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Tumor heterogeneity and strategies to overcome kinase inhibitor resistance in cancer: Lessons from melanoma

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Historically, cancer therapy has centered upon surgery to physically remove tumor, or modalities such as radiotherapy and chemotherapy that indiscriminately kill rapidly dividing cells. While often effective, these approaches lack specificity and are associated with severe off-target effects. Following the “oncogene revolution” of the 1980s there is now a realization that activating mutations in and overexpression of kinases can drive the initiation and progression of many types of cancer. These mutations are typically restricted to cancer cells thereby offering the possibility of therapeutically targeting the malignancy while sparing normal tissues. Most of the aberrant kinase activity reported so far occurs in receptor tyrosine kinases (RTKs), such as *c-KIT* (GIST), Bcr-ABL (chronic myeloid leukemia) and the epidermal growth factor (EGF) receptor (breast cancer, colorectal carcinoma, some sub-groups of lung cancer) as well as in serine/threonine kinases, such as *BRAF* (melanoma, colorectal carcinoma and thyroid carcinoma). All of these kinases are susceptible to targeting by small molecule inhibitors and there is now good evidence that kinase inhibitors constitute an important new class of anti-cancer therapies. The therapeutic targeting of kinases is not without its problems, and like conventional chemotherapy drugs, there is accumulating data suggesting that chronic kinase inhibitor treatment leads to the acquisition of resistance¹.

Melanoma is the deadliest form of skin cancer, for which no adequate treatments currently exist. Although a relative newcomer to the world of kinase inhibitor therapy, the melanoma field is already shifting the paradigm for targeted therapy in solid tumors. Scarcely 8 years have passed between the discovery of activating *BRAF* V600E mutations in >50% of melanomas and the recent phase I clinical trial of the *BRAF* kinase inhibitor PLX4032 (RG7204) in which >80% of patients whose melanomas harbored the V600E mutation showed significant tumor shrinkage². Unfortunately, the responses seen were only transient (PFS ~7 months) with most patients ultimately showing signs of resistance^{2,3}. In other cancers where kinase inhibitors have been more extensively evaluated (CML, GIST, NSCLC) resistance often occurs via the acquisition of secondary mutations at sites within the kinase's ATP binding site that prevent the binding of the drug to the hydrophobic pocket at so-called “gatekeeper” residues. This however does not seem to be the case in melanoma. Analysis of tumor samples from patients failing PLX4032 therapy using both deep and ultra-deep sequencing were unable to identify *de novo* mutations in *BRAF*, suggesting that the

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acquisition of a gatekeeper mutation is not the mechanism of resistance⁴. Further studies, where the BRAF kinase was immunoprecipitated from *in vitro* cultures of PLX4032 resistant melanoma specimens showed the BRAF to retain its sensitivity to PLX4032, again suggesting the absence of secondary *BRAF* mutations³. Instead, it appeared that resistance was mediated by signals arising upstream of mutated *BRAF* (Figure 1A). In support of this, recent data from two independent groups has shown BRAF inhibitor resistance to be mediated through increased RTK signaling (PDGFR β and IGF1R, respectively)^{4,5}. In the case of IGF1R, downstream MAPK signaling was reactivated following the re-routing of signaling from mutated *BRAF* to ARAF and CRAF⁵. In those melanomas with increased PDGFR β expression, the nature of the rebound signaling is currently unclear⁴. The observation that melanoma cells quickly compensate for the lack of a mutated *BRAF* signal is also supported by studies showing that MAPK signaling recovers very rapidly (often within 48 hrs) following treatment with BRAF inhibitors⁶⁻⁸. There is now a growing list of mechanisms by which melanoma cells can reactivate their MAPK signaling when BRAF is inhibited. Another recent study demonstrated that increased COT (MAP3K8) expression drives BRAF inhibitor resistance through the RAF-independent activation of ERK⁹. The clinical relevance of increased COT expression in the resistance phenotype was confirmed in a limited number of melanoma samples from patients failing BRAF and MEK inhibitor treatment^{7,9}. Taken together, these data suggest that the signaling network of *BRAF* V600E mutated melanoma cells is highly robust and favors a state in which MAPK signaling is maintained (Figure 1A). These findings provide the rationale for how BRAF inhibitor resistance may be managed, with a number of groups now suggesting that dual BRAF/MEK inhibition may prevent or delay the onset of resistance^{6,9,10}. This hypothesis is currently being evaluated clinically in a phase I/II clinical trial of the BRAF inhibitor GSK2118436 in combination with the MEK inhibitor GSK1120212 in *BRAF* V600E mutated melanoma patients who are treatment naïve (NCT01072175).

The idea of network robustness, where parallel signaling pathways rapidly compensate for those targeted by kinase inhibitors is well known in other tumor systems. This is best exemplified in the case of glioblastoma, where EGFR inhibition using erlotinib is associated with intrinsic resistance following a rapid switch from EGFR to c-MET signaling¹¹. Although the studies so far have implicated the potential role of upregulated IGF1R and PDGFR β signaling in BRAF inhibitor resistance in melanoma, there are likely to be other candidates, particularly as melanoma cells are known to express multiple RTKs.

Increased RTK signaling is not the only reported mechanism of BRAF inhibitor resistance in melanoma. There is also evidence that a minor group of patients failing PLX4032 therapy acquire activating *NRAS* mutations that were apparently lacking in the original tumor⁴ (Figure 1B). At this stage, it is unclear if these mutations arise *de novo* following PLX4032 treatment, or whether these patients harbored a pre-existing minor population of *NRAS* mutated cells within their otherwise *BRAF* V600E mutated tumor. Although *BRAF* and *NRAS* mutations are known to be mutually exclusive in individual melanoma cells, there is limited evidence demonstrating the presence of individual clones containing either *BRAF* V600E or *NRAS* mutations within the same tumor^{12,13}. If confirmed in larger patient samples, the presence of *NRAS* mutant clones in *BRAF* mutated tumors could have implications for melanoma treatment and may be particularly important given that BRAF inhibitors, such as PLX4032, actually stimulate the growth and invasion of *NRAS* mutated melanoma cell lines^{14,15}. In addition to *NRAS* mutations there is evidence from colon carcinoma that differences in BRAF gene copy number may also mediate resistance (this time to the allosteric MEK inhibitor AZD6244)¹⁰. Analysis of treatment naïve colon carcinoma cell lines and samples using fluorescence *in situ* hybridization (FISH) identified a minor population of cells harboring high-level amplifications in BRAF¹⁰. Chronic treatment of these cell lines with AZD6244 led to the acquisition of resistance and an expansion of the

highly BRAF amplified population¹⁰. A role for BRAF amplification in the acquired resistance phenotype was confirmed through shRNA studies and by overexpression of BRAF¹⁰.

The idea that kinase inhibitor resistance results from the inherent phenotypic and genetic heterogeneity within cancer cell populations is gaining traction. Another recent paper from the melanoma field identified a slow-cycling minor population of melanoma cells, expressing the H3K4 demethylase JARID1B (KDM5B/PLU-1/RBP2-H1), that appear important for the maintenance of tumor growth¹⁵. The potential role of the JARID1B+ sub-population of melanoma cells in drug resistance was suggested by their slow rate of growth that appeared to render them insensitive to therapy¹⁶ (Figure 1C). Other recent work has provided evidence that a drug tolerant state may emerge transiently and reversibly within individual cells of a tumor through epigenetic means¹⁷. Here the transiently drug resistant state seems to be dependent upon IGFR1 signaling and an altered chromatin state that required the histone demethylase RBP2/KDM5A/Jarid1A¹⁷. The identification of IGFR1 signaling and histone modification as being critical to the adoption of the drug-tolerant phenotype suggests that therapeutic escape may be amenable to pharmacological intervention and evidence was provided showing that inhibition of either IGFR1 or HDAC eradicated the drug resistant clones¹⁷. Taken together, all of this supports the paradigm shifting idea that kinase inhibitor resistance, rather than evolving slowly, occurs almost instantly, perhaps even as soon as the drug is first administered. If this idea holds up to further scrutiny, the possibility emerges of new strategies to manage drug resistance centered upon epigenetic regulation and phenotypic switching.

Expert opinion

Every kinase inhibitor evaluated in cancer so far has followed a course of initial response, relapse and resistance. Treatment failure and the acquisition of resistance are the major limiting factors that prevent kinase inhibitors from being effective long-term cancer treatments. There is a growing realization that tumors are made up heterogeneous populations of cancer cells that show considerable variations in their intracellular signaling, phenotypic behavior and possibly even their genetic make-up. It is likely that different therapeutic approaches will be needed to effectively target these sub-populations and determining the optimal drug combinations to achieve this is a key challenge for the future. Notwithstanding these caveats, it is already clear that small molecule BRAF kinase inhibitors represent a major therapeutic breakthrough for disseminated melanoma. The critical issue now facing the field is how to manage resistance and turn responses that last ~7 months to something more durable. Combination therapy seems to be the answer, and the search is already on for the key escape pathways that are activated when BRAF signaling is blocked. It is likely that the optimal drug combinations will be determined by the underlying genetics of the individual tumor and further study is required to match drug combinations to tumor genotypes¹⁸. Melanomas show a great deal of inter-tumor heterogeneity and it is perhaps unsurprising that all of the published studies to date have implicated different resistance mechanisms. Although RTK signaling seems to be important, this may not necessarily be the best combination therapy strategy. Melanomas express multiple RTKs and a situation can be envisaged where even combined targeting of BRAF and an RTK (e.g. IGFR or PDGFR) may prompt a switch to other RTKS. For the sake of simplicity, and ease of clinical management, the best strategy may instead be to focus upon signaling pathways that are common to all of the resistance phenotypes. This is likely to be therapeutically more realistic as there are only a finite number of growth and survival signaling pathways that a melanoma cell can utilize. There is already encouraging pre-clinical evidence that dual BRAF and MEK inhibition can delay the onset of resistance in *in vitro* models and enhance apoptosis in BRAF mutated melanoma cells^{6,9,10}. Additionally there seems to be an

important role for PI3K and AKT signaling in resistance and therapeutic escape, with preclinical data already supporting the combination of BRAF/MEK inhibitors with AKT/PI3K inhibition^{8,19}. Strategies to target minor sub-populations of slow proliferating melanoma cells may be more of a challenge, but they could potentially be eradicated by highly specific approaches, such as immunotherapy. Clinical studies, in which immunotherapies are combined with BRAF inhibitors are already at the advanced planning stage and are due to commence in 2011. There is now good reason to believe a corner has been turned in the therapy of disseminated melanoma. A future can be envisaged where highly personalized therapy approaches are tailored to the underlying genetics of each patient's melanoma allowing us to extend the effectiveness of small molecule BRAF inhibitors and improve the quality of life and survival of the patients.

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Figure 1. Schematic representation of possible BRAF inhibitor resistance mechanisms in melanoma

A). Pathway Switching: *BRAF* V600E melanoma cells chronically treated with BRAF inhibitors acquire drug resistance via switching between the three isoforms of RAF to activate the MAPK pathway. Establishment of IGFR1 and PDGFR signaling may also allow mutated *BRAF* to be bypassed. B) Pre-existing Clones: The presence of pre-existing mutations may affect the response of a tumor to BRAF kinase inhibition. *NRAS* mutated melanoma cells are not inhibited by BRAF inhibitors and may actually show increased growth, allowing the tumor to be repopulated. C) Intratumoral heterogeneity: Melanomas are highly heterogeneous tumors; a small subset of slow growing, JARID1b expressing melanoma cells have been observed with the ability to evade treatment, and may repopulate the heterogeneity of the tumor that existed prior to treatment. Targeted therapy toward the multiple phenotypes within a tumor may be required to adequately eliminate the whole tumor.