cells to apoptosis is largely abrogated by MST2 depletion (24). Although these observations show that ASK1 and MST2 play a role in the apoptosis induced by the deletion of c-raf, it must be kept in mind that the kinase activity of c-raf is not required for the suppression of either ASK1 or MST2. It is therefore unclear if either of these kinases plays a role in the apoptosis induced by the pharmacologic inhibition of raf activity.

Angiogenesis Blockade through raf Inhibition

Independent of their direct effects on tumor cells, drugs that inhibit the MAPK pathway may prove highly effective in vivo because of their ability to inhibit angiogenesis. The notion that this signaling pathway might be essential for vascular development arose from the results of knockout experiments demonstrating early embryonic lethality, largely due to angiogenic failure, in raf-1−/− mice (26). These studies, however, showed that the raf-1− deficient phenotype could be rescued through the introduction of a mutant raf-1 that does not activate the MAPK pathway (i.e., raf-1−YY340/341FF), suggesting that the effect of raf on endothelial cell survival could not be mediated through the conventional MAPK (i.e., Raf-MEK-ERK) pathway. Regardless of the identity of the particular raf substrate involved in the promotion of endothelial cell viability, it is clear that c-raf inhibition affects not only normal vascular development but tumor angiogenesis as well. Investigators in the Cheresh laboratory showed that a dominant-negative raf construct selectively targeted to the endothelium results in angiogenesis inhibition and tumor involution (29), establishing the paradigm of raf inhibition as a potentially effective treatment modality. Preclinical studies with the raf inhibitor sorafenib have shown antitumor activity in vivo using tumor cells that are completely resistant to the drug in vitro (17), suggesting that, in these cases, the antitumor activity might be entirely based on the antiangiogenic activity of the drug. The extent to which the inhibition of raf is responsible for these effects is unclear, because the drug directly inhibits several membrane tyrosine kinase receptors (e.g., vascular endothelial growth factor receptors I and II, and platelet-derived growth factor receptor-β) involved in angiogenesis (17). Recent studies with melanoma cells engineered to conditionally express a small interfering RNA for B-rafV600E suggest that in established tumors, the dominant antitumor effect associated with the loss of B-raf activity may be the result of diminished vascular endothelial growth factor production by the tumor cells and reduced angiogenesis (30). Thus, it is likely that the administration of raf inhibitor to cancer patients will have both direct effects on endothelial cell viability and indirect effects based on impaired growth factor production by tumor cells.

Discussion

The remarkable diversity of molecular mechanisms enlisted by melanoma cells to ensure the constitutive activation of the MAPK pathway attests to the extent to which the integrity of this signaling cascade is essential to tumor cell growth and viability. The critical role played by the MAPK pathway in cell survival is further supported by multiple studies with small interfering RNAs and pharmacologic inhibitors demonstrating the adverse consequences of raf or MEK blockade. Several agents that target this pathway are already undergoing clinical testing (reviewed elsewhere in this supplement) and some have already shown promise in clinical trials involving melanoma patients.

Despite our increasing knowledge of the regulation of the MAPK pathway, it remains unclear how its inhibition results in melanoma cell death. In the case of raf inhibitors such as sorafenib, it is unclear whether the critical lethal event induced by the drug involves MEK/ERK, one or more of the recently identified non-MEK raf-binding kinases, or an as yet uncharacterized factor. It is even unclear whether the in vivo antitumor activity of sorafenib is based on its antineoplastic or antiangiogenic activities and to the extent its effects on the tumor neovasculature predominate, whether they are mediated through the inhibition of raf or upstream tyrosine kinase receptors, such as those for vascular endothelial growth factor and platelet-derived growth factor. It is hoped that ongoing laboratory and clinical studies will not only lead to the clarification of these mechanistic details but also place us in a position to take optimal advantage of this emerging class of novel therapeutic agents.

Open Discussion

Dr. Atkins: What would you consider combining a raf inhibitor with if the mechanism of action is, for example, through apoptosis-inducing factor or the platelet-derived growth factor receptor?

Dr. Mier: It may be best to answer that through blind high-throughput screening, in which drug X plus sorafenib are
simply thrown in together and you pick a few variables, such as Annexin staining, and look for the combination that maximally affects the chosen variable. That kind of unbiased approach may lead to an answer long before a reasoned approach would. What we found to be most helpful and what amplifies the lethality of this drug most significantly are things that block survival pathways that are activated by the drug.

Dr. Gajewski: What is the structural basis for sorafenib blocking RAF function, and why do the constitutively active RAF mutants remain susceptible to inhibition?

Dr. Mier: As far as I know, sorafenib goes after the ATP-binding domain.

Dr. Flaherty: The crystal structure was solved and reported with the drug bound in the ATP-binding site. It pushes the activation loop of the kinase out of the fold that it would otherwise sit in. The question that has not been answered is how RAF interacts with its substrates; in other words, what domains of RAF interact with which substrates.

Dr. Sondak: I understand that RAF is also important in mitosis and mitotic spindle assembly, which would obviously point a finger directly at things such as taxanes and other mitotic spindle assembly inhibitors.

Dr. Mier: Survivin has a similar association. It likes to associate with microtubules, especially the spindle apparatus. Survivin is easily down-modulated by sorafenib. In fact, if you transfect in enough Survivin to get a thumb-wide band on your gel and you throw sorafenib into the culture, it all vanishes within minutes.

Dr. Atkins: Is there a way that we can potentially distinguish one RAF inhibitor from another by a pattern of proteins that it might activate in vitro that might allow us to better select which ones should be studied? Can we learn enough from this type of cell line data to help us determine which drug to use?

Dr. Mier: I hope so, but the easiest way of getting at that information may be just to do a high-throughput screen with a spheroid model or even in vitro cultures where the cells are growing on polystyrene. We can randomly add drug combinations and see which is the most effective. Those that look especially promising could then be put into an animal model and validated. The things that work best are the drugs that antagonize survival pathways that are activated when the cell is stressed with a RAF inhibitor, such as the JNK pathway. RAF is known to suppress the kinase MST2. There is a possibility that this may be more important than we know. However, this inhibition is not known to be influenced by the kinase function of any of the RAFs, but it is a definite proapoptotic pathway. One of the reasons why it may not be terribly important in melanoma is that many of the downstream components are genes that are epigenetically silenced in melanoma such as LATs.

Dr. Flaherty: As we develop new RAF inhibitors, we need a readout of what each one does and a profile of what the downstream effects are. It is clear that when we look at MAPK and ERK, we’re probably not seeing the full spectrum of what they can do.

Dr. Mier: One of the potential Achilles’ heel of sorafenib is that it’s a p38 blocker. The activation of p38 is an absolutely essential component to the lethality of cytokines. Here we’ve got a drug that blocks p38 with nearly the same IC50 as the RAF isoforms. Some of these other RAF inhibitors don’t have that feature and may work better for that reason alone.

Dr. Sosman: What happens to these different cell lines when you knockout MEK, ERK, BRAF, and CRAF? Do you actually kill all those cell lines?

Dr. Mier: The ones we’ve chosen to knockout are ones that we felt might be involved in the lethality of the drug, for example, apoptosis-inducing factor and BAD. If you use a flow cytometer to gage lethality by assessing Annexin or propidium iodide staining, you can cotransfect with red fluorescent protein to identify the cells that pick up your small interfering RNA and just transfect in red fluorescent protein alone for the control.

Dr. Sosman: If we get the ultimate drug, is it going to be effective in the most simplistic system? If we really knockout all of BRAF or MEK, is that enough?

Dr. Mier: I would think not. There’s plenty of reason to think that the lethality of a RAF knockdown has nothing to do with the disruption of the MEK pathway. The experiment that most illustrates this is the fact that if you knockout CRAF, you get an embryonic lethality at around day 9. The critical abnormality, when a pathologist looks at the embryos, is failure to develop blood vessels. The problem is that you can completely rescue the knockdown with a CRAF that has no MEK activating capability whatsoever. At least in the heart, for example, there is no effect on MEK when you introduce a CRAF knockdown. The lethality of the knockdown is entirely due to the loss of suppression of ASK1. I’m not sure that the MEK pathway is the only thing that is relevant to RAF.

References

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