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The Raf Inhibitor Paradox: Unexpected Consequences of Targeted Drugs

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Three papers in *Cell* and *Nature* now report that dimeric RAF is a plastic enzyme: blocking one ATP-binding site paradoxically stimulates the kinase activity of the other protomer. This occurs only in "primed" cells bearing activated RAS and WT RAF, explaining the selective efficacy of RAF inhibitors for RAF mutant cells.

Cancer is an obstinate beast. RAS is one of the most feared oncogenes, driving close to a third of all tumors, and one of the most difficult to target. The discovery that RAF kinases are direct effectors of RAS raised hopes that RAF kinase inhibition would afford an effective tool to slow RAS-driven cancers. The subsequent discovery of BRAF mutations in human cancers (Davies et al., 2002) inspired a new wave of searches for BRAF-selective inhibitors. RAF inhibitors block proliferation of BRAF mutant cell lines in vitro and in vivo but are surprisingly ineffective against RAS mutant cells and do not block ERK activation in such cells (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). Paradoxically, structurally-diverse ATP-competitive RAF kinase inhibitors can cause activation of the RAF-MEK-ERK pathway.

This paradoxical activation of RAF by a RAF kinase inhibitor was documented long ago (Hall-Jackson et al., 1999) and proposed to result from inhibitor-mediated inactivation of negative feedback loops. Three recent publications now offer new mechanistic explanations for the RAF inhibitor paradox (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010), based on homo- and heterodimerization of CRAF and BRAF and recognition of their roles in activating RAF kinase activity (Rajakulendran et al., 2009).

Crystal structures of BRAF show asymmetric dimerization (Tsai et al., 2008; Wan et al., 2004), and mutations that stabilize this asymmetric "side-to-side" dimer are growth-stimulatory whereas mutations that destabilize it counteract an oncogenic mutation (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Rajakulendran et al., 2009). Thus, alterations in RAF dimerization or its consequences can influence RAF signaling (Figure 1).

The new studies found that paradoxical RAF pathway activation by RAF inhibitors requires RAF binding to activated RAS, but only when RAF activation is dependent on RAS. Mutations disrupting RAF/RAS binding (Heidorn et al., 2010) or expression of dominant-negative RAS (Hatzivassiliou et al., 2010) abrogated paradoxical activation. However, an N-terminally truncated and RAS activation-independent CRAF mutant was hypersensitive to inhibitor-mediated ERK activation (Poulikakos et al., 2010). These critical findings suggest that the RAF inhibitor paradox is relevant only in a "primed" or pathological state. Collectively, these results may both explain the finding that RAF inhibitors are generally well tolerated and also potentially argue against the use of RAF inhibitors in RAS mutant tumors.

While all three studies showed that RAF inhibitors can activate the RAF-MEK-ERK pathway in the context of oncogenic RAS and that activation of CRAF is required, the proposed mechanisms are provocatively different. Heidorn et al. suggest that selective inactivation of BRAF is critical, basing this in part on data showing that low doses of BRAF-selective inhibitors PLX4720 and 885-A enhanced ERK activation in the presence of mutationally active NRAS, whereas pan-RAF inhibitor sorafenib did so only if putatively prevented from binding to CRAF by introduction into CRAF of the T421N gatekeeper mutation. In contrast, Hatzivassiliou et al. and Poulikakos et al. argue that binding to BRAF is not necessarily required. Indeed, knockdown experiments showed that MEK activation requires CRAF, whereas knockdown of BRAF did not diminish compound-dependent activation. Poulikakos et al. also found an inverse correlation between induction of ERK activation and the dose of six chemically distinct ATP-competitive RAF inhibitors, including sorafenib. They suggested that higher doses are required to shut down CRAF-mediated signaling, whereas lower doses might induce ERK activation by poorly blocking CRAF, regardless of RAF isoform specificity. Further mechanistic studies will be required to resolve these discrepancies.

Direct comparisons among these studies are difficult given their different drugs, doses, endpoints, and cells. Conflicting results have emerged regarding the roles of inhibitor off-rates, RAF membrane targeting, stabilization/destabilization of RAF heterodimer formation, requirements for RAS interaction, and the interplay between them. There may also be several means to achieve the same end. For example, PLX4720 (whose clinical analog is PLX4032 [Garber, 2009]) has a distinct manner of interaction with the BRAF-CRAF heterodimer and consequence for RAF translocation compared to 885-A or GDC-0879, yet all these inhibitors induced paradoxical activation of MEK.

A central mystery is how ATP-competitive inhibitors can bind to the catalytic binding pocket in place of ATP and yet activate RAF kinase activity. Poulikakos et al. elegantly addressed this issue and showed that inhibitor binding to kinase-dead CRAF transactivates its dimerized

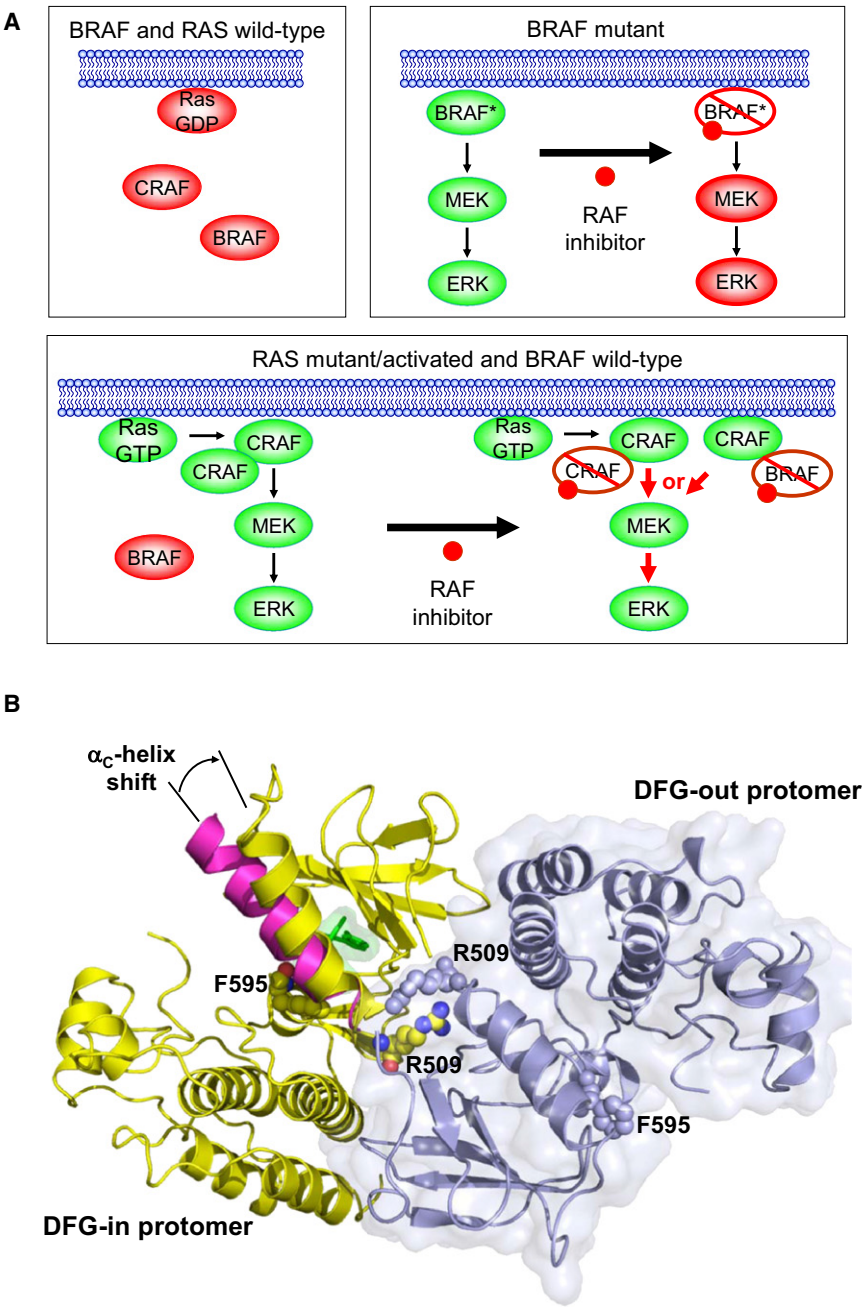

Figure 1. Cell Context-Dependent Consequences of Raf Inhibitor Treatment

(A) In BRAF wild-type tumors where RAS is inactive (top left panel), tumor cell growth is not dependent on RAF-MEK-ERK and, hence, is insensitive to RAF inhibitors. In BRAF mutant cells (top right panel), RAF inhibitors, particularly those with BRAF selectivity, potently block mutant BRAF activity. Without RAS activation, no transactivation of CRAF occurs. In untreated RAS mutant/activated tumor cells, RAS recruits CRAF, but not BRAF, to the plasma membrane, leading to CRAF activation of MEK and ERK (bottom panel). This model predicts that Ras activation by mutation or upstream signaling can be a mechanism of Raf inhibitor resistance in BRAF mutant tumor cells. Under treatment conditions with a RAF inhibitor where only partial inactivation of CRAF is achieved, CRAF activation of MEK is enhanced by RAS-GTP-dependent membrane recruitment and homo- or heterodimer formation with inhibitor-free CRAF. Treatment with a RAF inhibitor that fully blocks all RAF functions would prevent transactivation, resulting in effective ERK inhibition.

(B) A critical molecular interaction required for inhibitor-mediated transactivation of CRAF is the dimeric structure of RAF. Shown here is the structure of the three-dimensional asymmetric dimer of BRAF (generated by G. Bollag and C. Zhang using previously published coordinates [Tsai et al., 2008]). One protomer (yellow) is in the DFG-in conformation and the other (blue) is in the DFG-out conformation. The surface outline of the DFG-out protomer is shown lightly shaded, in part to indicate the dimeric interface. R509 from each protomer is also highlighted to reflect its importance in anchoring the dimer. F595 from the DFG motif is also highlighted to reinforce the significant differences in the conformations of the protomers.

partner, a kinase-competent CRAF that cannot bind RAF inhibitor. Perhaps in normal cells, in an energy-rich environment primed for proliferation, elevated RAS-GTP levels induce cooperative binding of ATP to RAF. In cancer cells, this process might be exploited for proliferative advantage.

These findings have important implications for designing next-generation RAF inhibitors. Regardless of mechanism, all three studies suggest that blocking CRAF is important to avoid activating ERK signaling in RAS mutant cells. An inhibitor that does not induce RAF dimerization or transactivation, or that inhibits both dimerization partners simultaneously, would preclude the paradoxical activation. Coadministration of BRAF and MEK inhibitors may prevent pathway activation.

What are the ramifications for using existing RAF inhibitors in the clinic? If decisions are based simply on the ability to impair MEK-ERK activation, then RAF inhibitors should be applied to BRAF, but not RAS mutant cancers. However, previous studies have found that MEK inhibitor blockade of ERK activation did not correlate with inhibition of either anchorage-dependent or -independent growth (Solit et al., 2006; Yeh et al., 2009) and, thus, phospho-ERK may not be the most predictive endpoint for antitumor efficacy. However, Heidorn et al. also explored a possible basis for the clinically relevant finding that, while kinase-activating BRAF mutations (e.g., V600E) and activating NRAS mutations (e.g., G12D) are found in a mutually exclusive manner in human cancers, kinase-inactive BRAF mutants are also found in human tumors and sometimes in association with mutant RAS. Using a transgenic mouse model of melanoma in which both mutant KRAS (G12D) and active RAF are required for malignancy, they demonstrated that catalytically inactivating mutations such as D594A, presumably mimicking the effects of BRAF kinase inhibitors, can allow BRAF to become more transforming in collaboration with mutant KRAS. These results provide another cautionary note for the use of BRAF inhibitors in cancers with mutant RAS.

Many questions remain. What is the real RAF isoform selectivity of "BRAF-selective" inhibitors? Are MEK inhibitors better? What about non-RAF effectors of

Ras? The relative lack of toxicity seen with RAF inhibitors argues that these remain very much worth pursuing and worth determining if there are conditions allowing treatment of tumors with mutant RAS. Additional, rigorous determinations should be done in appropriate genetically engineered mouse models of cancer to more accurately predict patient responses. Until more decisive information is obtained, patients enrolled in ongoing clinical trials should be selected on the basis of a confirmed BRAF mutation in their tumors. Also, the risk of skin lesions if BRAF inhibition acts as a tumor promoter when oncogenic RAS is present (Heidorn et al., 2010) indicates the importance of careful dermatological monitoring. Plasticity of the RAF pathway may contribute to BRAF inhibitor resistance. Finally, for patients treated with drugs targeted to these and other pathways, it will be crucial to collect additional data to better evaluate the complex interrelationships of these signaling events that together determine the success or failure of such novel and promising therapeutics.

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