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Review

Molecular Cancer Therapeutics

Durability of Kinase-Directed Therapies—A Network Perspective on Response and Resistance

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Abstract

Protein kinase–directed cancer therapies yield impressive initial clinical responses, but the benefits are typically transient. Enhancing the durability of clinical response is dependent upon patient selection, using drugs with more effective pharmacology, anticipating mechanisms of drug resistance, and applying concerted drug combinations. Achieving these tenets requires an understanding of the targeted kinase's role in signaling networks, how the network responds to drug perturbation, and patient-to-patient network variations. Protein kinases create sophisticated, malleable signaling networks with fidelity coded into the processes that regulate their presence and function. Robust and reliable signaling is facilitated through network processes (e.g., feedback regulation, and compensatory signaling). The routine use of kinase-directed therapies and advancements in both genomic analysis and tumor cell biology are illuminating the complexity of tumor network biology and its capacity to respond to perturbations. Drug efficacy is attenuated by alterations of the drug target (e.g., steric interference, compensatory activity, and conformational changes), compensatory signaling (bypass mechanisms and phenotype switching), and engagement of other oncogenic capabilities (polygenic disease). Factors influencing anticancer drug response and resistance are examined to define the behavior of kinases in network signaling, mechanisms of drug resistance, drug combinations necessary for durable clinical responses, and strategies to identify mechanisms of drug resistance. *Mol Cancer Ther; 14(9); 1975–84. ©2015 AACR.*

Introduction

Kinase-targeted therapies block signaling processes critical to tumor cell biology and have become routine components of clinical practice for many types of cancer. To date, most of these drugs achieve only moderate survival benefits due to either poor initial clinical response (innate/endogenous drug resistance) or disease relapse (acquired/secondary resistance; Fig. 1). Central to these phenomena are the drug-specific perturbations of tumor cell signaling networks and their responses to drug treatment. Because the knowledge of protein kinase molecular biology, tumor cell signaling, and tumor biology is rapidly expanding, incorporating a molecular perspective of network biology into drug discovery processes and clinical practice should facilitate the design of therapies with more durable clinical responses.

Understanding cellular signaling begins with the appreciation of its components, organization, functions. Tumor cells function in the context of complex tissues by translating extracellular cues into internal responses and sophisticated communication between intracellular processes. This requires creating order and tolerance to stochastic fluctuation in these highly concentrated protein reservoirs (~200 mg/mL; ref. 1). Inherent in the underlying processes are elements that define the duration of drug response as well as the mechanisms of resistance. One enzyme class that is essential to cellular regulation and disease is the protein kinase family (538 members) that comprises approximately 2% of the human genome (2). Protein kinases catalyze simple chemical reactions that transfer the γ-phosphate of ATP to the hydroxyl group of an amino acid residue to create phosphoproteins (2). These posttranslational modifications change the function of the substrate protein in many ways (e.g., enzymatic activity, subcellular location, stability, and protein–protein interactions). Because the specificity needed to orchestrate coherent signaling is not derived from the reaction chemistry, it is coded into the mechanisms that regulate the kinase's presence (i.e., expression, degradation, trafficking, activation, substrate recognition), function (catalytic, noncatalytic), and the availability of substrate proteins. The challenge to understanding kinase functions on a molecular level has been the complexity of the system. Recent technical and scientific advancements are enabling a more complete comprehension of the roles protein kinases serve in physiology, cancer biology, and drug performance.

Protein kinases are regulated through modulation of the dynamic catalytic kinase domain structure. The catalytic domain ("kinase domain") has a smaller N-terminal subdomain and a larger C-terminal subdomain that are linked by a peptidic strand (hinge) to form an active site cleft with a front pocket containing the catalytic residues and a regulatory back pocket (Fig. 2; ref. 2). Access to the back pocket is controlled by two residues—a conserved lysine residue and a "gatekeeper" residue. The catalytic domain can access a range of conformations. In the active conformation (closed), the N-terminal subdomain's αC-helix and the C-terminal subdomain's activation loop DFG tri-amino acid motif rotates inward toward the active site to orient active site residues for catalysis (DFG_{in} conformation). For many kinases, the active conformation is achieved through phosphorylation of the activation loop

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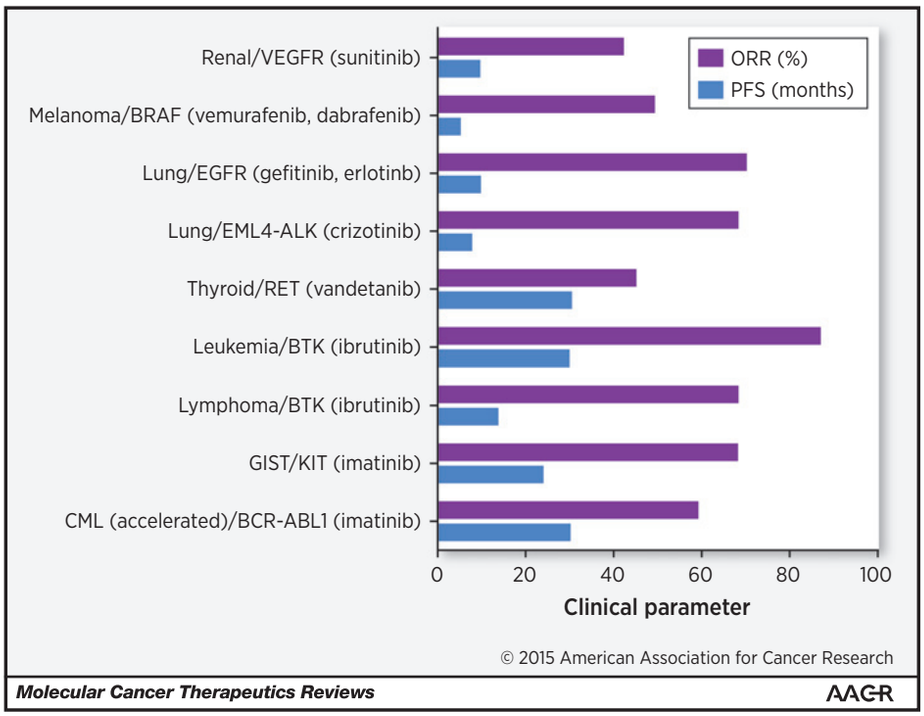

Figure 1.
Evaluation of the durability of response to approved kinase-targeted therapies for patients with advanced disease. Overall response rates (ORR, purple bars) to kinase-directed therapies is plotted relative to the durability of response as measured by PFS (blue bars)—sunitinib, VEGFR/renal cell carcinoma (99); vemurafenib, BRAF-V600E/melanoma (46); erlotinib and gefitinib, oncogenic EGFR/NSCLC (100, 101); crizotinib, EML4-ALK/NSCLC (102); vandetanib, RET/medullary thyroid cancer (103); ibrutinib, BTK/chronic lymphocytic leukemia (104); ibrutinib, BTK/mantle cell lymphoma (105); imatinib, KIT/gastrointestinal stromal tumors (106); imatinib, BCR-ABL1/blast crisis, chronic myeloid leukemia (90).

whereas others (e.g., EGFR) invoke an allosteric, intermolecular mechanism (3). Catalytic domain structural dynamics is also regulated by hydrophobic spines—internal amino acid residues that transverse the two subdomains (4). In addition, kinases have noncatalytic domains that facilitate substrate docking, subcellular trafficking, and recruitment of other signaling proteins (5–7). Taken together, sophisticated mechanisms have evolved to regulate protein kinases that can be coopted by tumor cells to evade kinase-directed therapies.

Multidimensional arrays of protein kinases create signaling networks with modular subunits and hierarchical structures that squelch unnecessary signaling (robustness), yet can respond to environmental changes (evolvability; ref. 8). Ingrained in these networks are essential behaviors that enable complex properties such as ultrasensitivity (switch-like behavior), bistability (two stable states which store one bit of information), and hysteresis (dependence on current and previous inputs). Many processes work in concert to create these network properties (e.g., feedback regulation, multistep activation, and trafficking). With such an intricate signaling fabric, the role of a kinase in a given network will define the effects from drug modulation. Protein kinases mutated in oncogenesis (e.g., BRAF-V600E, BCR-ABL1, BCR-ABL-T315I, and EGFR-L858R) have higher evolvability scores because they are central to the dysregulation that causes a phenotypic change in cell behavior. Nononcogenic driver kinases (e.g., stromal VEGFR2) are also important for cancer progression (9) and typically score higher in robustness because redundancy is built into the signaling network. Patient-specific network biology is also important because therapies that target a specific oncogenic kinase (e.g., BRAF-V600E) have a spectrum of clinical responses from robust, prolong responders to nonresponders (innate/endogenous resistance; refs. 10–12). As such, the network context of the targeted protein kinase is critical to identifying patients "wired" to respond as well as mechanisms that can be engaged to evade therapy.

Intrinsic Mechanisms of Drug Response and Resistance

Clinical response and resistance to kinase-directed therapies depend on properties of the targeted kinase (intrinsic factors). Altered protein kinases (e.g., mutated, amplified) are known to be critical to tumor cell signaling through their modified structures, activities, and molecular associations (Fig. 3). The strong dependence on these kinases for tumor cell survival is thought to be from either required oncogenic signaling ("oncogene addiction"; refs. 13, 14) or as counter balances to proapoptotic pressure ("oncogenic shock"; ref. 15). In addition, the properties of an altered kinase can cause "highly optimized tolerance"—acquired tolerance to conventional perturbations (e.g., hypoxia) but fragility to other perturbations (16). For example, the fusion of BCR to ABL1 that fundamentally changes the capabilities of a chronic myelogenous leukemia (CML) cell. When BCR-ABL1 function is lost for a short time (20–60 minutes), the tumor cell commits to apoptosis (17, 18). The presence of an altered kinase in a tumor can, but not always, enables an innate response toward the associated kinase-directed drug. For example, single-agent targeted therapies to BRAF-V600E in melanoma have high response rates and significant clinical benefit (19). In contrast, BRAF-V600E mutations also occur in colorectal cancer but BRAF-targeted therapies are not clinically effective (discussed later in this review in the context of extrinsic resistance; refs. 20, 21). Identification of sensitizing on-target mutations can stratify patient populations and accelerate clinical trials. Initial clinical studies of EGFR drugs toward unselected non–small cell lung cancer (NSCLC) patient

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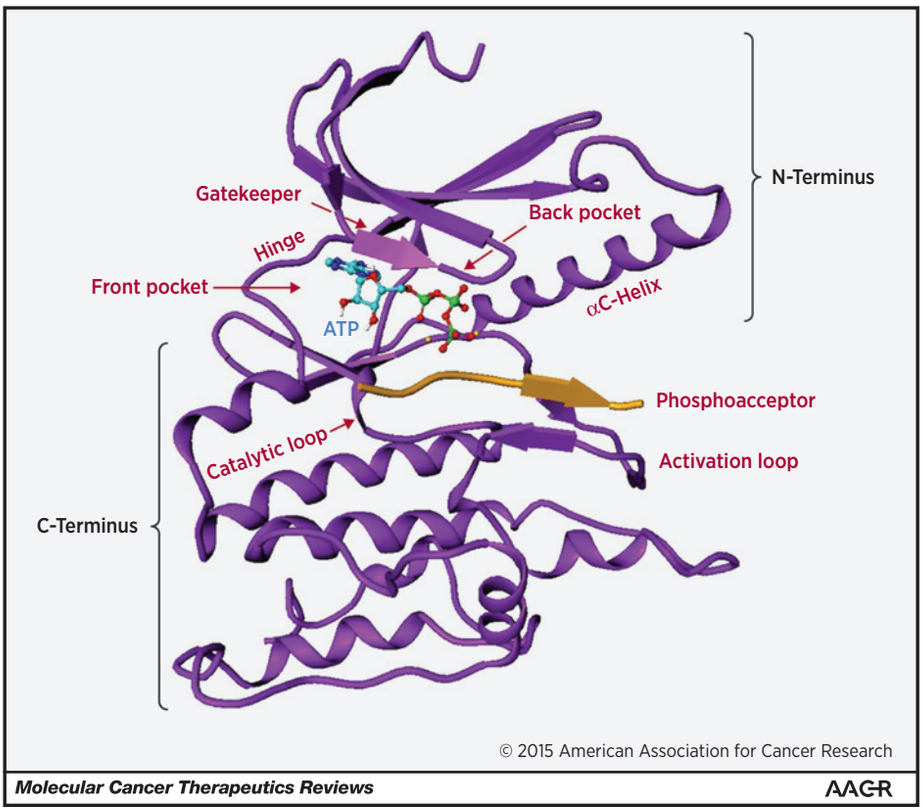

Figure 2.
Structure and key features of a protein kinase catalytic domain (Protein Data Bank ID code 2PHK). In purple is the backbone structure of a kinase catalytic domain with the phosphoacceptor substrate colored yellow. ATP is bound in the active site cleft between the N- and C-terminal subdomains. Critical structural and regulatory elements are labeled in red.

populations did not reveal statistically significant efficacy. However, retrospective analysis revealed a subset of patients responded to treatment—those with EGFR activating mutations (L858R, exon 19 deletions; ref. 22). These studies illustrate the utility of identifying factors that define intrinsic response to enable selection of effective therapies and design of efficient clinical trials.

Durability of response to kinase-directed therapies can be diminished by intrinsic (on-target) resistance—an altered form of the intended drug target that attenuates clinical performance (Fig. 3). Intrinsic resistance was initially observed with the first approved kinase-targeted drugs (e.g., imatinib/BCR-ABL1, erlotinib/EGFR; ref. 2). These resistance mutations can be distributed throughout the protein kinase (e.g., BCR-ABL1, refs. 23, 24); EML4-ALK, ref. 25) or highly localized (e.g., EGFR T790M; ref. 26) Many mechanisms have been proposed to explain how they cause drug resistance—steric hindrance to inhibitor binding (27), altered active site topography (28), disruption of favorable inhibitor interactions (29), altered protein dynamics (30), and increased oncogenicity (28), alteration of the ATP affinity (31). One of the simplest types of intrinsic resistance is steric hindrance—when the van der Wall radii of the drug and a kinase residue overlap to block access to the binding site. A recent example is an NSCLC patient with an oncogenic ROS fusion protein who responded to crizotinib but became resistant through a G2032R-mutant variant that directly interferes with crizotinib binding (32). Mutations distal from the ATP-binding site cause drug resistance by different mechanisms. A subset of these mutations indirectly blocks drug binding by changing the active site topography or conformational dynamics. Imatinib resistance was thought to result from a steric clash with the bulkier I315 mutated gatekeeper residue and the loss of a hydrogen bond to the T315 hydroxyl group (33). Subsequent molecular modeling and structural studies reveal that T315I causes a specific conformation that unlocks the DFG motif from an auto-inhibited orientation to facilitate the inactive-to-active states transition (30, 34). Another form of intrinsic resistance is from distal mutations that increase catalytic activity and provide compensatory activity, thus abrogating enzymatic activity lost to drug binding (i.e., 5-fold more enzymatic activity would compensate for 80% active site occupancy by a drug). Intrinsic resistance is not exclusively attributable to point mutations—alternative BRAF-V600E splice variants (35) and gene amplification (36) can confer drug resistance (Fig. 4). Gatekeeper mutations are reported mediate resistance by lowering the $K_{m,ATP}$ (higher active site occupancy of ATP; ref. 31). This may not be a dominant contribution to drug resistance because drugs can bind with much higher affinity than ATP ($K_i < 100$ pmol/L; refs. 37, 38), have much slower off-rates relative to ATP, and outcompete ATP for the common binding site or even achieve functional irreversibility (inhibitor residence time exceeds the kinase lifetime; ref. 2). Taken together, there are varied modes of intrinsic resistance to kinase-directed therapies, which affect clinical performance.

Extrinsic Mechanisms of Drug Response and Resistance

Response and resistance to kinase-directed therapies can depend on network structure and dynamics beyond the targeted kinase (extrinsic factors)—compensatory signaling (rewiring of networks) and polygenic tumor biology (alternative, compound

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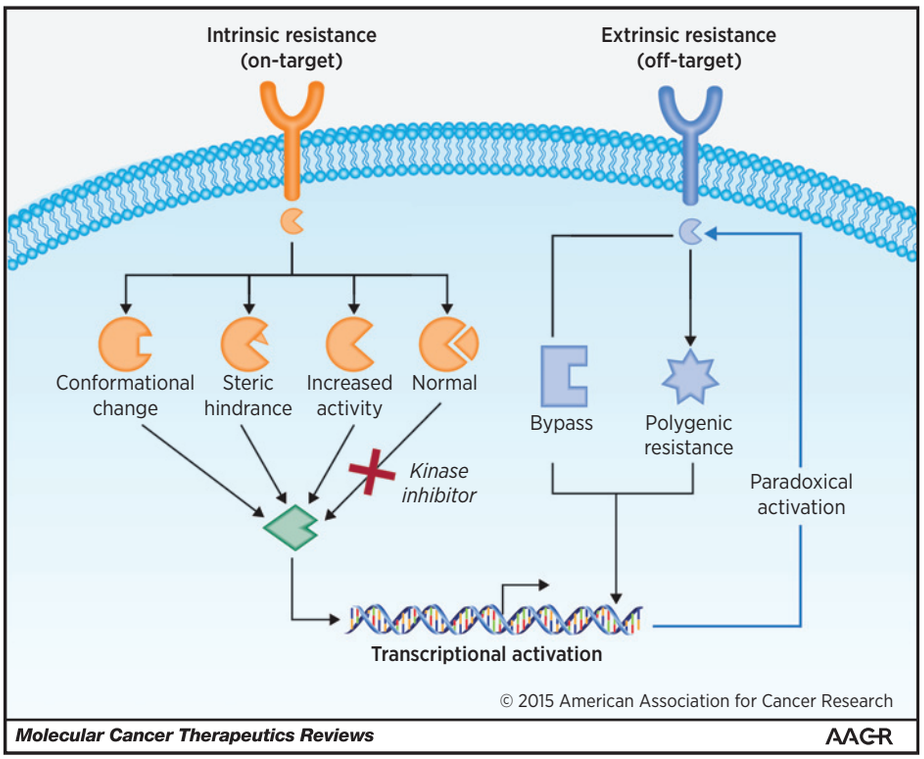

Figure 3.
Resistance mechanisms to kinase-directed therapies. Intrinsic, on-target resistance (pictured on left, orange) occurs when the drug target is mutated (red). Types of intrinsic resistance include steric hindrance, conformational change, and increased activity. These mutations result in an altered target protein kinase (green), which is no longer inhibited by the therapeutic and can resume its oncogenic signaling function to regulate transcriptional events. Extrinsic, off-target resistance (shown on right, blue) includes contributions to network signaling that decrease the durability of response—(i) bypass/compensatory signaling, (ii) additional mutations (polygenic tumor biology) within the network can restore oncogenic function, or (iii) paradoxical activation through inhibition of negative regulators of oncogenesis.

mutations; Fig. 3). Tumor cell biology underlies the historic approach of matching patients with therapies—classification of tumors by their organ of origin to group patients with similar underlying cancer biology. But the signaling networks are dynamic and vary at the patient level that leads to differential dependencies on a particular kinase, and therefore a spectrum of clinical responses. Tumor signaling networks can have multiple oncogenic mutations with a distribution of common and rare mutations that varies by tumor type (polygenic tumor biology), which can present as either innate or acquired resistance. The array of mutations in a patient population can be highly variable. In breast cancer, there are 40 loci of mutation, which occur at statistically significant rates (39). Only eight of the 40 driver mutations are observed in more than 10% of cancers. This distribution has been termed a "long tail" distribution and is observed in other types of cancer (19, 40). The complexity of tumor biology brought on by a distribution of somatic mutations is further complicated by the inherent genetic diversity of the patient population with germline genetic differences (40). In addition, epigenetic regulation adds to the complexity of kinase signaling networks. These variations result in a diversity of signaling networks in a given tumor type based on the assembly of mutations and the protein expression patterns. As such, diverse tumor cell contexts found in the clinic result in a spectrum of responses to kinase-targeted drugs. In addition, during treatment (acquired resistance) tumors can undergo clonal evolution to develop highly fit subclones with new driver mutations that cause relapse (41). These findings illustrate that a tumor cell's organ of origin imparts a degree of biologic order, but there are many patient-specific factors that make tumors biologically unique.

Extrinsic factors (those beyond the targeted kinase) make a tumor responsive to a kinase-directed therapies. These alterations can affect the signaling network architecture and render it vulnerable to kinase inhibition. For example, a metastatic bladder cancer patient who achieved remission in a failed mTOR-targeted drug clinical trial (everolimus) was shown to have a loss of function mutation in an mTOR pathway regulatory protein (tuberous sclerosis 1) responsible for the innate drug response (42). For this reason, the National Cancer Institute has initiated an "Exceptional Responders Initiative" to identify the molecular basis of strong clinical response (CR or PR for more than 6 months in <10% of the patients) even though a clinical trial did not meet its endpoint (trial failure; ref. 43). In addition, cancer genomic analyses are being used routinely to prospectively define the tumor biology beyond the targeted kinase to identify patients sensitive to a given therapy (discussed later in this review).

Compensatory signaling in response to drug treatment (i.e., extrinsic drug resistance) should be expected because kinase networks have evolved to contain built—in redundancies and cross-talk (e.g., signaling bypass, negative feedback loops, cross-inhibition, cross-activation, and pathway convergence; Figs. 3 and 4). An example of signaling bypass is BRAF-V600E melanoma treatment in which the effective drug blockade of BRAF-V600E is abrogated by the engagement of the downstream kinase MEK (acquired resistance; ref. 44). This bypass requires the addition of a MEK inhibitor to block the signaling pathway essential to the melanoma cell (45, 46), but the BRAF–MEK drug combination is also subject to subsequent bypass (35). Tumor signaling networks can extend to the surrounding stroma to evade blockade of a dominant oncogenic kinase (47). With BRAF-V600E–targeted drug treatment of melanoma, tumor cells can stimulate the

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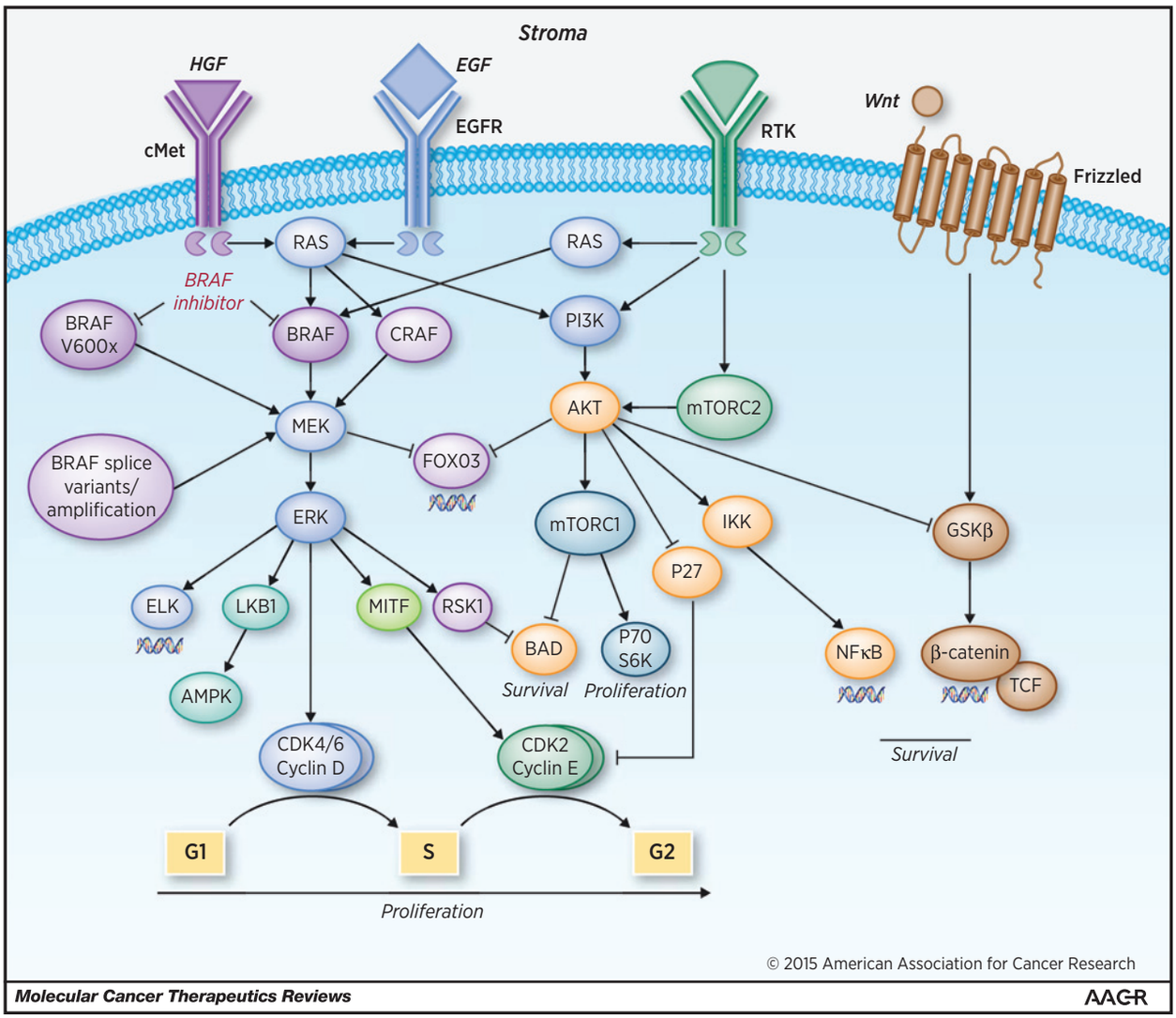

Figure 4.
Simplified BRAF signaling network. Growth factor receptors and RTKs on tumor cells signal through RAS to BRAF to activate the MEK–ERK signaling pathway resulting in oncogenic transcriptional events and loss of cell-cycle control. Intrinsic resistance to BRAF inhibitors occurs by splice variants or amplification of BRAF-V600x mutations (x = E, K). Extrinsic resistance to BRAF drugs can occur by engagement of other receptor tyrosine kinases (EGFR and RTK) to re-engage MEK and PI3K signaling downstream of the BRAF blockade (bypass mechanism).

stromal production of HGF ligand to activate cMet on tumor cells thus abrogating the blockade by re-engaging PI3K and MAPK signaling (Fig. 4; ref. 48). As mentioned above, targeting the BRAF-V600E mutation in colorectal cancer is ineffective as a single agent because of bypass signaling through EGFR (innate resistance; refs. 20, 21). Effective therapies are emerging that combine BRAF-, MEK-, and EGFR-directed drugs. A related resistance mechanism is through feedback loops, which normally regulate signaling processes to enable network robustness, but can be exploited by tumor cells to regain essential signaling (49). Kinase inhibition itself can derepress compensatory feedback mechanisms, which restores critical tumor cell signaling (50). Another level of complexity is derived from differential transcriptional control, which can cause drug insensitivity by changing both signaling networks and the role of an oncogenic protein kinase in the tumor cell (phenotype-switch drug resistance; refs. 51, 52). In addition, cancer is not a static disease. Tumor cell signaling networks and the microenvironment change during disease progression, which influences a drug's effectiveness. An early reliance of a tumor on angiogenesis is supplanted by new capabilities to invoke vasculogenesis or co-opt existing vasculature, circumventing the benefit derived from VEGFR blockade by targeted kinase therapies (53). These findings underscore the importance of understanding the dynamic nature of signaling networks.

Methods of Detecting Mechanisms of Response and Resistance

Predicting molecular mechanisms of innate sensitivity and acquired drug resistance are important components to

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developing durable therapeutic regimens. Preclinical analysis of tumor cell drug response can identify potential resistance mechanisms in months rather than in years of clinic studies to proactively identify appropriate patient populations and effective drug combinations. Hypotheses for factors that correlate with innate tumor cell sensitivity or resistance can be generated by screening panels of tumor cell lines *in vitro* using an appropriate assay endpoint (e.g., proliferation for cytotoxic therapies; refs. 54, 55). These cell lines are well characterized (e.g., gene transcription expression, DNA copy number, DNA methylation, single nucleotide polymorphisms, and mutations). Genomic analysis of resistant and sensitive cell lines can produce candidate markers for innate sensitivity or resistance that can be confirmed chemogenomically through cellular studies (54, 56–58). Novel methods for gene inactivation *in vitro* (e.g., CrispR to make isogenic cell lines) have significantly accelerated the biomarker validation process (55, 59). Findings from the *in vitro* analysis are integrated with patient genomic data (e.g., The Cancer Genome Atlas) to refine a clinical hypothesis. Early efforts to identify sensitive patient populations and effective drug combinations were pioneered with the National Cancer Institute's NCI60 panel of immortalized tumor cell lines from nine types of cancer (54). Sensitivity of BRAF-V600E–driven melanoma to MEK inhibitors was discovered by this approach (44). A limitation of the NCI60 is the small number of cell lines for each tumor type. More recently, broader tumor cell line collections and services have emerged, which more thoroughly characterize drug response in different tumor types [e.g., Cancer Cell Line Encyclopedia (ref. 60), Genomics of Drug Sensitivity in Cancer (ref. 61), and commercial venders]. Contributions from network biology to innate resistance can be challenging to predict because many factors affect how preclinical models respond to therapies (e.g., efflux; ref. 62). Another challenge is that the tumor cell panels are typically comprised of immortalized cell lines that are adapted to grow in 2D culture, which alters the tumor cell biology (55, 63). Recently, more clinically relevant tumor cells have been used to enhance the predictive power of the approach. For example, drug sensitivity screening of circulating tumor cells (CTC) studied *ex vivo* in nonadherent culture (64) or isolated leukemia tumor cells from patients (65) have been used to identify effective therapies for specific patients. *In silico* modeling can be integrated with knowledge of tumor kinase network signaling to provide valuable tools for assessing potential combinations and resistance mechanisms. Predictive, mathematical models are used to predict tumor growth and metastasis (66, 67). Studies using computational modeling used known activities of PI3K/AKT and MAPK/ERK on tumor cell proliferation and death to design synergistic drug combinations in $HER2^+$ breast cancer (67). Complex modeling of phenotype switching (e.g., epithelial–mesenchymal transition) have been performed to identify critical signaling nodes and bypass mechanisms (66). Taken together, the identification of innate sensitivity and resistance *in vitro* has benefited from better characterized tumor cell lines, more precise validation tools, and more predictive model systems.

Innate sensitivity and resistance are also routinely evaluated in preclinical *in vivo* models. The most widely used models are derived from immortalized human tumor cell lines implanted into immune-compromised mice (allogeneic xenograft models), which lack the appropriate tumor microenvironment. The predictive capabilities vary as a function of drug modality (e.g., validated for cytotoxic drugs) and tumor types (55, 68). These models are more useful to explore the pharmacokinetic–pharmacodynamic relationship, pharmaceutical properties (e.g., distribution), and safety liabilities. Patient-derived xenograft (PDX) models are an improvement because they more accurately model the complexity of patient tumor biology (69, 70). Limitations of this approach are that each PDX model represents the tumor biology of a single patient, not all biopsies can be engrafted, the host immune system is compromised, and the tumors loose heterogeneity upon engraftment (55, 71, 72). Genetically engineered mouse models and other syngeneic models are necessary to evaluate a therapy in a system with an intact immune system, but these models rely on murine physiology and form tumors stochastically. Taken together, *in vivo* models can be used to confirm *in vitro* findings and enable a more complete understanding of potential innate resistance mechanisms.

Exploring mechanisms of acquired resistance to targeted therapies is different than innate resistance because a change in response is sought, which allows for comparisons with unaltered control cells. Preclinical approaches that predict underlying mechanisms of resistance encompass *in vitro*, *in vivo*, and *in silico* analyses. With *in vitro* methods, tumor cells are treated in culture and resistant cells are clonally expanded and analyzed for alterations in pathways (e.g., genetic, protein expression; ref. 73). Many selection strategies have been used for creating resistant cell lines with variable results (73). Conceptually, the doses selected to treat cells should produce clinically relevant exposures (concentration and kinetics) for treatment of cells, but *in vitro* cell growth is distinct from patient tumor physiology and bolus/pulse, continuous, and escalating dosing schedules can all yield resistant tumor cell lines. Using mutant EGFR NSCLC tumor cells (PC-9, exon 19 deletion), escalating doses of erlotinib-treatment resulted in acquired resistance through the emergence of the clinically relevant T790M and BRAF mutations (74, 75). Studies of NSCLC H1650 cells (exon 19 deletion) made resistant to erlotinib by continuous exposure revealed a different mechanism – the induction of cancer stem cell–like mesenchymal properties (76). Long-term treatment equivalent to clinical regimens also can be used to generate drug resistance (77). A caveat to all of the *in vitro* approaches is that characteristics of the parental cell line (intrinsic genomic factors, prior patient treatment exposure, and culture methods) influences the selection of resistance mechanisms. Increased use of primary cell lines, *ex vivo* samples, more physiologic culture conditions (e.g., 3D culture, extracellular matrix), and coculture systems are improving the likelihood of predicting resistance in patients. For example, cell culture systems derived drug-resistant patients can be used *in vitro* to select the patient's therapies, which are confirmed using related *in vivo* models (34, 78). *In vitro* preclinical methods are relatively simple, use well-characterized systems, encompasses a broad array of tumor biology's, and are amenable to genetic manipulation allowing for rapid identification of potential acquired resistance mechanisms.

Preclinical prediction of intrinsic resistance mechanisms of drug resistance has been reported. For kinase-directed therapies that would be expected to elicit intrinsic acquired resistance, structural analysis during drug design can identify mutations that would interfere with drug binding. Co-crystal structures and molecular modeling studies can identify amino acid residues essential to drug binding (79–81). The range of mutations that

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could cause intrinsic resistance can be identified by considering single-nucleotide substitutions to the amino acid codons that would change the amino acid. Steric interference can be confirmed by biochemical analysis of the mutant protein and cellular analysis in engineered cell lines. A caveat to this approach and other preclinical methods is that they do not necessarily translate to the clinic. Nonetheless, preclinical studies for predicting response and resistance are becoming more accurate and are critical for creating insight into clinical performance of kinase-directed therapies.

Drug Combinations Can Enhance Clinical Response to Kinase-Targeted Therapies

Although "magic bullet" single-agent therapies are desirable, a wealth of findings underscore the need for the combination of drugs to effectively terminate oncogenic signaling and overcome resistance. Examples of overcoming innate drug resistance (CDK4/6-selective drugs), acquired bypass drug resistance (BRAF drugs), and polygenic disease (BCR-ABL1 drugs) illustrate the principle. Hyperphosphorylation of the cell-cycle checkpoint protein retinoblastoma (Rb1) by CDK4 or CDK6 inactivates the G_1 restriction checkpoint and allows cell proliferation. Preclinical studies show that a significant proportion of breast cancer patients have defects in cell-cycle regulation such that a selective CDK4/6 inhibitor would be expected to be efficacious (82). Surprisingly, single-agent clinical studies of a CDK4/6-selective drug (palbociclib) had modest signs of efficacy—two of 30 breast cancer patients had a partial response (innate resistance; ref. 82). ERα antagonists (e.g., tamoxifen) and estrogen-suppressing enzymes (e.g., letrozole) are known to block cell proliferation through a different cell-cycle process, G_0–G_1 cell-cycle arrest. Preclinical studies revealed synergy between CDK4/6-selective inhibitors and ERα antagonists in blocking proliferation of ERα$^+$ breast cancer tumor cells (83). A clinical trial of metastatic ERα$^+$ breast cancer patients with palbociclib combined with an estrogen biosynthetic inhibitor (letrozole) showed a statistically significant (P = 0.0004) increase in progression-free survival (PFS; 20.2 months) relative to letrozole alone (10.2 months; ref. 84). For colorectal cancer patients with a wild-type KRAS/BRAF-V600E background, BRAF-directed drugs are not effective because of bypass EGFR signaling (innate resistance) and combinations of BRAF and EGFR drugs are necessary (Fig. 4; ref. 21). Activation of the EGFR/MAPK signaling pathway also occurs as an acquired resistance mechanism in BRAF-V600E melanoma and requires the addition of a MEK drug to prolong clinical benefit (45). Drug combinations have been used to target multiple intrinsic mutations present in a tumor (polygenic disease). NSCLC studies show that the drug resistant form of EGFR (gatekeeper T790M mutant) can be present before EGFR drug treatment (e.g., erlotinib, gefitinib) and is selected for by treatment (acquired resistance) requiring therapies that target both oncogenic and drug resistance forms of EGFR (85). Multiple mutant forms of BCR-ABL1 can be present in a single CML patient that need to be blocked for a therapy to have a durable response. In one patient, axitinib was shown to potently inhibit the T315I variant of BCR-ABL1 but not the unmutated form, requiring combination with a broad-spectrum BCR-ABL1 drug (e.g., bosutinib) to achieve a sustained clinical benefit (34). These studies illustrate that multiple drugs that target common or related processes may be necessary to achieve durable clinical responses by overcoming drug resistance.

Cancer Diagnostic Analysis Enhances Survival

Cancer diagnostics affects patient outcome in many ways—selection of optimal therapies, monitoring for drug response, detecting mechanisms of drug resistance, characterizing tumor heterogeneity, and better surveillance for disease occurrence and recurrence. For solid tumors, in-depth cancer biology is defined at diagnosis and after surgery because tumor biopsies are readily available. CLIA-certified (Clinical Laboratory Improvement Amendments) cancer genome profiling services are available to identify mutated oncogenic drivers and tumor suppressors from these biopsies. Whole-genome sequencing and exon sequencing (RNA-seq) provides a more complete understanding of a patient's cancer biology landscape. This enhanced knowledge of a patient's biology has been valuable for selecting optimal therapies. For example, if a colorectal cancer patient has to choose between an anti-EGFR mAb or an anti-VEGF monoclonal antibody biologic to add to the backbone chemotherapy (e.g., XELOX, FOLFOX, FOLFIRI), awareness of activating mutants that abrogate EGFR blockade (e.g., KRAS, BRAF, NRAS, PI3K; ref. 86) is critical to selecting a therapy with a durable response. Although assessing tumor biology through biopsies is valuable, it has limitations. Obtaining biopsies is an invasive procedure that is inconvenient for the patient and adds significant health risks (87). For solid tumors, the procedure is not typically used to measure dynamics of a patient's tumor biology or its response to therapy. Instead, less informative imaging techniques (e.g., MRI, PET, and CT) are used to characterize response to treatment and disease relapse. Noninvasive assessments of patients with solid tumor cancers have been shown to be clinically effective, but are limited to a few tumor types with known circulating protein antigens (e.g., CA-125, ovarian cancer; carcinoembryonic antigen, and colorectal cancer) and provide little detail of the patient's tumor biology. Advances in proteomic techniques are being applied to identify new protein markers of disease, but are not sufficient for routine clinical application (88). Therefore, to deploy more effective therapies that specifically target a patient's disease, a deeper understanding of its cancer biology is necessary.

Noninvasive, "liquid" biopsies can expand the knowledge of a patient's tumor biology and affect clinical outcome. This approach is standard practice for hematologic malignancies because the tumor cells are readily available for *ex vivo* studies. This analysis enables cancer detection at an earlier disease progression stage, characterization of the cancer biology, and assessment of the response to treatment. Early detection increases the chance of therapeutic success because there are fewer tumor cells and less genetic diversity which lowers the probability of drug resistance tumor cells being present (89). Noninvasive biopsies have enabled the overall survival of CML patients treated with imatinib to increase from 33 months in advanced disease (blast crisis) to 93% 5-year survival with early disease (chronic phase) because the bcr-abl translocation can be readily detected enabling early therapeutic intervention (90, 91). The approach is being extended to solid tumor cancers because of the emergence of new approaches to monitor blood-borne biomarkers—circulating DNA (ctDNA) and CTCs (92, 93). These diagnostics may enable near real-time monitoring the efficacy of therapy, capture the extent of tumor cell heterogeneity, measure changes in tumor biology in response to treatment (resistance mechanisms), and identify residual disease. Circulating tumor DNA are released

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into the blood from tumor cells during apoptotic or necrotic cell death (94–97). Quantitation of ctDNA is complicated by cell-free DNA (cfDNA) fragments of 180 to 200 base pairs are released into whole blood as part of normal physiology (e.g., phagocytosis) and comprises up to 99% of the circulating DNA in a patient's blood. Because the half-life of ctDNA is short (~2 hours), it can provide fine temporal analysis of tumor biology. The development of highly sensitive methods for detecting ctDNA is responsible for its emergence in the clinic and synergizes with tumor biopsy genomic analysis, because monitoring for tumor-specific DNA enhances the technique's sensitivity. Analyzing CTCs is another promising diagnostic approach because these cells contain a wealth of information about a patient's tumor biology (92, 93, 98). Creating a CTC diagnostic is challenging from many reasons—CTCs are extremely rare as well as heterogeneous. The most advanced methods use an enriching step before detection (e.g., filtration, microchips, automated microscopy). The only FDA-approved diagnostic (CellSearch, Veridex) is based on bead capture of CTCs by an EpCAM epitope and is approved for metastatic breast, colon, and prostate cancers. More recently, direct methods of CTC detection are emerging (ISET, RareCells CO; ScreenCell, Co.). To date, a universal approach to detect CTCs is elusive but many new methodologies are being assessed in the clinic (92, 98). The next frontier for the noninvasive biopsy approach is for surveillance in the adjuvant and posttreatment settings. An issue to overcome is that the analyte is present at much lower levels than when the disease first presents requiring more assay sensitivity. Nonetheless, these emerging diagnostic technologies are enabling more effective treatments for patients and should have a profound effect on patient survival.

Summary

Oncology research, drug discovery, and patient treatment are in a transformational period due to the rapid expansion of data, knowledge, medicines, and diagnostics. Cancer genomic analysis, tumor cell biologic studies, and clinical feedback from kinase-directed therapies all show that protein kinase signaling is critical to many aspects of tumor biology in ways only recently unveiled. One recurring theme is that the intricate, malleable signaling networks in which protein kinases operate are far more complex than originally thought. Integration of a network perspective into exploring disease biology and therapeutic intervention will result in more effective therapies for the growing number of cancer patients in need of durable treatments.

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No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Durability of Kinase-Directed Therapies—A Network Perspective on Response and Resistance

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