

Chapter 2

The Dynamics of the Cell Signaling Network; Implications for Targeted Therapies

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Keywords Signal transduction • Signaling network • Feedback • Resistance • Molecular targeted therapeutics • Systems theory • PI3K • AKT • mTOR • RAS • RAF • MEK • ERK • S6K

Introduction

There are four major mechanisms of resistance to molecular targeted therapies that have been identified thus far (Fig. 2.1). Mutations in the ATP binding pocket result in resistance to small molecule ATP mimetics [1] and this mechanism is discussed in Chap. 1 of this book [2]. The other identified mechanisms are consequences of changes in the cell signaling network. Intrinsic resistance to molecular targeted therapies may exist due to the activity of redundant signaling pathways within the network at the time of initial treatment. Similarly, there can be preexisting activating mutations in effector proteins downstream of the targeted kinase. Compensatory signaling events within the cell signaling network can also result in acquired resistance. These include upregulation of alternate signaling pathways controlling growth and survival as well as the loss of feedback control, which triggers activation of secondary growth and survival signaling pathways. The mechanisms that are dependent on changes within the cell signaling network are discussed in this chapter. The references cited are in no way comprehensive of the field, rather they illustrate the major points presented; apologies for any omissions.

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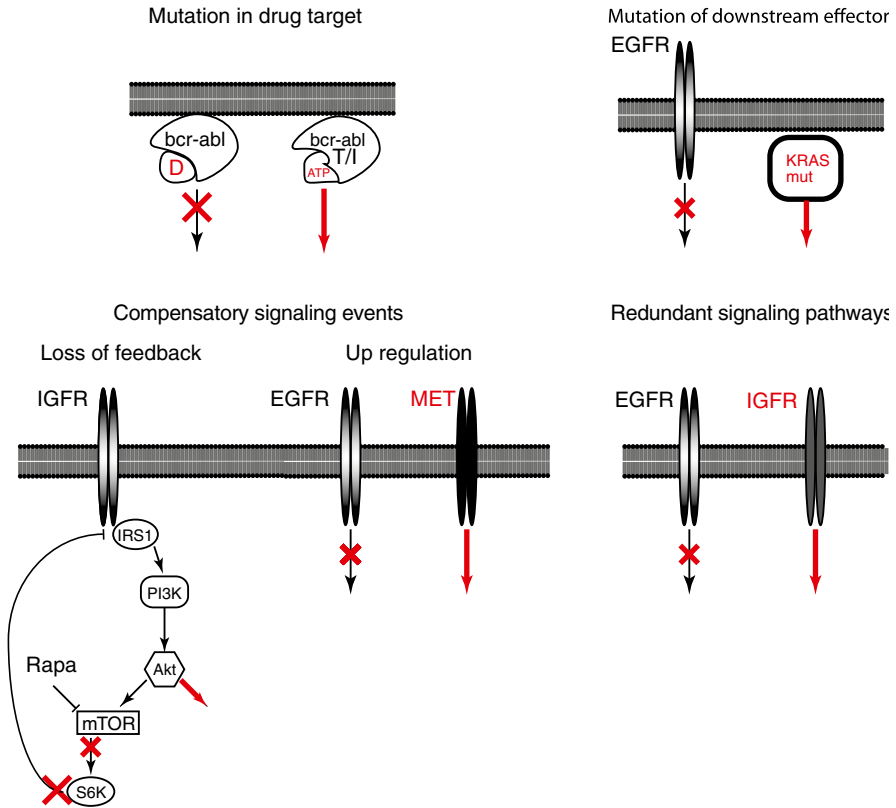


Fig. 2.1 The major mechanisms of resistance to molecular targeted therapies. These include (1) mutation in the drug target preventing binding of the ATP competitive inhibitor but still allowing for ATP binding. (2) Mutation of a downstream effector rendering inhibition of an upstream activator ineffective. (3) Loss of feedback control when inhibiting a downstream effector facilitating activation of signaling. (4) The presence of redundant signaling pathways regulating growth

A Network Model for Cell Signaling

The majority of new molecular-targeted therapies are directed against proteins involved in signal transduction [3, 4]. The classical model for signal transduction is that of a signaling pathway consisting of many compartmentalized, hierarchical, and independent proteins. Although it has long been recognized that connections between multiple pathways exist, the understanding of this crosstalk is relatively limited, as many studies designed to analyze these connections do not consider the dynamic, quantitative, or iterative nature of the cell's signaling system [5]. Most research focuses on describing the detail complexity of particular aspects of the total system; for example, how a given kinase cascade relays a growth control signal. However, it is the dynamic complexity, how the detail complexity from a multitude of pathways interacts in time and space, that regulates biologic processes,

including carcinogenesis. This concept is supported by data demonstrating that extracellular signals are transmitted through a network of proteins rather than through cross-talk between hierarchical signaling pathways [5–8]. This network model is not at the exclusion of canonical signaling pathways that likely make many of the significant connections within the signaling network.

Additional data in support of a signaling network in cancer come from studies suggesting that cancer cells acquire multiple mutations during the process of cancer development that are necessary for full malignancy [9]. Wide scale sequencing efforts for breast and colorectal cancer genomes projected that there are 81 and 105 mutant genes, respectively [10]. Of these, an average of 14 and 20, respectively, are causative. Interestingly, signal transduction and transcriptional machinery components are common functional categories that are mutated in these cancers. This work has been further supported by more recent sequencing studies of human cancers [11]. A similar story has emerged thus far from The Cancer Genome Atlas; one example is in glioma where sequencing of 206 tumors uncovered dysregulation in RB, p53, and RTK signaling including ERBB3, RAS, and PI3K [12]. These cancer genome sequencing studies suggest that many mutations are needed to undermine the robust wild-type cell signaling network and give rise to cancer.

Given the multitude of causal mutations in cancer, it is surprising that any one molecular-targeted agent would have any effect. The “oncogene addiction” model for cancer speculates that the many genetic changes during cancer development generate a fragile system that is vulnerable to the stress caused by one perturbation – the inhibition of a causal oncogene [13]. Sharma et al. presented experimental evidence suggesting that the pro-apoptotic outcome of oncogene inhibition results from coordinated differences in the decay of pro-apoptotic and pro-survival/pro-proliferation signals [14]. The phenomenon of oncogene addiction is well documented in multiple mouse tumor models and cancer cell lines [15]. The clinical effectiveness of imatinib, which targets the BCR/ABL fusion protein in chronic myelogenous leukemia (CML), implies that leukemia cells are addicted to the ABL oncogene [16]. Initial responses of other molecular-targeted therapies in patient subsets that are defined by a particular molecular signature are also consistent with the oncogene addiction model; for example, trastuzumab in ERBB2 positive breast cancer. Further evidence for oncogene addiction is seen in the discovery of resistance mutations following treatment with molecular-targeted therapies that are kinase inhibitors [1, 17, 18]. Unfortunately, the clinical reality is that patient responses to most molecular-targeted therapeutics are not universal and are not long lasting [19]. The oncogene addiction model postulates that the clinical ineffectiveness of these therapies is due to tumor cells having the ability to mutate around their addiction, known in this model as oncogenic escape or “addiction switching” [13, 20]. Oncogenic escape or addiction switching are inherent in the signaling network model. The dynamic complexity of the cell signaling network accounts for (1) the functional redundancy in the robust cancer system generating intrinsic resistance and (2) the feedback control generating acquired resistance to molecular-targeted agents. It is the functional redundancy and the feedback control

that contributes to the robustness of cancer as a system. The concept of a signaling network is critical to the development of effective molecular-targeted therapeutics as it suggests why inhibition of a single component of a canonical pathway is insufficient to have dramatic effects for the treatment of cancer. Moreover, the signaling network model illustrates how difficult it is to empirically develop effective combinatorial therapies and suggests that finding such fragilities in the cancer system will require an in-depth understanding of the dynamics of the cell signaling network in tumor cells.

There is a concept in Systems Theory termed highly optimized tolerance (HOT), which suggests that robust systems tend to be fragile to unexpected perturbations; resistance to a broad range of perturbations is paid for with extreme sensitivity to seemingly innocuous perturbations [21, 22]. In the context of cancer, HOT theory postulates that identifying fragile loci and therapies that can target these points should lead to more efficacious treatments. This is akin to oncogene addiction but does not require that the fragile loci be a driving mutation. When considering HOT theory and the cell signaling network, fragile loci may exist that are not yet identified.

Systems Theory also suggests another possibility; targeting multiple nodes within the cell signaling network could create a fragile state and thus a more efficacious treatment, especially if the combinations of molecular-targeted agents used have independent selective pressures [22]. These combinations would effectively reduce tumor heterogeneity (as well as heterogeneity within the cell signaling network) and the corresponding robust state, thereby overcoming the limited effectiveness of molecular targeted agents.

Redundant Signaling Pathways

There is a high frequency of receptor tyrosine kinase (RTK) expression and activation in human cancers [23]. RTKs are critical regulators of signaling pathways that regulate diverse cellular functions including growth, survival, differentiation, and motility. Consequently, molecular targeted therapies directed at RTKs have been a major focus and represent the majority of FDA approved molecular targeted therapies to date [19]. Different families of RTKs are activated concurrently through redundant inputs that effectively maintain downstream signaling when any single RTK is inhibited. In nonsmall cell lung carcinoma (NSCLC) there is elevated epidermal growth factor receptor (EGFR) expression, making the EGFR an attractive therapeutic target [24]. However, while EGFR activity is necessary for a response to RTK inhibitors targeting the EGFR, expression of the EGFR does not predict therapeutic response. It is the presence of an activating EGFR mutation or gene amplification that predicts a clinical response to EGFR inhibitors [25–27]. Unfortunately for this subset of patients, the clinical response is not durable. The majority of NSCLC patients are not responsive to EGFR inhibitors or inhibitors that target other RTKs. The simplest explanation is that there are redundant signaling

pathways regulating cancer cell growth and survival, and that inhibition of any one pathway is insufficient for a biological effect. Consistent with this, studies have found the expression of many different RTK families at both the mRNA and protein level in primary NSCLC tumors [28, 29]. This would suggest that combinations of RTK inhibitors might be necessary for effective inhibition of NSCLC growth. One study has shown that combinations of EGFR and fibroblast growth factor receptor (FGFR) inhibitors resulted in synergistic growth inhibition [30].

Activation of multiple RTK families is also common in glioblastoma multiforme (GBM). Analysis of GBM cell lines, xenografts, and primary patient tumors showed concurrent activation of multiple RTKs including EGFR, ERBB3, PDGFRA, MET, RET, MST1R, and CSF1R [31]. In this study, expression of a dominant negative EGFR did not alter PI3K activity, suggesting redundant RTK activation of PI3K. The authors go on to show that combinations of RTK inhibitors, such as erlotinib targeting the EGFR and SU11274 targeting MET, were required to disrupt PI3K association with GAB1 as well as downstream AKT and S6 phosphorylation. The erlotinib and SU11274 combination effectively inhibited adherent and anchorage-independent growth. Similar observations were made when RNAi was used to inhibit either MET or PDGFRA expression; knockdown of either MET or PDGFRA conferred sensitivity to EGFR inhibition [31]. These studies are consistent with concomitant activation of multiple RTKs limiting cancer dependency on any one RTK, thereby rendering tumors refractory to a single molecular targeted therapy. Combinations of either multiple RTK inhibitors, or molecular targeted therapies with activity against multiple RTKs could more effectively inhibit downstream intracellular signaling and tumor cell growth and survival.

While redundancy in RTK signaling results in intrinsic resistance to any single RTK inhibitor, another mechanism of intrinsic resistance is the presence of a constitutively active mutation in a signaling protein downstream of the targeted protein. This is essentially an epistasis phenomenon, where there is a regulatory hierarchy such that inhibition of the “upstream” component has no effect since there is mutational activation of the “downstream” component, even when the upstream component is overexpressed and activated. This is an oversimplified argument given what we know now about the cell signaling network (discussed earlier) and feedback loops within the signaling network (discussed later). However, there is clinical evidence for mutational activation of downstream pathway components that render RTK inhibitors ineffective [32, 33].

For both lung and colon carcinoma the presence of KRAS mutations is an important predictive factor for determining which patients will respond to EGFR inhibitors [32, 33]. In lung carcinoma, there was a perfect association with KRAS mutations and resistance to EGFR inhibitors; none of the KRAS mutant tumors responded to either gefitinib or erlotinib [32]. As observed in other studies, EGFR mutations were highly predictive of response to EGFR inhibitors. Similar observations were made in colorectal cancer examining KRAS mutational status [33, 34]. In one study, the KRAS mutational status of 394 patients, half of whom were on the EGFR inhibitor cetuximab and half on best supportive care, was examined [33]. Essentially equivalent numbers of KRAS mutations were found in the two groups.

Cetuximab was effective only in patients with wild-type KRAS where it was associated with improved overall and progression free survival. For patients with a mutant KRAS, there was no difference in overall or progression free survival between cetuximab treatment and best supportive care. Collectively, these studies suggest that the clinical decisions to treat patients with lung and colorectal cancer would be improved by mutational profiling of KRAS and EGFR, since the effectiveness of EGFR inhibitors is limited in the presence of mutational activated KRAS and show a response, albeit limited, in patients with EGFR mutations.

Compensatory Signaling Pathways

Upregulation of Alternative Signaling Pathways

Treatment with molecular targeted agents can lead to the activation of alternative signaling pathways that result in resistance. Resistance to RTK inhibitors often correlates with reactivation of the PI3K-AKT signaling pathway. Gefitinib resistant lung cancer cells were generated by exposure to increasing concentrations of gefitinib [35]. Genome-wide copy number analyses and mRNA expression profiling of the parental and resistant lung cancer cell lines identified MET amplification correlating with gefitinib resistance. Amplification of MET led to MET association with and activation of ERBB3, which in turn reactivated PI3K-AKT signaling. In the gefitinib resistance cells, treatment with either an EGFR or MET inhibitor alone had no effect on cell viability. However, the combination of EGFR and MET inhibitors resulted in cell death. While this resistance mechanism was uncovered using an experimental model, it appears to function in patients who have developed resistance to gefitinib; 22% of lung cancer patients who developed gefitinib resistance had MET gene amplification [35]. In two patients with pre- and postgefitinib treatment paired tumor samples, only the posttreatment sample showed MET amplification, consistent with MET amplification being a compensatory response to EGFR inhibition as opposed to a redundant signaling pathway. It also suggests that MET amplification is a driver of gefitinib resistance. This is further supported by a study using array-based comparative genomics to examine differences in untreated patients or those patients with acquired resistance to either gefitinib or erlotinib [36]. In this analysis, 21% of patients with acquired resistance had MET amplification in contrast to only 3% of untreated patients having amplification of MET.

In other examples of gefitinib resistance in lung cancer models there was an increase in PI3K-AKT signaling [37]. Again, cancer cell lines were exposed to increasing concentrations of gefitinib (lung cancer and HNSCC) or the anti-EGFR antibody therapy, cetuximab (lung cancer), until clinically relevant doses and resistant cell lines emerged. In the resistant cell lines gefitinib continued to inhibit EGFR and EGFR-ERBB3 dimerization as well as activation of downstream ERK signaling. However, under these conditions PI3K-AKT signaling persisted. Gene expression

analysis identified decreases in IGFBP3 and/or IGFBP4 expression depending on the specific model of RTK inhibitor resistance. These IGF binding proteins inhibit IGF activation of the IGFR [38]. The decrease in IGFBP expression in the gefitinib and cetuximab resistant cell lines facilitates IGFR signaling to PI3K-AKT [37]. Subsequent combined inhibition of IGFR and EGFR inhibited PI3K-AKT signaling and cell growth in the resistant cells, whereas inhibition of IGFR alone was insufficient. Furthermore, when IGFBP3 was added back to resistant cells, sensitivity to gefitinib was restored, suggesting IGFR signaling as causal in gefitinib resistance.

Collectively, these studies suggest that molecular profiling of patient tumors that have failed molecular targeted therapies could identify mechanisms of resistance and subsequent effective second line therapies. From a systems perspective, the continual targeting of a central pathway such as PI3K-AKT signaling, even when different activators of PI3K-AKT are being targeted, will likely not provide durable clinical responses. There is clearly strong selective pressure for reactivation of the PI3K-AKT signaling pathway and multiple mechanisms to achieve it. Sequential or concurrent treatments of inhibitors can effectively prevent resistance in experimental models. It is not yet known whether such treatments effectively reduce heterogeneity of the cell signaling network and induce a fragile state in patients; however, the lack of independent selective pressures suggests that a fragile state will not be achieved.

Interestingly, the mechanism of resistance to RTK inhibitors is not restricted to compensatory signaling of PI3K-AKT triggered by upregulation of additional RTKs [39]. Short-term treatment of breast cancer cell lines with lapatinib led to an increase in estrogen receptor (ER) dependent cell growth. In this system, increases in the Forkhead transcription factor family member, FOXO3, and caveolin-1 enhanced ER transcriptional activity. This resulted in a switch from ERBB2 dependent regulation of survivin, an important anti-apoptotic regulator, to ER dependent regulation of survivin. Combining lapatinib with the ER antagonist ICI 182,780 abrogated acquired lapatinib resistance. Importantly, lapatinib treatment enhanced the expression of ER-regulated gene products in a rare subset of patients with ERBB2 and ER positive breast cancers suggesting that this mechanism of resistance may be clinically important.

One intriguing example of compensatory signaling in response to EGFR inhibition was observed in a breast cancer system [40]. Gefitinib treatment of breast cancer cells *in vitro* and *in vivo* led to a shift in the equilibrium state of ERBB3 phosphorylation and downstream activation of AKT. In cultured cells this increase in ERBB3 phosphorylation was due to increased ERBB3 membrane association and possibly the downregulation of ERBB3 phosphatase activity. Inhibition of EGFR and ERBB2 signaling results in a decrease in AKT activity and subsequent membrane localization of ERBB3. It appears that the loss of negative feedback from AKT may trigger this relocation since gefitinib treatment does not trigger ERBB3 phosphorylation in the presence of a constitutively active AKT. Feedback control is a major regulator of the cell signaling network. How perturbations in feedback control result in resistance to molecular targeted therapies are discussed below.

Loss of Feedback Control

In normal and cancer cells, activation of the cell signaling network is regulated by feedback control. Molecular targeted therapeutics can disrupt feedback signaling pathways and attenuate the therapeutic response. A fundamental understanding of the intricacies of the cell signaling network is required to design effective therapies that do not trigger unintended consequences. The best examples described to date on how loss of feedback control drives resistance to molecular targeted therapies centers around the PI3K-AKT signaling pathway (Fig. 2.2). In the canonical PI3K pathway, ligand activation of RTKs (e.g., IGFR) leads to activation of PI3K through adapter proteins such as IRS1 [41]. Alternatively, PI3K can be activated through a direct interaction with activated EGFR or RAS. This results in the production of phosphorylated phosphoinositides (e.g., PIP3) and membrane recruitment of phosphoinositide-dependent kinase 1 (PDK1) and AKT. Once at the membrane, AKT is fully activated by PDK1 and mTORC2. Activated AKT then phosphorylates multiple substrates resulting in the activation of mTORC1 by inhibiting negative regulators of mTORC1 activity. Elevated mTORC1 activity has been observed in multiple cancers [42]. The downstream positioning of mTORC1 in the PI3K pathway, its activation state in cancer, along with the availability of a natural product inhibitor, rapamycin, quickly led to a focus on mTORC1 as a therapeutic target.

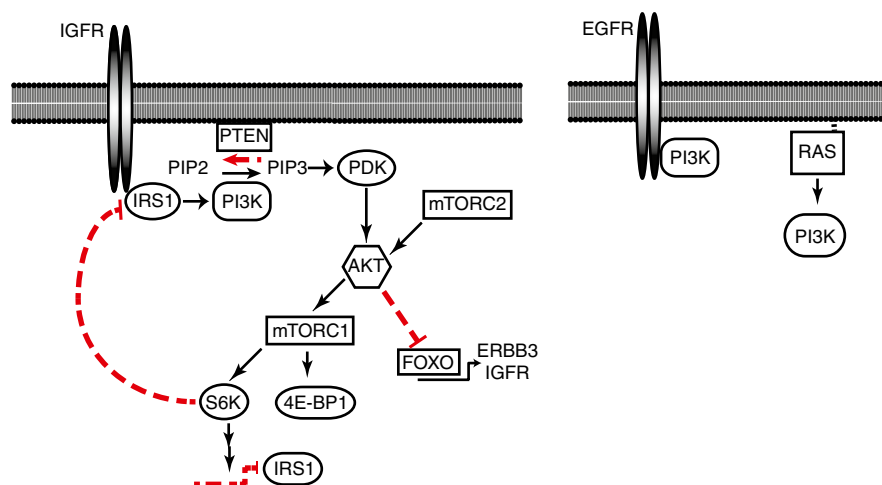


Fig. 2.2 The PI3K – AKT signaling pathway. Positive signals are represented by black arrows. Negative signals are represented by red dashed lines with squared ends. PI3K can be activated by RTKs and small GTPases. PI3K signals to S6K, which inhibits IRS1 through both phosphorylation and transcriptional down regulation. AKT inhibits FOXO, which positively regulates RTK transcription. Inhibition of mTOR with Rapamycin alleviates the negative regulation of IRS1 further stimulating PI3K-AKT signaling. Inhibition of AKT relieves the negative regulation of FOXO resulting in the up regulation of RTKs, including ERBB3 and IGFR

mTORC1 inhibitors, including rapamycin and its derivatives, have shown robust activity in model systems. However, the clinical trial results with mTORC1 inhibitors have been more modest than predicted [42]. The mechanism of resistance to mTORC1 inhibitors is a subject of major therapeutic significance.

Studies have shown that while inhibiting mTORC1 activity in cancer cells effectively suppressed the phosphorylation of downstream effectors such as p70S6K and 4E-BP1, it increased the phosphorylation and activation of AKT. A feedback loop between PI3K, mTORC1, and IRS1 was first described in adipocytes [43]. In this study, two structurally unrelated PI3K inhibitors and rapamycin inhibited insulin-induced phosphorylation and degradation of IRS1; however, MEK inhibition had no effect on IRS1. Interestingly, rapamycin could block the insulin triggered IRS1 phosphorylation and degradation even in the presence of a constitutively active PI3K, placing mTORC1 feedback to IRS1 downstream of PI3K.

Mechanistic details of this feedback loop were uncovered in mouse embryo fibroblasts where tuberous sclerosis 2 (TSC2) was identified as a critical regulator of PI3K signaling [44]. TSC2 suppresses S6K, both an effector and negative regulator of PI3K signaling. S6K transcriptionally represses IRS1 and phosphorylates IRS1 protein on S302, which inhibits its association with activated insulin receptors. S6K also phosphorylates IRS1 on S1101 and loss of this phosphorylation enhances insulin signaling and AKT activation [45].

The clinical implications of this feedback loop are crucial for understanding how inhibitors of PI3K-AKT signaling can be used most effectively in the clinic. In an *in vivo* cancer model, mice heterozygous for TSC2 develop tumors with alterations in the mTORC1-IRS1-AKT feedback loop and this is accelerated by the loss of PTEN [46, 47]. More importantly, in human cancer biopsies, pharmacological inhibition of mTORC1 leads to AKT activation [48–50]. mTORC1 inhibition induces IRS1 expression and abrogates feedback inhibition, resulting in AKT activation in patient tumors treated with rapamycin or RAD001, a rapamycin derivative. Similar observations were made in breast and prostate cancer cell lines [48]. Inhibition of IGFR blocks rapamycin induced AKT activation thereby sensitizing cancer cells to mTORC1 inhibition. These data suggest that in cancer cells with mTORC1 activation, the feedback loop downregulates RTK signaling. Inhibition of mTORC1 blocks the negative feedback to IRS1, resulting in activation of AKT and attenuation of the growth inhibition by rapamycin. Combination therapy inhibiting both mTORC1 and the IGFR receptor results in additive inhibition of growth *in vitro* [48].

Similar observations were made in another study using lung carcinoma cells where rapamycin inhibition of mTORC1 activated survival signaling, which attenuated the therapeutic response [51]. Rapamycin effectively inhibited S6K and 4E-BP1 phosphorylation, which are indicative of mTORC1 inhibition; however, rapamycin also increased AKT and eIF4E phosphorylation. Concurrent treatment with a PI3K inhibitor blocked the rapamycin induced activation of AKT and eIF4E and enhanced inhibition of growth and colony formation of lung cancer cells. Collectively, these studies suggest that an increase in AKT activity attenuates the effect of mTORC1 inhibition and facilitates cancer cell growth and survival. Only upon discovery of this mTORC1-IRS1-AKT feedback control system could

effective combinatorial treatments be determined; inhibition of IGFR-1 in breast and prostate cancer cell lines and of PI3K in lung cancer cell lines sensitized cells to mTOR inhibition [48, 51].

The advent of polypharmacology, the focus on multitarget drugs, has led to compounds that inhibit both PI3K and mTOR, thereby inhibiting both the PI3K-AKT signaling pathway and the mTORC1-IRS1-AKT feedback loop [52]. Screening a panel of isoform-selective PI3K inhibitors against genetically diverse glioma cell lines identified PI-103 as the most effective inhibitor of growth. The potent growth suppressive activity of PI-103 was due to its ability to inhibit both PI3K and mTORC1, resulting in maximal inhibition of AKT. The combinatorial inhibition of PI3K and mTORC1 by PI-103 was efficacious against glioma xenografts. This is consistent with the studies described above and further suggests that inhibition of PI3K and mTORC1 would be effective in treating cancer driven by aberrant PI3K-AKT signaling. Similar observations have been made with a dual PI3K and mTOR targeted therapeutic in clinical trial, NVP-BEZ235 [53].

The direct role of AKT in the feedback from RTK signaling was recently examined. In multiple cancer cell lines, an allosteric AKT inhibitor induced both the expression and phosphorylation of a subset of RTKs, including ERBB3, IGFR, and Insulin receptor [54]. This results in an increase in ERBB2-ERBB3 heterodimers and increased ERBB3 phosphorylation. The increase in RTK mRNA expression is dependent on the Forkhead transcription factors (FOXO1/3/4) that are negatively regulated by AKT. Inhibition of AKT facilitates activation of FOXO, leading to upregulation of RTKs. This too, results in an increase in ERBB2-ERBB3 heterodimers and increased ERBB3 phosphorylation. Blockade of AKT can thus lead to an increase in RTK signaling, reducing therapeutic benefit. Combined inhibition of ERBB and AKT signaling in two experimental models led to partial tumor regression. Thus, PI3K-AKT signaling is regulated by both FOXO and mTORC1 feedback loops and co-targeting RTKs and AKT can effectively inhibit tumor cell growth. This is further evidence that a thorough understanding of signaling pathways facilitates the design of more efficacious drug combinations.

While the best described alterations in feedback control to date have been observed in the PI3K signaling cascade, recent insights into feedback controls of the RAS-ERK signaling cascade are beginning to impact how inhibitors of this pathway can be used in the clinic (Fig. 2.3). RAS-ERK signaling is a central regulator of growth factor induced cell proliferation and survival [55]. Activation of RAS leads to signaling along several RAS effector pathways, the best studied of which are RAF, PI3K, and RALGDS. The RAS-RAF-MEK-ERK signaling pathway is constitutively activated in cancer through multiple mechanisms, including activating mutations in RAS, RAF, and MEK, loss of the tumor suppressor NF1 (a RAS GTPase), and upstream activation of receptor kinases through mutation, amplification, or ligand activation [55].

Feedback loops in the RAS signaling pathway are thematically similar to those that occur in PI3K signaling. Sprouty (SPRY) and MAP kinase phosphatase (MKP) are proteins that are transcriptionally upregulated by ERK [56, 57]. SPRY binds to GRB2 and prevents GRB2 and SOS1 from interacting with and activating RAS. Additionally, SPRY can directly interact with RAF and prevent RAF activation of

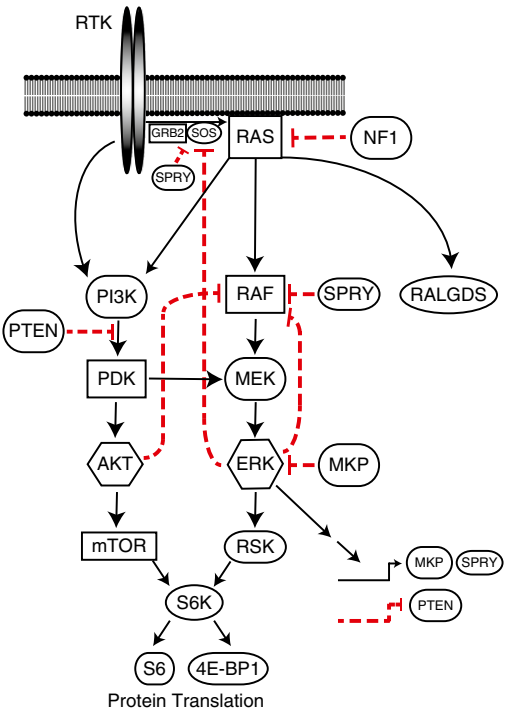


Fig. 2.3 The RAS signaling pathway. Shown are the RAS-ERK signaling pathway and PI3K-AKT cross talk with RAS-ERK signaling. Positive signals are represented by black arrows. Negative signals are represented by red dashed lines with squared ends. SPRY and MKP are negative regulators of RAS-ERK signaling. When MEK is inhibited, the negative feedback from ERK to SOS and ERK to RAF is lost, leading to an up regulation of RAS and RAF activity

MEK. MKP is an ERK phosphatase that dephosphorylates and inactivates ERK. Moreover, ERK itself directly phosphorylates SOS and RAF on inhibitory residues [58, 59]. Thus, there are both transcriptional and posttranslational feedback loops in the RAS-ERK signaling pathway. When ERK activity is blocked by MEK inhibition, the loss of feedback inhibition increases both RAF and MEK phosphorylation [59, 60]. Additionally, MEK inhibition activates EGFR signaling through the loss of ERK feedback to SOS [61]. Thus, inhibition of RAF and MEK can have unintended consequences on upstream signaling.

Several different strategies for inhibiting the RAS-ERK signaling pathway have been explored [62]. The most efficacious to date target RAF. Recent research has revealed insights on how selective RAF inhibitors can be most effectively used in the clinic (Fig. 2.4). Three recent studies demonstrate that while selective BRAF inhibitors effectively inhibit the growth and signaling in mutant BRAF tumors, these inhibitors can also activate CRAF through the formation of RAF dimers [63–65]. Moreover, this phenomenon is potentiated in the presence of mutationally active RAS. These observations have shed critical mechanistic insight into the clinical performance of RAF and MEK inhibitors and are facilitating patient selection

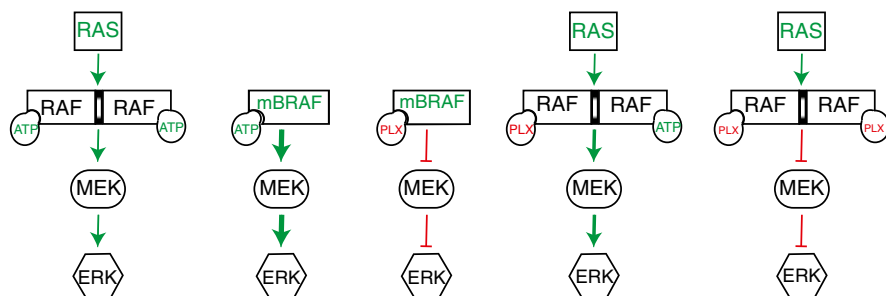


Fig. 2.4 The effects of RAF inhibitors on wild-type and mutant cells. In normal cells RAS-ERK signaling regulates cell growth. Positive signals are represented by green arrows. Negative signals are represented by red lines with squared ends. RAF inhibitors, such as PLX4032 (PLX), effectively inhibit signaling in BRAF mutant cells. However, in the presence of a active RAS low doses of RAF inhibitors leads to activation of RAF via transactivation where the catalytic activity of one dimer partner bound to PLX is inhibited and the wild-type partner bound to ATP is activated by transactivation. High doses of RAF inhibitors effectively inhibit RAF signaling independent of RAF mutational status

for the clinical use of drugs targeting the RAF-MEK-ERK pathway. There is considerable excitement over the recent clinical success of PLX4032, a selective RAF inhibitor, for the treatment of melanoma [66]. PLX4032 caused potent inhibition of signaling and proliferation in mutant BRAF melanoma, with a response rate of 78% by response evaluation criteria in solid tumors (RECIST). What is particularly surprising about the efficacy of PLX4032 is that it contrasts with the lack of clinical efficacy observed with MEK inhibitors. The selective MEK inhibitor, PD325901, had minimal clinical activity in melanoma patients; only 3 patients had RECIST responses and the drug was not pursued further due to neurological toxicity [67, 68]. If MEK is the major RAF effector in cancer cells, then how does inhibition of these two kinases yield such disparate clinical results? The answer may reside with three studies exploring how the inhibition of RAF can lead to the activation of MEK in cells harboring a KRAS mutation [63–65].

The report by Heidorn et al. demonstrated that the selective BRAF inhibitor, 885-A, could bind to wild-type BRAF in mutant KRAS cells, promote the formation of BRAF-CRAF dimers, and facilitate CRAF activation [65]. This study also demonstrated that kinase dead BRAF can similarly form BRAF-CRAF dimers and facilitate MEK activation in the presence of mutant KRAS. This observation that the kinase dead BRAF behaves similarly to the small molecule inhibited mutant BRAF provides an explanation for the apparent paradox of why kinase dead BRAF mutants are found in melanoma, especially since upward of 70% of melanomas carry an activating mutation of BRAF [69].

The report by Hatzivassiliou et al. focused on determining the mechanism of action for two distinct selective BRAF inhibitors, PLX4720 (a compound similar to PLX4032) and GDC-0879 [64]. This group found that GDC-0879 activated MEK in mutant RAS cells through promotion of RAF dimers, including BRAF-CRAF,

BRAF-ARAF, and CRAF-CRAF dimers. Through crystal structure analysis they found that drug binding caused a conformational change in RAF that promotes dimer formation. Interestingly, PLX4720 inhibits BRAF-CRAF dimer formation while still activating MEK signaling. The data reported in this study suggest that PLX4720 induces an alternative conformational change in BRAF that compromises BRAF dimer formation and activated MEK by promoting CRAF homodimer formation.

Further evidence for CRAF homodimer formation in response to PLX4720 in mutant RAS cells comes from Poulikakos et al [63]. Using a series of both gatekeeper and dimerization mutants, they demonstrated that transactivation of RAF dimers is responsible for MEK activation by RAF inhibitors. Importantly, MEK was activated in cells where RAS was activated by upstream oncogenes, including ERBB2. Thus, RAS need not be mutationally activated to facilitate MEK activation by RAF inhibitors. Activated RAS promotes RAF homo- and heterodimer formation, explaining the enhanced ability of RAF inhibitors to activate MEK in cells with active RAS. In cells carrying a mutant BRAF, RAS activity is typically low and transactivation of RAF dimers is not required for activation of MEK. Thus, RAF inhibitors are selectively effective at inhibiting mutant BRAF cells. Low doses of pan RAF inhibitors, including Sorafenib, could also activate MEK signaling. The mechanistic explanation for this is that at low doses of ATP-competitive inhibitors, activation of RAF occurs via transactivation where the catalytic activity of one dimer partner is inhibited and the partner is activated by transactivation. At high doses, the catalytic activity of both partners is inhibited, thus preventing signal transduction to MEK.

Collectively, these studies provide significant insight into the clinical observations with RAF and MEK inhibitors [63–65]. While the excitement over the clinical success of PLX4032 is justified, there are some potential concerns about the long-term treatment of patients with a drug that activates RAF-MEK-ERK signaling in normal tissues. A subset of patients on PLX4032 developed keratoacanthomas and squamous cell carcinomas, possibly due to activation of MEK [66]. Hatziaassiliou et al. observed hyperactivation of ERK and hyperproliferation in the skin of mice treated with GDC-0879 consistent with activated ERK signaling driving skin malignancies in patients [64]. The prevalence of RAS mutations in squamous cell carcinoma and the presence of RAS mutations in actinic keratosis are also consistent with active RAS facilitating pathway activation in cells with wild-type BRAF [63]. This suggests caution in the long-term management of patients treated with selective RAF inhibitors, especially in patients where exposure to environmental carcinogens may be higher – either through lifestyle choices or vocation.

These studies also suggest why RAF inhibition may be more effective clinically than MEK inhibition. RAF inhibitors selectively inhibit RAF signaling in cells carrying mutationally activated BRAF and do not inhibit RAF in normal tissues [63, 64]. In fact, the RAF inhibitors may activate ERK in normal tissue. This provides a greater therapeutic window allowing for administration of high doses of RAF inhibitors that effectively inhibit mutant BRAF in tumor cells [63]. This contrasts with MEK inhibitors that inhibit ERK activity in both cancer and normal tissues; any therapeutic activity is derived from the tumor dependence on ERK over

normal tissues. Although we must keep in mind that PLX4032 is selective for mutant BRAF over the other RAF proteins; this may contribute to or even explain the improved therapeutic for PLX4032 over PD325901. This may be of critical importance since the Poulikakos study demonstrated that low doses of pan-RAF inhibitors are also able to activate MEK through RAF dimer transactivation [63]. This observation raises the additional clinical caveat that selectivity for mutant BRAF, or the mutant form of any oncoprotein for that matter, will be critical for the clinical success of a drug. It also illustrates that suboptimal doses of a molecular targeted therapy may not only be ineffective but also have deleterious effects. Thus, the effective clinical use of these types of therapies may depend on determining pharmacodynamic endpoints in individual patients.

Feedback controls in the RAS-ERK signaling pathway may also impact the effectiveness of RAF (and MEK) inhibitors. In BRAF mutant melanoma, SPRY is unable to interact and inhibit mutant BRAF, however SPRY feedback at the level of RAS is intact as is the negative feedback phosphorylation of SOS by ERK [70]. This intact feedback loop may account for the low RAS activity levels in mutant BRAF cells, which may be important for the clinical efficacy of PLX4032. In support of this, exogenous expression of activated RAS in mutant BRAF cells renders PLX4720 ineffective [63]. This suggests that activation of RAS in mutant BRAF tumors, either by RAS mutation or activation from an upstream activator, could lead to resistance to PLX4720. Similarly, overexpression of CRAF generates resistance to PLX4720 [63]. However, to date neither of these potential mechanisms has been observed clinically.

The Convergence of PI3K-AKT and RAS-ERK Signaling

Considerable evidence exists for the concurrent mutational activation of PI3K-AKT and RAS-ERK signaling pathways in human cancers. These pathways are intricately linked. PI3K is a major RAS effector and PI3K activation by RAS has been studied extensively. PI3K activity is required for RAS mediated tumorigenesis [71]. In addition to direct activation of PI3K by RAS, RAS-ERK signaling can also modulate PI3K signaling through transcriptional regulation. Overexpression of mutant RAS downregulates PTEN expression in an ERK dependent manner [72]. Moreover, PI3K signaling can directly modulate RAF; AKT phosphorylates both CRAF and BRAF on inhibitory sites reducing RAF activity [73, 74]. Additionally, the kinase upstream of AKT, PDK1 (PDPK1), regulates MEK activity by phosphorylating MEK on the canonical RAF sites, Ser222 and Ser226 (Fig. 2.3) [75].

An additional feedback loop linking the RAS-ERK and PI3K-AKT signaling pathway involves the mTORC1. Inhibition of mTORC1 with rapamycin and its analogs in both experimental models and patient samples led to an increase in ERK activity [76]. This increase in ERK activity was dependent upon RAS, MEK, and PI3K. Expression of a dominant negative RAS construct that blocks RAS signaling or pharmacologic inhibition of MEK with UO126 abrogated rapamycin induction of

ERK as did a constitutively active rapamycin-insensitive S6K and pharmacologic inhibition of PI3K. The linkages between these two signaling pathways may explain in part why concurrent mutational activation is often observed and why inhibition of either pathway alone has insufficient effects on tumor growth and survival.

In addition to the feedback linkages between the PI3K-AKT and RAS-ERK signaling pathways, these signaling pathways converge downstream of ERK and AKT. ERK activates RSK, which in turn phosphorylates and activates S6K [77]. S6K phosphorylates several residues on S6 ribosomal protein and activates protein translation. RSK also phosphorylates eIF4B, which enhances its interaction with eIF3 and promotes translation [78]. AKT promotes the activation of the mTORC1 pathway, which activates protein translation through direct phosphorylation of S6K and 4E-BP1. The phosphorylation of 4E-BP1 results in its dissociation from eIF4E and activation of mRNA translation. A study by She et al, further suggests that 4E-BP1 mediates the effects of mutational activated PI3K-AKT and RAS-ERK signaling [79]. Tumors with mutations in both KRAS and PI3K are resistant to inhibition of either AKT or MEK alone. However, these tumors are sensitive to the combinatorial treatment of AKT and MEK inhibitors. As mentioned above, 4E-BP1 is a downstream effector for both AKT and ERK signaling that regulates protein translation through interaction with eIF4E. Concomitant inhibition of AKT and MEK effectively blocks 4E-BP1 phosphorylation leading to inhibition of tumor growth [79]. Blockade of 4E-BP1 directly by RNAi also decreased tumor growth. This work emphasizes the importance of knowing the mutational status of multiple genes and considering the redundancy present in the cell signaling network when choosing therapies, including combinations of molecular targeted agents.

The Rational Design of Effective Drug Combinations

The focus on combinations derived from the PI3K-AKT and RAS-ERK pathways is logical given the research to date, as is combining inhibitors of these pathways with RTK inhibitors. Uncovering feedback loops within these signaling pathways enables the prediction of effective combinations of molecular targeted therapies. Underlying the malignant phenotype is the extensively rewired cell signaling network. Therefore, the key to successfully treating cancer is identifying critical, functional nodes in the aberrant cancer cell signaling network whose inhibition will result in a fragile state leading to system failure. However, the analysis of feedback loops within these signaling pathways to date also suggests that unintended consequences of drug combinations may yet be uncovered which limit therapeutic efficacy. Thus, the challenge remains to functionally identify effective combination therapies.

One unbiased approach to identify effective drug combinations is analogous to synthetic lethal screening used in yeast genetics [80]. Two genes qualify as synthetic lethal if mutation or loss of either one alone is compatible with cell viability; but the simultaneous loss of both genes results in cell death. While the majority of synthetic

lethal interactions have been described for loss of function alleles, synthetic lethality also occurs with gain of function genes. One gene may become essential when another gene is mutationally activated or overexpressed, a phenomenon known as synthetic dose lethality [80]. This concept can be applied to developing cancer therapies – identifying genes that are synthetic lethal with oncogenes or tumor suppressor genes could be effective therapeutic targets. The therapeutic window for these drug targets should also be greater since in cancer one partner of the synthetic lethal pair is generated from an oncogenic or tumor suppressor mutation. Disruption of the pathway containing the second partner in the synthetic lethal pair will kill the cancer cells. However, in normal cells the function of that pathway is intact and will remain unaffected.

This concept of synthetic lethality may underlie clinical observations that are consistent with oncogene addition; CML, which responds well to gleevec, carries mutations in addition to the BCR/ABL fusion. Furthermore, EGFR mutant NSCLC that are initially responsive to gefitinib also carry mutations in addition to EGFR. Tumors may be responding to therapies targeting early mutations in cancer development and progression since subsequent mutations carry a selective advantage in the context of the preceding transforming mutations. These later mutations then may be deleterious in the absence of the earlier mutations. Thus, inhibiting gene targets that are altered early in cancer development may uncover synthetic lethal relationships with genes that are mutated late in cancer progression. This model may also help explain how mutant BRAF is such an excellent target in melanoma, even though mutations in BRAF are an early event found in premalignant nevi [81]. Inhibition of BRAF may be effective initially due to synthetic lethal relationships that are present in frank melanoma carrying additional oncogenic and tumor suppressor mutations.

Unbiased chemical and genetic screens can be used to uncover synthetic lethal relationships. Using RNAi to perform genetic screens, three studies identified STK33, PLK1, and TBK1 as synthetic lethal partners with mutant KRAS [82–84]. Scholl et al targeted approximately 1,000 genes across eight cell lines with half of those cell lines carrying an activating mutation in KRAS as well as normal human fibroblasts and immortalized human mammary epithelial cells [83]. The shRNAs targeted protein kinases, phosphatases, and known cancer related genes. Analysis of shRNAs that caused growth inhibition selective to cell lines harboring a mutant KRAS resulted in a small list of candidate synthetic lethal partners with STK33 ranked as the top hit. Subsequent experiments confirmed the synthetic lethal association of KRAS and STK33. Intriguingly the relationship was specific to KRAS; neither HRAS nor NRAS are synthetic lethal with STK33.

Using a pooled shRNA approach, Luo et al targeted over 30,000 unique transcripts in two isogenic cell lines with one carrying a mutant KRAS and the other a wild-type KRAS [84]. From both the initial screen and subsequent analysis, including experiments using a second isogenic pair of cell lines, a list of 77 genes with a synthetic lethal relationship with KRAS were ultimately identified. Computational analysis suggested that mutant KRAS cells have an increased dependency on genes regulating the proteasome and the mitotic machinery. This led

to preferential killing of KRAS mutant cells by drugs that target the mitotic machinery including a preclinical PLK1 inhibitor or a proteasome inhibitor (bortezomib). This effect was specific to mutant KRAS as analysis of isogenic lines with and without an activating PI3K mutation showed the opposite effect; PI3K mutants were less sensitive to mitotic machinery inhibitors.

Barbie et al used a meta-analysis of RNAi screens targeting protein kinases, phosphatases, and cancer related genes in 19 cell lines to identify mutant KRAS synthetic lethal partners [82]. They identified TBK1, which is a noncanonical IKB kinase as the top ranked KRAS synthetic lethal partner. In follow up studies, apoptosis was induced when TBK1 was suppressed in cancer cell lines where mutant KRAS is a driver gene. Mechanistic analysis revealed that TBK1 activated NFkB survival signals involving REL and BCLXL. Interestingly, REL as well as STK33 and PLK1 were identified in this study but fell below the cutoff in secondary analysis. Collectively, these studies establish the paradigm for rationally identifying combinatorial targets for inhibiting cancer growth. Validation of this approach will come as inhibitors of these synthetic lethal partners for mutant KRAS cancers are evaluated in the clinic.

It is plausible that these synthetic lethal partners are the fragile nodes within the cell signaling network predicted from HOT theory. Inhibition of these single nodes generates cytotoxicity that is dependent on the aberrant cell signaling network in the cancer cells. This is consistent with the system robustness, driven by mutational activated RAS signaling, being paid for with fragile nodes such as STK33, PLK1, and TBK1. However, the plasticity of the cell signaling network will likely facilitate resistance. STK33, PLK1, and TBK1 may be fragile nodes in cancer cells, but it is not yet determined if these are fragile nodes for the cancer system. Consistent with this concern is the clinical data, which thus far suggests that the response to single agent molecular targeted therapies is not durable. Thus, it remains likely that drug combinations will still be required. While the clinical effectiveness of these synthetic lethal targets is not yet known, it would be interesting to test if combinations of STK33, PLK1, and TBK1 inhibition are more effective and less likely to give rise to resistance than inhibition of any one synthetic lethal partner in KRAS mutant cancer.

The general paradigm used to date for identifying combinations of molecular targeted agents has relied on our understanding of the cell signaling network. This has led to the preclinical development of drug combinations targeting members of the RAS-ERK, PI3K-AKT, and RTK signaling pathways that have been selected empirically. The synthetic lethal approach has functionally identified targets that conceptually align with combination therapy; inhibition of the synthetic lethal partner is dependent upon combination with the activating oncogene. Another unbiased approach not yet published would be screening for drug or RNAi combinations that display synthetic lethality measured by synergistic growth inhibition. The hope is that the confluence of studies focused on particular subsets of the cell signaling network (e.g., PI3K-AKT and RAS-ERK) and the unbiased screening methods being employed will yield new and effective drug combinations for preclinical development and clinical trials.

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