Targeting oncogene dependence and resistance

Our expanding experience with imatinib mesylate provides instructive lessons on the power and pitfalls of targeted therapy. The often impressive initial clinical responses seen with imatinib in a variety of malignancies inevitably give way to the emergence of resistant disease. Recent findings reveal several mechanisms of resistance and suggest ways to overcome them.

Targeted therapies promise to revolutionize the care of cancer patients. The most dramatic examples of the potential power of this approach come from successes with the small molecule imatinib mesylate, also known as Gleevec or STI571. Imatinib effectively inhibits the aberrant signaling properties and malignant consequences of oncogenic *BCR-ABL*, *KIT*, and *PDGFRA* in patients with chronic myelogenous leukemia (CML) (Druker et al., 1996, 2001b), gastrointestinal stromal tumor (GIST) (Demetri et al., 2002; Tuveson et al., 2001), and hypereosinophilic syndrome (HES) (Cools et al., 2003a), respectively. Treatment with imatinib as a single agent, however, also reveals an inherent weakness of monotherapy—the emergence of tumor resistance (Cools et al., 2003a; Demetri et al., 2002; Druker et al., 2001a). Almost invariably in each of these malignancies, disease resistance develops after differing degrees of initial response and latency. Recent articles by Cools et al. (2003b [this issue of *Cancer Cell*]), La Rosée et al. (2002), and Huron et al. (2003) describe preclinical studies of structurally distinct tyrosine kinase inhibitors against imatinib-resistant *PDGFRA* and *BCR-ABL* alleles, and suggest that tumor resistance to targeted therapeutics is not insurmountable. In the context of these studies, we briefly discuss potential mechanisms that dictate responsiveness and resistance to therapies directed against oncogenes and consider the challenges that lie ahead.

First, why does targeted therapy work? To date, none of the targeted therapeutics in use, or in development, is specific for a mutated state of a protein. Rather, they additionally inhibit wild-type functions of one or more cellular proteins. Thus, cancer cells appear to demonstrate a particular sensitivity to these agents. Indeed, central to the success of imatinib is the vulnerability of tumor cells to the disruption of dominant oncogenic pathways, a phenomenon aptly referred to as "oncogene dependence" or "oncogene addiction" (Weinstein, 2002). Oncogene dependence is hypothesized to result from the subversion and reorganization of normal cellular signaling networks by an introduced dominant oncogene; removal or modulation of the oncogene leads to alterations in the aberrant signaling network that are detrimental to cell proliferation and/or survival (Fan et al., 2002; Weinstein, 2002). The protooncogenic counterparts do not form aberrant signaling networks, which may partially explain the therapeutic index of targeted therapeutics.

Preclinical evidence supporting this model of oncogene dependence has been provided by both cell culture experiments and transgenic mouse tumor models. Druker and colleagues demonstrated in their landmark study that IL-3-dependent, imatinib-resistant M07 human megakaryocytic cells and 32D murine myeloid cells subsequently became IL-3 independent and imatinib sensitive following transfection with *BCR-ABL* (Druker et al., 1996). Interestingly, M07 and 32D cells that were transformed with *BCR-ABL* and cultured in the absence of IL-3 were still sensitive to the apoptotic effects of imatinib even after the media were supplemented with IL-3, suggesting a new and unexplained requirement of the transfected cells for the introduced oncogene. An elegant series of experiments by Weiss and colleagues extended these observations to immortalized NIH 3T3 fibroblasts transfected with *v-erbB* or *v-src*. Subsequent inhibition of these oncogenes with allele-specific chemical inhibitors did not merely revert the cells from a fully transformed to an immortalized state, but rather led to the arrest of cellular proliferation (Fan et al., 2002). Furthermore, this arrested phenotype was at least partially attributable to diminished Raf kinase signaling and cyclin D1 levels, known downstream mediators of *v-erbB2* and *v-src*. Additional insight has come from inducible transgenic mouse models of melanoma (Chin et al., 1999), leukemia (Felsher and Bishop, 1999; Huettner et al., 2000), breast cancer (Moody et al., 2002), and lung cancer (Fisher et al., 2001) have provided powerful systems to demonstrate the requirement of "tumor maintenance" on the initiating oncogenes *H-ras*^{G12V}, *c-myc*, *BCR-ABL erbB2*, and *K-ras*^{G12D}, respectively. In many of these systems, when expression of the relevant oncogene was subsequently turned off, the malignant phenotype regressed rapidly, accompanied by widespread apoptosis and growth arrest. Of particular relevance to targeted therapeutics in human malignancy, these initiating oncogenes were similarly required for tumor maintenance in mouse models that additionally harbored multiple tumor suppressor gene mutations (Chin et al., 1999; Fisher et al., 2001).

Clinically, oncogene dependence has been observed in both biochemical and genetic analyses in patients undergoing treatment with imatinib. A direct correlation has been demonstrated between disease responsiveness and the interruption of downstream Bcr-Abl biochemical pathways, such as Crkl phosphorylation (Gorre et al., 2001). Interestingly, investigations into the nature of relapsed and resistant disease in CML patients treated with imatinib led to the discovery of mutations in the *BCR-ABL* locus, substantiating the essential role of this oncogene in maintaining the diseased state (Gorre et al., 2001).

So, why does targeted therapy fail? Several mechanisms of resistance to imatinib in CML patients have been described (Gorre et al., 2001; Hochhaus et al., 2002; Shah et al., 2002). The majority of CML patients with resistance to imatinib have a clonal expansion of leukemic cells harboring novel mutant *BCR-ABL* alleles. These mutations confer reduced sensitivity to inhibition by imatinib and segregate either as direct contact site mutants within the kinase domain or as allosteric modifiers of the preferred conformational state of Bcr-Abl for imatinib binding. *BCR-ABL* gene amplification provides yet another mechanism of resistance seen in patients, and results in sufficient unbound, active kinase to maintain the neoplastic state. Although clinical resistance to imatinib

Table 1. Mechanisms of resistance to Imatinib

Type	Oncogene dependence	2° Oncogene dependence	Locus	In vivo	Alternative therapy
Target					
Mutations	+	-	Bcr-Abl; PDGFRA	+	PKC412, PD166326, PD180970
Amplification	+	-	Bcr-Abl	+	Geldanamycin
Pharmacokinetic					
Extracellular	+	-	α-1 glycoprotein	-	ND
Cellular	+	-	P-glycoprotein	-	ND
Biological					
Alternate pathway	-	+	ND	-	ND
Innate	±	-	CML stem cell	+	ND

In vivo, observed in vivo or in patient-derived material; ND, not determined.

usually develops after an initial response, mutation and amplification of the *BCR-ABL* locus can be detected in the leukemic cells of some patients even prior to the initiation of therapy (Shah et al., 2002). The preponderance of mutations at the *BCR-ABL* locus in patients with CML is currently unexplained, and postulated mechanisms include an increased mutational rate in progenitor cells and neomorphic properties of mutant Bcr-Abl proteins (Shah et al., 2002). Mutations or amplification of the dependent oncogene that directly impede the bimolecular interaction between a critical fraction of the oncoprotein and the targeted therapeutic at clinically relevant drug concentrations may be best characterized as forms of "target resistance."

Interestingly, in some imatinib-resistant CML patients, neither mutation nor amplification of the *BCR-ABL* locus can be detected, indicating that other forms of resistance must also occur in vivo (Gorre et al., 2001; Shah et al., 2002). Indeed, preclinical data have suggested the presence of at least two additional mechanisms for the lack of response to targeted therapeutics. First, "pharmacokinetic resistance" to targeted therapeutics might occur by a variety of mechanisms that decrease drug delivery to or increase drug efflux by the cancer cells. For example, α-1 acidic glycoprotein has been shown to bind imatinib and reduce serum drug levels in a murine CML xenograft model (Gambacorti-Passerini et al., 2000). Although there have been as yet no reports of such resistance in patients treated with imatinib, careful pharmacokinetic studies in serum and target tissue are required in patients who do not respond to imatinib and who do not have target resistance. Finally, in patients with "biological resistance," the cancer cells are no longer dependent on the targeted oncogene. Biological resistance may occur due to the presence of additional dominant oncogenes in the tumor cells that are not inhibited by the targeted therapeutic (secondary oncogene dependence), or may be an innate property of some cancer stem cells that make them impervious to disruption of targeted oncogenic pathways. Evidence for the latter possibility has been found in the form of pluripotent, quiescent CML cells that have been isolated from patients and are capable of sustained survival during incubation with imatinib in vitro (Graham et al., 2002).

Given the ever increasing number of patients with a variety of malignancies that are demonstrating resistance to imatinib following initial response, the articles by Cools et al. (2003b), La Rosée et al. (2002), and Huron et al. (2003) are especially timely. La Rosée et al. showed that a novel pyrido[2,3-d]pyrimidine derivative (PD180970), originally developed as a Src kinase inhibitor, had potent activity in cell culture against a number of *BCR-ABL* mutants commonly found in imatinib-resistant patients. A structurally related compound (PD166326) that effectively inhibits signaling downstream of another imatinib-resistant allele, *BCR-ABL*^{E255K}, was recently described by Huron et al. (2003). As the backdrop to their work described in this issue, Gilliland and colleagues recently demonstrated that the development of hypereosinophilic syndrome (HES), and its sensitivity to imatinib, appear to result from an intrachromosomal deletion that creates a novel fusion tyrosine kinase, *FIP1L1-PDGFRA*, in a majority of patients (Cools et al., 2003a). Moreover, an HES patient that developed target resistance to imatinib was found to harbor a mutant *FIP1L1-PDGFRA*^{T674I} allele, and structural considerations predicted that this mutation would interfere with imatinib binding to the PDGFRA kinase domain. In the current issue, Cools et al. now show that PKC412, a staurosporine derivative with widespread activity against tyrosine kinases, can effectively inhibit signaling by the PDGFRA^{T674I} kinase in cell culture and prevent *PDGFRA*^{T674I}-induced myeloproliferative disease in a mouse transplant model. This represents the first in vivo demonstration of overcoming target resistance with a second targeted therapeutic. Collectively, these results are significant because they show that, in principle, target resistance can be overcome with alternative, structurally distinct agents directed against the same enzymatic activity in an oncoprotein. Importantly, each of these alternative inhibitors is effective for specific sets of mutant alleles. For example, PKC412 does not inhibit the *PDGFRA*^{N659D} allele, and neither PD166326 nor PD180970 inhibits one of the most common imatinib-resistant alleles in CML, *BCR-ABL*^{T315I}. Indeed, the non-crossresistant nature of these agents is thought to reside in their predilection for binding different conformational states of the kinase domain. Thus, these studies also underscore the importance of understanding not only the mechanisms of emerging disease resistance, but also the detailed mechanisms of drug sensitivity. Ultimately, the clinical utility of such agents will depend not only on their spectra of activities against common mutant alleles, but also on their respective bioavailability and toxicity profiles. As PKC412 is currently undergoing evaluation in phase 1/2 trials, the results of Cools et al. should prompt the rapid approval of clinical pro-

tocols to evaluate efficacy in patients with imatinib-resistant HES, and perhaps other malignancies, who could potentially benefit.

Clinical experience with imatinib has provided invaluable insight into the processes necessary to sustain malignancy in vivo, and prompts consideration of the best way forward. Initially, wherever possible, candidate oncogene targets must be identified for each malignancy by establishing if oncogene dependency and the accompanying aberrant signaling network are operant in that cancer. Ironically, the malignancies we know the least about in this regard are also the most common causes of adult death from cancer, including lung, colorectal, breast, and prostate, although oncogenic alleles of *K-ras* and *BRAF* are potential candidates in some, and overexpressed *erbB2* has been targeted with the Herceptin monoclonal antibody in breast cancer (Vogel et al., 2002). Small molecule inhibitors, RNA interference (Brummelkamp et al., 2002), and proteomic techniques are amongst the methodologies that can be used to identify and characterize potential dominant oncogenes.

Resistance to molecular therapeutics that target oncogenes will likely occur by one or more mechanisms in most, if not all, patients (Table 1). By extension of the work discussed here (Cools et al., 2003a; Huron et al., 2003; La Rosée et al., 2002), target resistance will be common in advanced malignancies and several novel agents will be required to combat each oncoprotein. For example, some CML patients who relapsed after response to imatinib had at least four unique mutant *BCR-ABL* alleles identified in their leukemic cells, emphasizing the polyclonal potential of target resistance (Shah et al., 2002). Combination therapy with multiple targeted agents may lead to faster responses and more durable remissions. Additional methods to attack the primary target have been proposed, such as Geldanamycin, which diminishes Bcr-Abl protein levels by inhibiting the function of the molecular chaperone and heat shock protein, Hsp90 (Gorre et al., 2002). Importantly, Geldanamycin is equally effective against native and mutant Bcr-Abl protein and may be particularly useful in combination with kinase inhibitors in cases of *BCR-ABL* amplification. Pharmacokinetic resistance to imatinib has not been demonstrated thus far in patients, but may become more problematic due to drug interactions as combinations of targeted therapeutics are assessed in patients. The ability to assess drug levels and biochemical pathways in small tumor samples is critical to properly evaluate novel therapeutics and will require sensitive proteomic, genomic, and pharmacological approaches. Biological resistance that is due to secondary oncogene dependence may be directly treated with the appropriate targeted agents. However, biological resistance due to the innate features of primitive progenitor cancer cells, or "cancer stem cells," (Reya et al., 2001) may pose the most difficult challenge of all. Cancer stem cells may not be as responsive as progeny cells to the inhibition of dominant oncogenic pathways, and a deeper understanding of the properties of these cells is therefore critical. For example, the inability of imatinib to kill multipotential CML precursor cells in vitro (Graham et al., 2002) suggests that monotherapy will only be effective as a continuous therapy and, then, only until resistant disease appears. Indeed, curative intent by targeted agents may not be realized until the advent of specific strategies to combat the cancer stem cell.

The unfolding story of imatinib is reminiscent of experience over the past two decades with the development of effective anti-retroviral therapies for the human immunodeficiency virus. Resistance to reverse transcriptase monotherapy was addressed first with alternative individual drugs and then with combinations of agents that target two or more pathways. Reasonable combination therapeutic approaches would target additional pathways in cancer cells (Hoover et al., 2002; Nakajima et al., 2003; Yu et al., 2002), as well as non-cell-autonomous characteristics of the tumor microenvironment. The role of conventional chemotherapy and radiation therapy in combination with targeted therapeutics is being evaluated; however, in principle, these highly mutagenic modalities may inadvertently spawn increased resistance to targeted agents. Future success will depend upon the availability of multiple versions of targeted agents that modulate the function of dominant oncogenes and other important cancer genes, rapid methods to determine disease responsiveness and resistance, and willingness within the pharmaceutical industry to conduct collaborative clinical trials using combinations of novel targeted therapies. If these goals are pursued cooperatively, this new era of molecular oncology may indeed fulfill its long-awaited promise.

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FANCF methylation contributes to chemoselectivity in ovarian cancer

A new model of ovarian cancer tumor progression implicates aberrant *FANCF* promoter methylation that is associated with gene silencing and disruption of the Fanconi-anemia-BRCA pathway. Disruption of the pathway occurs de novo in ovarian cancers and may contribute to selective sensitivity to platinum salts.

Ovarian cancer cells accumulate genetic changes that allow them to evade chemotherapeutic drugs and become increasingly dangerous. In view of the high mortality rates associated with ovarian cancer, a better understanding of the molecular mechanisms underlying tumor progression in the disease could reveal novel pathways of high clinical relevance. A key feature of ovarian cancer is its sensitivity to platinum salts such as Cisplatin (CDDP) and Carboplatin, two drugs that have been the mainstay of therapy for decades. Unfortunately, ovarian cancer cells, with their unstable genomes, are initially sensitive to this class of drugs, but the cells invariably become resistant.

In a recent study, Taniguchi et al. (2003) describe a model for ovarian tumor progression in which the initial methylation of *FANCF*, a gene associated with Fanconi anemia, is followed by *FANCF* demethylation and CDDP resistance. *FANCF* is one of seven recently cloned Fanconi anemia genes whose protein products were found to interact with proteins involved in DNA repair pathways, including BRCA1, RAD51, ATM, and NBS1 (D'Andrea and Grompe, 2003). Five of the *FANC* gene products (FANCA, FANCC, FANCE, FANCF, and FANCG) are subunits of a nuclear complex (FA complex) that is required for the monoubiquitination of the downstream FANCD2 protein (Figure 1A). The seventh gene, *FANCD1*, was recently shown to be identical to *BRCA2* (Howlett et al., 2002). Defects in the Fanconi-anemia-BRCA (FA-BRCA) pathway are associated with genomic instability and increased sensitivity to DNA-damaging agents such as ionizing radiation (IR), mitomycin C (MMC), and CDDP. In response to ionizing radiation-mediated double-strand breaks, ATM phosphorylates the NBS1 protein. Phosphorylation of NBS1 is required for FANCD2 phosphorylation at serine 222, leading to activation of an S phase checkpoint. In response to DNA damage, the FA complex mediates ubiquitination of FANCD2 at lysine 561. Activated FANCD2 is translocated to chromatin and DNA repair foci, which contain the BRCA1 protein and BRCA2/FANCD1 protein complex. BRCA2/FANCD1 binds to RAD51 and to DNA, promoting a DNA repair response. The ubiquinated FANCD2 also colocalizes with NBS-MRE11-RAD50 complex in DNA damage nuclear foci. Germline mutation of several genes in the pathway result in impaired response to DNA damage and increased cancer susceptibility.

FANCD2 exists as two isoforms in normal cells, nonubiquitinated FANCD2-S and monoubiquitinated FANCD2-L. Inducible expression of monoubiquitinated FANCD2 in response to DNA damage requires an intact FA-BRCA pathway. Taniguchi et al.(2003) screened 25 ovarian cancer cell lines with varying sensitivities to CDDP and found two cell lines, 2008 and TOV-21G, without the FANCD2-L isoform. Compared to other ovarian cancer cell lines, both 2008 and TOV-21G are hypersensitive to CDDP with half maximal inhibitory concentration (IC50) less than 1.0 μm of CDDP. TOV-21G cells were retrovirally transduced with various *FANC* cDNAs (*FANCA*, *FANCC*, *FANCE*, *FANCF*, *FANCG*) in an attempt to correct any