

Chapter 6

Mechanisms of Resistance to PI3K and AKT Inhibitors



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Abstract Hyperactivation of the PI3K pathway is frequent in human cancer. Whether it occurs via overexpression/phosphorylation of upstream receptors that promote the binding and activation of PI3K, or as a consequence of activating alterations of the nodes of the signaling cascade, deregulated PI3K signaling can promote tumor growth and survival. This provided the rationale to develop inhibitors targeting virtually all the components of this pathway. Despite these efforts, however, the responses in the clinic have been anecdotal and short lived for most of these agents.

In the last few years, clinical studies have demonstrated that specific compounds can elicit strong antitumor activity if administered to selected patients. For example, AKT catalytic inhibitors and specific PI3K α inhibitors have shown promising clinical responses in patients with tumors bearing activating mutations of AKT and PIK3CA, respectively. Nevertheless, the intrinsic or acquired resistance to PI3K/AKT/mTOR inhibitors limits the activity of these agents. The mechanisms that tumor cells adopt to by-pass pharmacological inhibition of PI3K/AKT/mTOR are tissue-dependent and can be the results of either pre-existing conditions that rapidly compensate for the therapeutic pressure or the acquisition of genomic and/or epigenomic changes that confer fitness over time even upon PI3K full blockade. In both cases, combinatorial strategies seem to be necessary to prevent or delay the emergence of drug resistance, and many of these therapeutic options are currently being tested in the clinic.

Keywords Signaling pathway • Drug resistance • Targeted therapy • Protein kinase

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Abbreviations

| | |
|-----------|--|
| AGC | Protein Kinase A, G, And C Kinase Family |
| AKT | RAC-Alpha Serine/Threonine-Protein Kinase |
| AMP | Adenosine Monophosphate |
| AMPK | AMP-Dependent Protein Kinase |
| ARF | ADP Ribosylation Factors |
| ATP | Adenosine Triphosphate |
| BAD | BCL2 Associated Agonist of Cell Death |
| BCL2 | B-Cell Lymphoma 2 |
| BRD4 | Bromodomain And Extra Terminal Domain 4 |
| Cdc42 | Cell Division Cycle 42 |
| DEPTOR | DEP Domain-Containing Mtor-Interacting Protein |
| Eif4e | Eukaryotic Translation Initiation Factor 4E |
| ER | Estrogen Receptor |
| ERK | Extracellular Signal-Regulated Kinase |
| FOXA1 | Forkhead Box A1 |
| FOXG1 | Forkhead Box G1 |
| FOXO | Forkhead Box O |
| GAP | GTP-Ase Activating Protein |
| GDP | Guanosine Diphosphate |
| GTP | Guanosine Triphosphate |
| H3k4me1/2 | Histone 3 Lysine 4 Mono-/Di-Methylated |
| HER2 | Human Epidermal Growth Factor Receptor 2 |
| IGFR1 | Insulin-Like Growth Factor 1 Receptor 1 |
| IRS1 | Insulin Receptor Substrate 1 |
| KMT2D | Histone-Lysine N-Methyltransferase 2D |
| LKB1 | Liver Kinase B1 |
| MAPK | Mitogen-Activated Protein Kinases |
| MEK | MAPK/ERK Kinase |
| MLST8 | Mammalian Lethal with SEC13 Protein 8 |
| MYC | V-Myc Avian Myelocytomatosis Viral Oncogene Homolog |
| P16ink4a | 16 kDa Inhibitor of Cyclin-Dependent Kinase Type 4A |
| P21CIP1 | 21 kDa CDK-Interacting Protein 1 |
| P27KIP | 27 kDa Kinase Inhibitor Protein |
| PBX1 | Pre-B-Cell Leukemia Transcription Factor 1 |
| PKD1 | 3-Phosphoinositide Dependent Protein Kinase-1 |
| PGC-1 | Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 |
| PIF | PKD1-Interacting Fragment |
| PIM | Proviral Integration Site for Moloney Murine Leukemia Virus-1 |
| PKC | Protein Kinase C |
| PRAS40 | Proline-Rich Akt Substrate of 40 kDa |
| PROTOR | Protein Observed with Rictor-1 |
| PTEN | Phosphatase and Tensin Homolog |
| Rac1 | Ras-Related C3 Botulinum Toxin Substrate 1 |

| | |
|--------|---|
| RAF | Rapidly Accelerated Fibrosarcoma |
| RAPTOR | Regulatory Associated Protein of MTOR Complex 1 |
| RHEB | Ras Homolog Enriched in Brain |
| RICTOR | Rapamycin-Insensitive Companion of MTOR |
| RSK | 90 kDa Ribosomal S6 Kinase |
| SH2 | Src Homology 2 |
| SIN1 | Stress-Activated Map Kinase-Interacting Protein 1 |
| SMAD | Mothers Against Decapentaplegic Homolog |
| TSC2 | Tuberous Sclerosis Complex Protein 2 |
| VPS15 | Vacuolar Protein Sorting 15 |
| VPS34 | Vacuolar Protein Sorting 34 |

6.1 The PI3K/AKT Pathway

6.1.1 Overview

Receptor tyrosine kinases (RTKs) are large proteins that exist as monomers, dimers, or multimers and can be found embedded in the plasma membrane through a relatively short transmembrane-spanning domain. While the amino-terminal portion of these proteins is mainly involved in the recognition of extracellular ligands, the carboxy terminus is intracellular and has been shown to serve as a docking platform to many signaling molecules, especially upon phosphorylation [1]. Therefore, RTKs are generally associated with proteins that are responsible for triggering the downstream signal transduction when the receptor has been stimulated/activated. There are several signaling pathways that are activated by RTKs, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), Src, phospholipase C (PLC), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways, among others [2]. In this chapter, we will describe the basic knowledge regarding the biochemistry and signal transduction of the PI3K pathway, the current pharmacological strategies aimed to target this pathway, and we will discuss in detail the current mechanisms of resistance to this family of inhibitors.

The PI3K family is composed by eight members with catalytic lipid kinase activity classified in three groups according to their substrate specificity and structure. Class I PI3K use phosphatidylinositol (PI)-(4,5)-bisphosphate (PIP2) as a substrate in order to generate phosphatidylinositol-(3,4,5)-trisphosphate (PIP3). Both class II and III give rise to phosphatidylinositol 3-phosphate from the unphosphorylated substrate PI. While the Class I of PI3K is mainly involved in the signal transduction downstream of receptors such as RTK and G-protein coupled receptors (GPCR), class II and III appear to be related with vesicular trafficking. Intensive work in the field of class II and III PI3K is currently undergoing to better understand the physiological and pathophysiological roles of these lipid kinases [3–5]. For the purpose of this chapter, we will focus on the class I PI3K and use the term “PI3K” to refer specifically to this class of kinases (Table 6.1).

Table 6.1 The PI3K family: isoforms, substrates, and functions

| Class | Catalytic subunit | Regulatory subunit | Substrate | Function |
|-------|---|--|--------------|--|
| Ia | p110 α p110 β p110 δ | p85 α/β , p55 α , p50 α and p55 γ | PIP2 | Angiogenesis, cell growth, metabolism, motility, transformation, immune cell biology, etc. |
| Ib | p110 γ | p101 and p87 | PIP2 | |
| II | PI3K-C2 α PI3K-C2 β PI3K-C2 γ | | PI (P4P)? | Glucose metabolism, cilium function, migration, angiogenesis. |
| III | VPS34 | VPS15 | PI | Endosomal biology |

6.1.2 Biochemistry and Genetics

PI3K enzymes are defined by their lipid kinase activity, required to phosphorylate the 3-OH' residue of the inositol ring of PIP2. This enzymatic activity is carried out by the catalytic subunit (p110), which is normally associated with a regulatory subunit. Each subunit contains different protein domains [6, 7]. These domains are important for the biochemical and structural functions of the protein and, in the case of p110, include:

- **Kinase domain:** Required for the enzymatic activity of the protein.
- **Helical domain:** It is used as an interacting interphase not only with other proteins but also within the structure of p110.
- **C2 domain:** It is thought to participate in the phospholipid binding required for plasma membrane targeting.
- **Ras-binding domain (RBD):** This region exhibits high affinity towards the GTP-loaded small GTPase Ras.
- **p85-binding domain:** Also known as the N-terminal adaptor-binding domain (ABD), this region is responsible for the binding to the regulatory subunit.

The regulatory subunit of PI3K (p85) is characterized by the presence of two different SH2 domains (nSH2 and cSH2), an SH3 domain, a BH domain, and an inter-SH2 domain (iSH2). While the SH2 domains are required for binding to the activated phospho-Tyr residues of RTK, the inter-SH2 domain seems to be responsible for the interaction with the ABD domain of p110. The BH domain has been shown to interact to small GTP-ases Rac1 and Cdc42. The regulatory subunits of PI3K have multiple functions. They stabilize the catalytic subunit, inhibit the basal kinase activity, and they also engage the activation of the catalytic subunit downstream of the phosphorylated tyrosine motifs as a result of the interaction with their SH2 domains [8].

The crystal structure of PI3K α in complex with the regulatory subunit p85 α was initially solved in 2007 [9], and provided deep insight into the domain distribution, catalytic mechanism, and the template for rational drug design. X-ray crystallography and hydrogen-deuterium exchange mass spectrometry have shown that there are several inhibitory interfaces between p85 and p110. For instance, the p85 nSH2

domain creates inhibitory interfaces with the C2, helical, and C-lobe kinase domains of p110, the p85 iSH2 domain with the p110 C2 domain, and the p85 cSH2 domain with the p110 C-lobe kinase domain. This last interaction is, in fact, an isoform-specific regulatory mechanism, since only the p110 β and p110 δ exhibit this contact [10].

There are four different genes that encode the Class I PI3K family (*PIK3CA*, *PIK3CB*, *PIK3CD*, and *PIK3CG*). A lot of the knowledge regarding the functions of each catalytic and regulatory isoform of PI3K has been achieved through the generation of transgenic mice that either lack a gene (knock-out) or express a kinase inactive version of these genes (knock-in). These models are not only important to elucidate the contribution of each isoform into the normal biology, but also to gain insights into the possible secondary effects that prolonged inhibition of these kinases could lead to [5, 11]. For instance, using these genetic mouse models it has been elucidated that *Pik3ca* inhibition results in major defects in the generation of the vascular system [12]. *Pik3ca* knock-out mice die at the embryonic stage (E9.5) from severe vascular defects and impaired proliferation [13]. Similar results have been observed with *Pik3ca* knock-in mice, where the D933A mutation renders the kinase inactive. Although heterozygous mice are viable, vascular defects are still observed, together with a metabolic impairment [14]. In the case of *Pik3cb*, the difference between the two strategies to generate mouse models is more accentuated. While the knock-out mice die at E3.5, the knock-in version only remains partially lethal, suggesting a non-catalytic function of this isoform during the development [3, 14]. Additionally, these mice exhibit impaired insulin and GPCR-dependent signaling resulting in a metabolic phenotype. *Pik3cd* and *Pik3cg* mouse models evidence an important contribution of both kinases in the immunological response. None of the strains reported are embryonically lethal, but adults either carrying the kinase-inactive mutation or lacking the gene have impaired signaling and functions in the B and T cells, neutrophils, and macrophages [15–18]. Importantly, *Pik3cd* mice also display allergy and have been recently linked to decreased T-cell-dependent cancer immunotolerance, encouraging the use of PI3K δ inhibitors as immunotherapy [19].

6.1.3 Signal Transduction by the PI3K Pathway

A schematic of the PI3K pathway is depicted in Fig. 6.1. Upon catalytic activity of PI3K, the levels of PIP3 in the membrane raise drastically. The presence of PIP3 at the plasma membrane triggers the rapid activation of downstream effectors that are involved in cell survival, proliferation, motility, control of metabolism, and gene expression among others [3]. The key molecular features involved in the recognition of PIP3 are the phosphoinositide-binding domains, which are present in several proteins that are part of the PI3K pathway and involved in the subcellular localization or the activation of these proteins. Specifically, the pleckstrin homology domain (PH) has been shown to preferentially bind PIP3 over other phosphoinositides. There are over 250 proteins that contain identifiable PH domains, underscoring the complexity of the lipid signal cascade [20]. Among all these proteins, the better

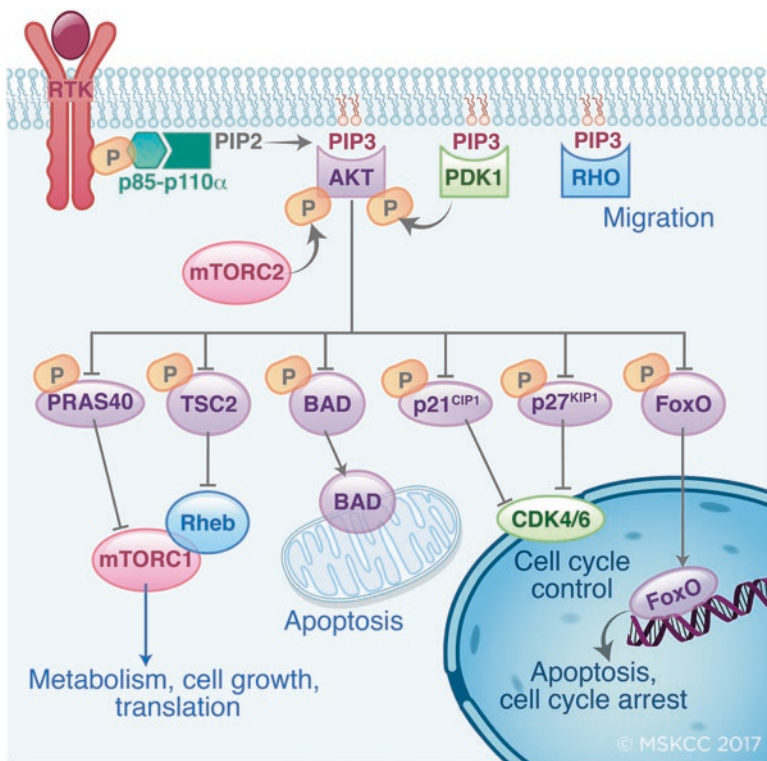


Fig. 6.1 The PI3K/AKT pathway. Activation of the PI3K/AKT pathway is the result of several stimuli. However, it is commonly associated to RTKs activation through the binding of the regulatory subunit p85 to RTK phosphorylated residues. The lipid kinase activity of the catalytic subunit of PI3K (p110) generates the second messenger PIP3, which recruits different effectors to propagate the downstream signaling cascade. PDK1 and mTORC2 activate AKT by two phosphorylations, allowing this kinase to phosphorylate an array of molecules involved in cell death, cell cycle control, metabolism, and other cellular effects. Here, we have represented some downstream effectors that include, but are not limited to, PRAS40 and TSC2 (negative regulators of mTORC1), BAD (pro-apoptotic BH3-containing protein), p21^{CIP1} and p27^{KIP1} (CDK inhibitors and negative regulators of the cell cycle), and FOXO transcription factor (apoptosis and cell cycle transcriptional regulator)

characterized PH domain-containing effectors of the PI3K pathway are the RHO and ARF Guanine Nucleotide Exchange Factors (GEFs), PLC, and the kinases AKT and 3-phosphoinositide dependent protein kinase-1 (PDK1), among others. In terms of the cellular signaling in cancer, PDK1 appears to play a major role. Experiments using *Pdk1* (the gene coding PDK1) knock-out mice have shown to reduce tumor burden when crossed with *Pten* (the gene coding for Phosphatase and tensin homolog, PTEN) heterozygous mice, which are prone to malignancies such as lymphoma and prostate cancer in a PI3K-dependent manner [21]. PDK1 contains a high affinity PH domain that has the ability to recognize PIP3 upon PI3K activity. Although PDK1 is constitutively active, the PH domain provides substrate specificity upon

translocation into the plasma membrane, where some of the PDK1 targets are recruited. This is the case of AKT, which contains a PH domain that requires the interaction with PIP3 in order to first unfold the kinase domain due to a conformational change and second interact with PDK1 in the plasma membrane [22]. The presence of the lipid phosphatase PTEN is sufficient to revert this event, by decreasing the levels of PIP3 in the plasma membrane [23].

At the plasma membrane, AKT is phosphorylated at the activation loop (T308 in AKT1) by PDK1 [24], and this appears to be sufficient to partially activate the kinase. Additionally, the mTOR complex 2 (mTORC2) phosphorylates AKT at the hydrophobic motif (S473 in AKT1), providing increased activity and/or substrate specificity [25]. mTORC2 is a large protein complex formed by the kinase mTOR and several proteins required for the proper assembly and substrate recognition, such as RICTOR, SIN1, MLST8, DEPTOR, and PROTOR among others [26]. mTORC2 is required for the hydrophobic motif phosphorylation of several protein kinases, including AKT, and some evidences also suggest that mTORC2 is able to phosphorylate AKT and PKC at the turn motif (T450) during protein translation [27, 28]. The mechanism of activation of mTORC2 remains elusive, although it has been proposed that the complex would be activated in the presence of PIP3 due to the presence of a PH domain in the subunit SIN1 [29]. It has been suggested that other kinases might be responsible for the phosphorylation of the hydrophobic motif of AKT; however the cumulative evidence using genetic and pharmacologic tools suggest that mTORC2 is the main upstream kinase, if not the only one.

The AKT family of serine/threonine protein kinases contains three isoforms that are encoded by the genes *AKT1*, 2, and 3. Activation of AKT is considered a key output of the PI3K pathway due to the large number of substrates interacting with this kinase, including mediators of apoptosis, cell cycle, metabolism, and others that contain the consensus motif RXXRX(S/T) [30, 31]. For instance, AKT is able to phosphorylate and inhibit BAD, a proapoptotic member of the BCL-2 family, and Caspase 9, two main regulators of the mitochondrial apoptotic pathway. It also inhibits the cyclin-dependent kinases (CDKs) inhibitors p21^{CIP1} and p27^{KIP}, directly related with the inhibition of cell cycle progression. Moreover, AKT can also inhibit the forkehead transcription factors FOXO1, 3, 4 and 6, involved in the transcriptional regulation of several genes including the proapoptotic *CD95L*, *BCL2L11* (BIM), *BBC3* (PUMA), *CDKN2A* (p21^{CIP1}) and *CDKN2B* (p27^{KIP}) [32]. In addition, AKT can phosphorylate PRAS40 and TSC2, two negative regulators of mTORC1 activity [33, 34].

Similar to mTORC2, mTORC1 is a large protein complex containing the kinase mTOR. However, in this case the complex is associated to the protein RAPTOR, which dictates substrate specificity [35]. mTORC1 senses and responds to environmental cues such as nutrient availability, stress, and mitogens to regulate protein synthesis through a highly orchestrated and complex mechanism. mTOR was originally identified in the early 1990s as a mutated protein that can confer resistance to the growth inhibitory effects of rapamycin in yeast and it was later considered a master regulator of cell growth and metabolism that signals to 4E-binding protein 1 (4EBP1) and 40S ribosomal protein S6 kinase (S6K), which are both important in

the physiological control of mRNA translation. In fact, mTORC1 promotes protein synthesis by phosphorylating 4EBP1, which in turn prevents 4EBP1 binding to the eukaryotic initiation factor 4E (eIF4E), enabling eIF4E to initiate cap-dependent translation. On the other hand, the activation of S6K1 by mTORC1 leads to an increase in mRNA biogenesis and cap-dependent translation. mTORC1 has also been demonstrated to activate RNA Pol I transcription and thus rRNA synthesis through a process involving the protein phosphatase 2A (PP2A) and the transcription initiation factor IA (TIF-IA) [36].

The AKT-mediated phosphorylations of PRAS40 and TSC2 inhibit their activity, leading to an increased mTORC1 signaling [33, 37]. Mechanistically, it has been suggested that phosphorylation of TSC2 by AKT promotes the translocation of the TSC complex away from the lysosomes, where the small GTPase RHEB is found, to activate mTORC1. In the absence of AKT activity, the Tuberous Sclerosis Complex (TSC) complex translocates to the lysosome, where TSC2 acts as a GTPase Activating Protein (GAP) towards RHEB. The resulting GDP-loaded RHEB is unable to activate mTORC1 leading to the inhibition of this complex [38].

6.2 PI3K/AKT Activation in Cancer

Many tumor types are characterized by constitutive activation of the PI3K/AKT pathway; however, here we will focus only on those malignancies for which the blockade of this signaling cascade may be a valid (and rational) therapeutic strategy. The net output of this pathway can be roughly defined as the algebraic sum of several alterations that contribute to activate the downstream effectors of PI3K/AKT/mTOR. Thus, any step from RTKs aberrant phosphorylation to sustained mTORC1 activity may be responsible for increased signaling and, possibly, represents an actionable therapeutic vulnerability.

HER2 overexpression (mainly by gene amplification) is a classical example of upstream activation of the PI3K pathway. It occurs in about 15–20% of breast cancer and, in lesser percentages, in other malignancies such as gastric, endometrial, ovary, salivary, colon. The same may apply for EGFR overexpression in triple negative breast cancer (lacking the expression of hormonal receptors and *ERBB2* amplification, TNBC), lung and colon, MET in gastric and lung and FGFR1 in breast.

More downstream, activating mutations of PI3K (either in the regulatory subunit p85 or, more frequently, in the catalytic subunit p110) are responsible for the aberrant PIP3 production and consequent increase of AKT/mTOR signaling. Data from the breast tumor samples analyzed by the TCGA and our internal cohort of patients at Memorial Sloan Kettering Cancer Center show that about 25% of breast cancers exhibit mutations in *PIK3CA*, the gene encoding the p110 α subunit of PI3K [39, 40]. These frequently involve hotspots that are characterized by mutations on the helical (E545K, E542K) and kinase (H1047R) domains of the p110 α catalytic subunit of PI3K. Besides breast cancer, *PIK3CA*

is frequently mutated also in head and neck cancer, endometrial cancer, ovarian cancer and hematological diseases.

Loss of expression/function of PTEN and/or INPP4B lipid phosphatases can also occur, also resulting in increase PIP3 production and activation of the pathway [41, 42]. PTEN loss, by genomic deletion or epigenetic silencing, is frequent in TNBC, prostate cancer, glioblastoma, endometrial cancer and stomach cancer.

AKT is also activated directly via mutations and copy-number alterations of the AKT isoforms. The most frequent AKT mutation is found in the PH domain of AKT1 where a glutamic acid is substituted with a lysine residue at amino acid 17 (E17K) [43], resulting in enhanced activity of the kinase. This mutation leads to a constitutive membrane localization of the kinase and increased phosphorylation on T308 and S473 in a PI3K-independent manner [43–45]. AKT1^{E17K} mutation is present in several tumor types, but is more frequently detected in invasive breast carcinoma with an overall somatic mutation rate of 2.5% (TCGA results from 1098 patients).

Less common non-hotspot mutations in AKT1 with varying transforming potential have been reported in human breast cancers [46]. AKT3 is the most frequently amplified AKT isoform in breast cancer, and has been mostly studied in the triple-negative subtype in the context of resistance to therapy [47].

Activating mutations have been also reported to occur in mTOR. These alterations can induce resistance to either rapalogs (see below) or mTOR catalytic inhibitors [48–50] and can be present in both therapy naïve patients or emerge as a consequence of mTOR blockade.

Canonically, it is thought that activating mutations in different effectors of the PI3K/AKT/mTOR pathway are mutually exclusive. This assertion is, however, based mainly on primary untreated tumors. It is in fact still early to know whether this scenario could change in tumors undergoing pharmacological pressure.

6.3 Inhibitors Targeting the PI3K-AKT Pathway

The first inhibitors of this pathway were isolated more than two decades ago and targeted other kinases such as mTOR and DNA-PK. These agents included wortmannin and LY294002 and, due to the lack of therapeutic windows, their use was confined to the laboratory as tool compounds [51, 52]. The first “modern” inhibitor of the PI3K pathway was BEZ235 (Novartis Pharmaceuticals). Initially thought to be a pan-PI3K inhibitor, they later discovered it targets also mTOR with equal or higher potency [53, 54]. Because of its strong antitumor activity in several preclinical models, either used as single agent or in combination with other targeted agents, BEZ235 was tested in a number of clinical trials. Unfortunately, despite anecdotal responses in a variety of tumor types, the toxicity profile and poor pharmacokinetic properties lowered the enthusiasm about this molecule [55]. Additional dual PI3K/mTOR inhibitors were successively developed and are now under clinical investigation.

Other molecules more selective for the PI3K enzyme were isolated shortly thereafter. GDC-0941 (Genentech Inc. [56]) and BKM120 (Novartis Pharmaceuticals [57]) target all the isoforms of PI3K (pan-PI3K) and are likely the most studied of this class. GDC-0941 showed good pharmacokinetic properties and has been tested in a variety of solid cancers. Despite anecdotal responses across many tumor types, its future clinical development is currently uncertain due to generally modest anti-tumor activity [58]. BKM120 has been extensively studied in many preclinical models and clinical settings, spanning from breast to glioblastoma, head and neck, lung and other cancers. A peculiar characteristic of this agent is the blood-brain barrier permeability [59], which on one hand renders it suitable for the treatment of brain tumors or brain metastases, but on the other can cause moderate to severe mood disorders [60]. BKM120 has recently shown interesting antitumor activity in both TNBC and estrogen receptor (ER)-positive breast tumors [60, 61]. Specifically, in TNBC BKM120 seems to induce DNA damage and sensitizes these tumors to the Poly (ADP-ribose) polymerase (PARP) inhibitor olaparib, independently of the BRCA status [62], whereas in ER-positive tumors BKM120 can synergize with the ER degrader fulvestrant. These combinatorial strategies have been tested in the clinic with promising preliminary results and, in the ER-positive setting, BKM120 showed a significantly increase activity in tumors bearing *PIK3CA* mutations.

Despite these encouraging evidences, the therapeutic window of these compounds is still a major limitation for their clinical development. In the attempt to obviate this bottleneck, pharmaceutical companies engaged in the development of isoform-specific PI3K inhibitors that, if given to the appropriate patients, can elicit strong antitumor activity with tolerable on-target toxicity. The two most promising compounds targeting specifically p110 α are BYL719 (Novartis Pharmaceuticals [63]) and GDC-0032 (Genentech Inc. [64]). The latter also targets p110 $\gamma\delta$ and is more potent against the H1047R and E545K p110 α mutants compared to the wild-type isoform. Used as monotherapy, both agents resulted in convincing clinical responses in a number of solid tumors bearing *PIK3CA* mutations, with particular activity in breast and head and neck cancers [65–67]. Not surprisingly, most of the efforts for the clinical advancement of these compounds are indeed focused in the treatment of these two tumor types. In breast cancer patients, both BYL719 and GDC-0032 are being tested in combination with anti-hormonal therapy [68] whereas head and neck patients are treated with single agent or in combination with the anti-EGFR antibody cetuxumab or with radiation.

Other isoform-specific PI3K inhibitors targeting p110 β or p110 δ have been developed for different indications. One of the most successful is CAL-101, which was approved by the Food and Drug Administration (FDA) for the treatment of chronic lymphocytic leukemia, small lymphocytic lymphoma and follicular lymphoma [69].

Downstream PI3K, catalytic (e.g. AZD5363, GDC-0068) and allosteric (MK-2206) AKT inhibitors have also been investigated in both preclinical and clinical settings. Although these agents have shown antitumor activity in several *PIK3CA*-mutant and PTEN-deficient experimental models [70–72], their clinical efficacy in non-selected patients have been anecdotal [73–76]. In patients with tumors bearing the AKT1 E17K mutation, however, the activity of AZD5363 has been remarkable [77].

Finally, the allosteric inhibitor of mTORC1 and the catalytic inhibitor of mTOR have been extensively tested in numerous laboratory models and in clinical trials. Everolimus is certainly the most known and clinically successful allosteric mTORC1 inhibitor, having achieved the approval by the FDA for several solid tumors, including ER-positive breast cancer when combined with aromatase inhibitors [78]. Catalytic mTOR inhibitors have been developed to obviate the paradoxical increase in AKT caused by anti-mTORC1 allosteric agents (see below) and because they can inhibit directly mTORC2 and, as a consequence, AKT. These compounds are being studied in the clinic [79, 80], but their relatively narrow therapeutic window will likely require an accurate patient selection and/or combinations with other therapeutic agents.

6.4 Mechanisms of Resistance to PI3K/AKT/mTOR Inhibitors

Similar to virtually all anticancer drugs, resistance to PI3K/AKT/mTOR inhibitors can be *de novo*, when cells are intrinsically refractory to the antitumor activity of these agents, or acquired, when tumors initially respond but eventually escape therapy over time.

Typically, intrinsically resistant tumors either carry genomic alterations that prevent or nullify the inhibition of the target or are capable to adapt to the pharmacological stress by triggering the activation of compensatory pathways. Acquisition of resistance, instead, usually occurs via positive selection of tumor clones that are (or become) genetically or epigenetically predisposed to survive even in the presence of PI3K/AKT/mTOR suppression.

In this part, we will discuss the most common mechanisms of resistance to PI3K inhibition described to date.

6.4.1 Resistance Mediated by RTK Activation

Pharmacological inhibition of the PI3K/AKT/mTOR signaling cascade can induce a rapid overexpression/activation of RTKs that, in turn, can fuel downstream signaling pathways and limit the effectiveness of this therapy (Fig. 6.2). More than a decade ago, it was reported that inhibition of mTOR can release a negative feedback phosphorylation of AKT mediated by insulin receptor substrate-1 and result in the activation of the PI3K/AKT pathway [81]. This work, very provocative at that time, pioneered the field in what it turned out to be a common occurrence in targeted therapy: activation of RTKs in response to downstream effectors inhibition. A few years later, three independent investigations converged to the same conclusion that inhibition of the PI3K/AKT pathway leads to overexpression and activation of HER3, HER2 and other RTKs [82–84]. The relevance of this cellular adaptation was underscored by the fact that the concomitant inhibition of both PI3K/AKT and the

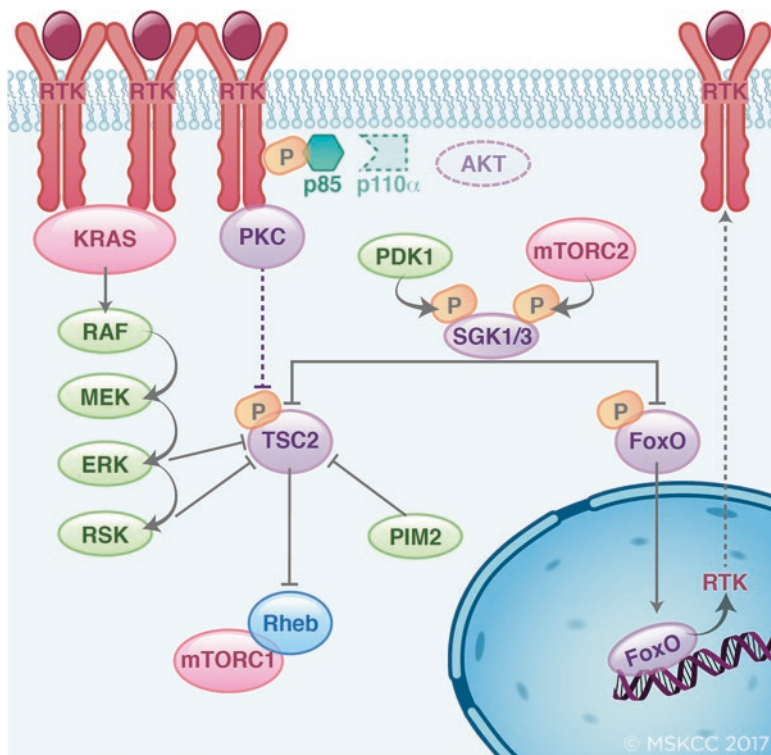


Fig. 6.2 Mechanisms of resistance mediated by kinases. Upon inhibition of PI3K or AKT the pro-survival mechanisms of the cancer cell are challenged, leading in multiple cases to cell death or cell cycle arrest. However, cancer cells can overcome these pharmacological stresses by relying on parallel signaling pathways that lead to the pro-survival phenotype. For instance, upregulation of RTKs is a common effect resulting from the transcriptional activity of FOXO transcription factors upon PI3K/AKT inhibition. Increased RTK signaling has been shown to activate the RAS/RAF/MEK/ERK mitogenic pathway. Two major signaling nodes, TSC2 and FOXO, contain several AKT phosphorylation consensus motifs that can be also phosphorylated by other kinases upon inhibition of PI3K/AKT. Some examples discussed depending on the tumor type include PIM2, SGK1/3, RSK3/4, ERK, and other putative kinases such as the PKC family. Importantly, most of these kinases could also be inhibited by selective pharmacologic inhibitors

upstream RTKs resulted in superior antitumor effects. As a matter of fact, a number of subsequent studies confirmed the validity of this therapeutic strategy in different preclinical models [72, 85–87].

Typically, RTK overexpression occurs rapidly in response to PI3K/AKT inhibition. However, in some instances this can also be the result of continuous suppression of the pathway. It is the case of AXL overexpression in response to acquired resistance to the PI3K inhibitor BYL719 in head and neck cancer models [88]. In this work, we describe that the increase expression of this RTK is sufficient to limit the sensitivity to PI3K inhibition by interacting with EGFR and circumventing PI3K pathway blockade. Although AXL expression is likely a multi-resistance

mechanism [89], at least another study identified AXL as a causative player for inducing resistance to PI3K inhibition [90].

More than one mechanism triggering RTK overexpression upon PI3K/AKT inhibition is likely to be at play; none of which, however, seems to be attributable to stable genomic amplification of the genes coding these receptors (Fig. 6.2). Chandarlapaty et al. reported that FOXOs transcription factors shuttle to the nucleus of the cells as a result of AKT inhibition and promote RTK expression [82]. Another group reported that the PIM-1 kinase regulates the increase expression of RTKs in response to AKT inhibition in prostate cancer [91]. In any case, the overall output is the activation of downstream signaling that compensates for the pharmacological PI3K/AKT blockade.

6.4.2 Dependency on Other PI3K Isoforms

As mentioned above, current pharmacological approaches in the field of PI3K appear to move towards the inhibition of specific isoforms of this enzyme. For example, inhibitors targeting the PI3K α have been shown to be more effective in malignancies harboring mutations in *PIK3CA* [65–67], while inhibitors targeting the PI3K δ isoform have been approved for the treatment of relapsed chronic lymphocytic leukemia [92]. However, a clear disadvantage for the use and development of this class of compounds is that other isoforms could participate in the re-activation of the pathway because of the differential regulation of the PI3K isoforms. In fact, several reports indicate that a crosstalk among the different isoforms occurs in the context of PI3K inhibition resistance.

For instance, it has been shown that the pharmacologic effect of PI3K α inhibitors is diminished as a result of increased PIP3 accumulation over time. This rebound is particularly evident in HER2-positive cells and is the result of an increased dependency on the p110 β isoform, since both inhibition and knockdown of such isoform reduce the levels of PIP3 [93]. Moreover, the combination of p110 α and p110 β inhibitors exhibits greater anti-tumor effects than single agent treatment in BT474 xenografts. Although the mechanism is not well-understood, some evidences suggest that both RTKs and GPCRs could participate in this phenotype [93]. In line with these findings, a similar phenotype has been reported for PTEN-deficient prostate tumors. Loss of PTEN is linked to increased dependency on different PI3K isoforms in a tissue-specific manner. In the case of prostate malignancies, p110 β appears to be the major player when PTEN is lost [94]. On the contrary, thyroid tumors, glomerulonephritis, and hamartoma syndrome appear to depend on both the p110 α and p110 β isoforms [95].

There is high prevalence of PTEN loss in prostate cancer, hence treatment with PI3K p110 β inhibitors has been considered as a possible therapeutic strategy. However, there are compensatory mechanisms as a result of increased p110 α signaling. In LNCAP prostate cancer cells, the increased p110 α signaling is mediated by IGF1R [96]. A plausible explanation is that the feedback mediated by S6K

phosphorylation of IRS-1 would be critical to re-activate the PI3K pathway through an alternative PI3K isoform.

We have described a genetic mechanism of resistance to p110 α inhibitors identified in the metastatic lesions from a patient that relapsed to the treatment with the p110 α inhibitor BYL719 [42]. In this case, the pharmacological pressure upon treatment with BYL719 selected for tumor cell populations carrying inactivating mutations and deletion in *PTEN*. Similar to the observations in prostate cancer, loss of *PTEN* expression results in increased dependency on the p110 β isoform, bypassing the therapeutic effects of BYL719. Continuous efforts by us and others in genotyping resistant lesions have evidenced that this is a fairly common mechanism of resistance also observed in other p110 α inhibitors such as GDC0032 (unpublished results). The combination of isoforms specific inhibitors is efficacious in treating patient-derived xenografts from resistant metastatic lesions and cell lines engineered to express shRNA against *PTEN* and also pan-PI3K inhibitors have been shown to be active in such context, because of their ability to target both isoforms [42]. The lack of predilection towards a specific isoform could also shed light on the fact that pan-PI3K inhibitors efficacy is not associated with the *PIK3CA* status in tumors and cell lines. In *PTEN*-negative breast cancer cell lines, a report has also shown that the mutations D1067Y/A/V in *PIK3CB* can drive resistance to the pan-PI3K inhibitor GDC0091 as a result of increased affinity to the lipid substrate PIP2 [97].

The strong dependency on the different PI3K isoforms, highlighted by the different mechanisms of resistance described above, suggest that these tumors are particularly addicted to this oncogenic pathway.

6.4.3 Resistance to PI3K/AKT Inhibitors by Ser/Thr Kinases

Because the PI3K pathway activates several Ser/Thr kinases, including AKT and S6K, to propagate the downstream signaling, it is plausible that other related kinases can compensate the inhibitory effects of targeting PI3K by phosphorylating overlapping substrates (Fig. 6.2 and reviewed in [22]). This effect is not exclusive of PI3K and AKT inhibitors, but common in most targeted therapies that block kinases involved in essential cellular processes, such as RAF and MEK, among others [98].

Different experimental approaches have been undertaken in order to identify alternative kinases that drive resistance to PI3K/AKT inhibitors. In general, screening technologies are useful and can address comprehensively the effect of every single kinase of the human kinome in the resistant phenotype. For instance, using open reading frame (ORF) gain-of-function screenings, sensitive cells are transfected or infected with libraries containing the cDNA of different kinases. Resistant clones are then selected upon exposure to therapeutic doses of the drug of interest and finally sequenced to identify the cDNA that drives resistance. Using this approach, it has been shown that the ribosomal protein kinases RSK3 and RSK4 have the ability to drive resistance to the pan-PI3K inhibitors BKM120 and GDC0941, the dual PI3K/mTOR inhibitor BEZ235, and the AKT inhibitor

MK2206 [99]. Although the mechanism was not clearly elucidated, it appears that RSK3/4 overexpression could rescue the cap-dependent translation activity even upon mTORC1 inhibition. Because RSK kinases require ERK1/2 phosphorylation at the hydrophobic motif for its maximal activity, it has been suggested that combination with inhibitors of the MEK/ERK pathway would revert the resistant phenotype [100].

Another recent large-scale ORF screening has addressed the role of some kinases in the resistance to BYL719, identifying PIM and PKC kinase isoforms and AKT as putative mediators of resistance to this drug. Overexpression of PIM1 appears to induce resistance, not only to BYL719, but also to the pan-PI3K inhibitor GDC0941 and the AKT inhibitors MK2206 and GDC0068 [101]. Although the effects of the overexpression in driving resistance to these agents is clear, the effects of inhibiting PIM kinases in resistant cell lines are somehow mild, indicating that additional mechanisms of resistance could co-exist. In hematological cancers, the levels of the PIM2 isoform are elevated probably as a result of the activation of the upstream transcription factors STAT, which act as effectors of multiple cytokine receptors commonly hyperactivated in liquid malignancies [102]. This high expression of PIM2 is particularly evident in multiple myeloma and has been suggested to lead to resistance to PI3K inhibitors in these cells. Mechanistically, PIM2 was shown to phosphorylate TSC2 and PRAS40 and activate mTORC1 [103]. Other well-known substrates of PIM kinases are the eIF4E binding protein 1 4EBP1 that would engage into protein synthesis independently of mTORC1, the FOXO transcription factors, and the apoptosis-related protein BAD [104].

Activation of mTORC1 is a key event in the resistance to PI3K inhibitors in many tumors types, probably because of its role downstream of PI3K [105]. Activation of mTORC1 predicts sensitivity to such inhibitors, as tumors that display residual mTORC1 activity upon acute PI3K blockade will not respond significantly to the therapy [106]. The concomitant inhibition of PI3K and mTORC1 has been proven to sensitize resistant cell lines in breast and head and neck cancer [88], proving that mTORC1 plays a causative role in limiting the sensitivity to PI3K inhibitors.

Many Ser/Thr kinases have the ability to regulate the activity of the mTORC1, by either activating or inhibiting different regulators of the complex. Perhaps, the most important negative regulator of mTORC1 is TSC2, which is part of the trimeric TSC complex [107–109]. Lack of TSC2 has been shown to activate mTORC1 independently of the PI3K/AKT axis and, most likely, tumors that exhibit downregulation of this protein are refractory to PI3K inhibitors.

TSC2 contains several phosphorylation sites with consensus motifs for kinases involved in the regulation of cell growth and survival. Despite the lack of a crystal structure, it has been speculated that most of the TSC2 phosphorylations would prompt an electrostatic repulsion with the lysosomal membrane due to the negative charges [38].

In general, kinases that phosphorylate and inactivate TSC2 have been linked with resistance to PI3K inhibitors. We have previously discussed RSK and PIM kinases, which phosphorylate TSC2 at highly conserved sites present in residues S939 and T1462, among others that contain the consensus motif RXXRX(S/T), where X is

any amino acid [110]. These sites have also been reported to be phosphorylated by the Serum Glucocorticoid-induced kinase (SGK) [111]. We reported that in cell lines that are intrinsically resistant to PI3K α inhibitors SGK1 is elevated at both protein and mRNA levels, as a result of promoter de-methylation [111]. As expected, these same cell lines were shown to be correlated with resistance to AKT inhibitors. Mass spectrometry analysis of SGK1 kinase assays using TSC2 as a substrate revealed increased phosphorylation sites in the same sites as those previously reported to be phosphorylated by AKT and RSK.

Additionally, the exposure of sensitive cell lines to PI3K and AKT inhibitors results in increased expression of SGK3 at both the mRNA and protein levels by a mechanism that is yet unknown. At the same time, these cells exhibit re-activation of the mTORC1 signaling as a result of TSC2 phosphorylation, measured by using an antibody against the phospho-RXRXX(S/T) motif [112]. SGKs share many other substrates involved in cell survival with AKT, such as the FOXO transcription factors. This may explain the ability of these kinases to promote survival upon PI3K/AKT inhibition [113].

Other kinases that have been proposed to mediate resistance to PI3K and AKT inhibitors in head and neck cancers are PKC's, a complex family of kinases that are classified between conventional (PKC α , β I, β II, and γ), novel (PKC δ , ϵ , θ , and η), and atypical (PKC ζ , and ι/λ) according to their cofactor requirements (Fig. 6.2). Despite the lack of a precise biochemical mechanism leading to mTORC1 activity, it has been shown that these enzymes are responsible to regulate such complex downstream of EGFR signaling [88]. A plausible explanation is that PKC isoenzymes are able to phosphorylate TSC2, since the consensus motif for the PKC substrates partially overlaps with those described for AKT, SGK, and RSK [22]. This is explained by the fact that these kinases are structurally similar in their kinase domains and belong to the AGC family of kinases, a highly conserved group of enzymes involved in cell growth, survival, and proliferation. The regulation and activation of AGC kinases require three critical phosphorylation events that take place in the turn motif, activation loop, and hydrophobic motif [22]. Phosphorylation at the hydrophobic motif is carried out by different kinases present in the cell. For instance, the hydrophobic motif kinases for all the RSK isoforms is ERK1/2, while for AKT, PKC, and SGK is mTORC2. This phosphorylation is considered to be a priming event, because once phosphorylated it serves as a docking site for PDK1 to phosphorylate the activation loop. PDK1 is a constitutively active kinase because it has the ability to auto-phosphorylate its activation loop at S241 and lacks a hydrophobic motif. In contrast, PDK1 contains a hydrophobic pocket termed the PIF-interacting pocket that serves as a docking site for phosphorylated AGC kinases hydrophobic motif [114]. Inhibition of PDK1 results on the inhibition of most of the AGC kinases, because in the absence of activation motif phosphorylation, these kinases are inactive [115]. Therefore, inhibition of PDK1 could be considered as a strategy to target all these kinases that drive resistance to PI3K and AKT inhibitors, such as RSK, SGK, and PKC. Consistently, we have found that in breast cancer cell lines intrinsically resistant to PI3K α inhibitors, PDK1 inhibition sensitizes to these therapeutic agents, as a result of SGK1 inhibition (Fig. 6.2 and [111]). Because

PDK1 also regulates RSK and PKC, it is tempting to speculate that this therapy would be highly beneficial in cases where resistance is driven by such kinases. Small molecule inhibitors of PDK1 have been reported in the literature, however their efficacy did not match the expectations and, in most cases, phosphorylation of AKT at T308 was used to read-out PDK1 inhibition. However, AKT is the only PDK1 substrate that does not require hydrophobic motif phosphorylation as a priming event and its interaction with PDK1 is the result of a translocation to the plasma membrane upon PIP3 synthesis. When PDK1 is inhibited using small molecule drugs, AKT still has the ability to be phosphorylated by mTORC2 at the hydrophobic motif and uses the high affinity interaction between this phosphorylation and the PDK1 PIF-binding pocket as a mechanism to secure its proper activation. Consistent with this mechanism, inhibition of mTOR or mTORC2 deletion increases the sensitivity to PDK1 inhibitors and it also explains why the combination between PDK1 and PI3K/AKT inhibitors is effective [111].

Based on the current knowledge, other mechanisms of resistance mediated by Ser/Thr kinases could also take place in some contexts. For instance, the 5' adenosine monophosphate-activated (AMPK) kinase has been shown to phosphorylate TSC2 at S1387 and T1271 leading to an increased GAP activity towards Rheb [116]. In this case, the activation of AMPK would lead to the inactivation of mTORC1, an expected outcome since AMPK is a sensor of low nutrients and high AMP/ATP ratio. AMPK is a trimeric complex formed by the catalytic core (α) and two regulatory subunits (β and γ) and is phosphorylated at the catalytic core by the upstream kinase LKB1 in the presence of AMP [117]. Since LKB1 loss is a fairly common event in cancer, as LKB1 acts as a tumor suppressor, it would be plausible that loss of LKB1 is a biomarker of resistance to PI3K and AKT inhibitors.

Finally, another group of kinases that is becoming attractive as a target in breast cancer is the cyclin-dependent kinase (CDK) family. The CDK4/6 inhibitor palbociclib has been recently approved for the treatment of metastatic ER-positive breast cancer in combination with anti-estrogen therapy [118]. Using a chemical library against PI3K inhibitor resistant cell lines, a study found that the inhibition of CDK4/6 sensitizes *PIK3CA*-mutated resistant cell lines in vitro and in vivo [119]. Although the exact mechanism by which this combination is beneficial has not been elucidated, it remains possible that different members of the PI3K/AKT/mTOR pathway regulate key players of the cell cycle, such as p16^{INK4A}, p27^{KIP1}, p21^{CIP1}, or CDK4/6 directly.

6.4.4 Hormone Receptor-Dependent Resistance

Mutations in *PIK3CA* are enriched in breast cancers that express the ER [120]. Therefore, it has been hypothesized that there is an important crosstalk between the PI3K and ER pathways in luminal breast cancers [121]. Several studies have shown that the PI3K pathway is a mechanism of resistance to anti-estrogen therapy, used in the treatment of hormone-dependent breast cancers [122]. Consistent with these

observations, the upstream receptor HER2 is also known to drive resistance to these agents and it is clinically considered as a biomarker of resistance to such inhibitors [123]. Consistently, clinical responses are observed when the combination of anti-estrogen therapy and the mTORC1 inhibitor everolimus was given to patients whose disease was refractory to previous treatment with the aromatase inhibitors letrozole or anastrozole [78]. Although the addition of everolimus prolongs progression free-survival, the adverse effects observed are considerable. A similar problem has been recently observed when combining anti-estrogen therapy with pan-PI3K inhibitors such as NVP-BKM120 [124], urging the development of selective p110 α inhibitors in the clinical setting.

Perhaps, for the relevance of this chapter, the opposite situation, in which the activation of ER signaling drives resistance to PI3K inhibitor, is more relevant. Our laboratory has previously demonstrated that cultured ER-positive breast cancer cell lines exhibit an increased luminal gene expression signature when exposed to therapeutic doses of PI3K and AKT inhibitors [125]. This signature is highly enriched in transcripts that are canonical targets of the ER transcription factor and, consistently, ER activity is increased upon PI3K inhibition. Chromatin-immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) have demonstrated increased binding of ER in a large proportion of genes and have revealed the presence of consensus binding motifs for FOXA1 and PBX1, two cooperative transcription factors previously found to be critical in the estrogen-dependent activation of ER [126]. Although increased levels of ER mRNA and protein have been found, it is plausible that this is the results of a positive feedback loop, since ER expression is known to be regulated by ER itself.

Despite being termed pioneer factors, FOXA1 and PBX1 require the presence of active methylated histone marks (specifically H3K4me1/2) in order to bind DNA. This is particularly interesting because it suggests that the activity of methylases/demethylases can actively modify the accessibility of the ER complex to the chromatin and, in agreement with this hypothesis, KMT2D was found to play a key role in the regulation of this process. Mechanistically, the kinase AKT phosphorylates KMT2D at S1331 inhibiting the methyl-transferase activity of the enzyme, suggesting that AKT activation negatively affects ER transcription (Fig. 6.3). In the presence of PI3K or AKT inhibitors, KMT2D S1331 phosphorylation is lost and the enzymatic activity increased, priming the recruitment of FOXA1, PBX1, and consequently ER, into the designated loci [126]. These studies add supporting evidences for the combination of agents that degrade ER with PI3K inhibitors and open a new avenue for the design of small molecules that target the epigenome.

In the context of prostate cancer, elegant studies using genetically-engineered mouse models of the disease have proven that PI3K inhibitors also result in the upregulation of the androgen receptor (AR) signaling [127]. As previously discussed, in this malignancy the p110 β isoform is responsible for the downstream signaling [94]. The inhibition of PI3K with pan-PI3K inhibitors has an important effect in the activity of AR, a parallelism with ER in breast cancer. However, it remains to be elucidated whether the mechanism is the same. Interestingly, several reports have shown the presence of FOXA1 mutations in this cancer, suggesting that these could cooperate with androgen signaling [128].

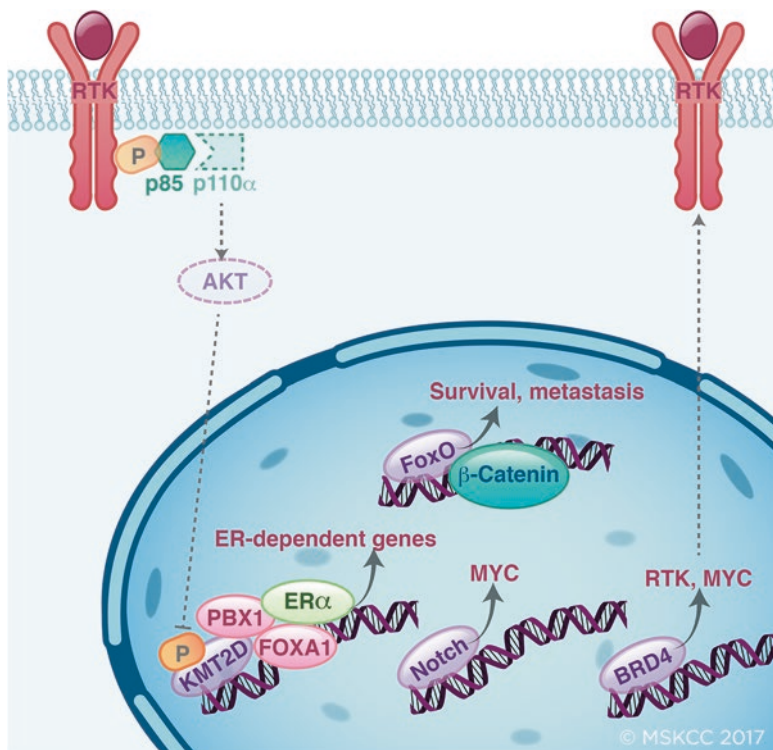


Fig. 6.3 Epigenetic mechanisms of resistance to PI3K/AKT inhibitors. Resistance to PI3K and AKT inhibitors can also be regulated transcriptionally by several transcription factors. Hormone receptors, such as ER and AR, have been shown to drive resistance to PI3K/AKT inhibitors in breast and prostate luminal cancers. In the case of ER, the regulation of the methylase KMT2D (MLL2) by AKT is required to allow the recruitment of ER cofactors to the chromatin. It is possible that some similarity might exist with the mechanism regulating AR activation upon PI3K inhibition. Other transcription factors involved in the resistance to these inhibitors include Notch, which appears to counteract the inhibitory effects of mTORC1 through the expression of MYC. FOXO has also been shown to interact with β -catenin and promote a gene expression output leading to cell survival and metastasis. The case of BRD4 is less studied, but appears that would regulate the expression of RTK and MYC

6.4.5 Resistance to PI3K/AKT Inhibitors by Transcription Factors

Cancer cells can also become resistant to targeted therapies such as PI3K and AKT inhibitors by changing their transcriptional landscape, a process that is generally mediated by the activity of transcription factors that either activate or repress the expression of target genes (Fig. 6.3).

One of the first studies that systematically addressed resistance to anti-cancer agents demonstrated that both Notch and C-MYC transcription factors are

markers of resistance when activated. In the case of Notch signaling, it was shown that overexpression of the intracellular active domain of NOTCH1 (ICN1) was sufficient to cause resistance in different breast cancer cells to BEZ235 [129]. ICN also caused resistance to the PI3K inhibitor PIK90, the mTOR inhibitor PP242 and mTORC1 inhibitor Everolimus, suggesting that the effect of this transcription factor was not specific to PI3K but it was rather driving resistance to the entire PI3K/mTOR pathway. In fact, cells overexpressing ICN have been shown to have similar levels of pS6K and p4EBP1, markers of activation of mTORC1, implying that the resistance is the result of an alternative pathway or a downstream effector, in this case, C-MYC. Knockdown of C-MYC in cells overexpressing ICN results in re-sensitization to these therapeutic agents, thus suggesting that C-MYC is the main downstream effector driving resistance. This is also consistent with the fact that the cap-dependent translation of C-MYC is dependent on mTORC1 [129]. Interestingly, in mouse models of T-cell leukemia, loss of Notch signaling was associated with resistance to the pan-PI3K inhibitor GDC-0941 [130]. Despite the involvement of NOTCH and C-MYC in PI3K/mTORC1 resistance has not been validated in clinical samples, there is a strong rationale to accept these transcription factors as putative modulators of resistance to such therapies.

Among the different transcription factors involved in the PI3K pathway, FOXOs is perhaps the better characterized due to its direct regulation by AKT and its role in cell survival [32]. Upon PI3K signaling, AKT phosphorylates FOXO at several residues and causes the binding with the 14-3-3 proteins in the cytoplasm, releasing it from their DNA-binding sites. Inhibition of AKT promotes a rapid dephosphorylation and translocation to the nucleus, where FOXO's engage into their transcriptional program. There are different transcription factors that have been shown to interact with and inhibit FOXO transcription factors, such as SMAD, FOXG1, PGC-1, and β -catenin, and could be potential mediators of resistance to PI3K inhibitors by blocking the cell death and cell cycle arrest mediated by FOXO's. As a matter of fact, β -catenin drives resistance to PI3K and AKT inhibitors in colorectal cancers by modulating the transcriptional output of FOXO into driving metastasis [131].

6.4.6 Other Mechanisms Involved in the Resistance to PI3K/ AKT Inhibitors

There are a number of novel topics that have become interesting for the treatment of cancer such as epigenetic inhibitors and nanoparticles in drug delivery. Although these fields are still recent, they have an interesting potential in the field of PI3K inhibition. For example, in order to decrease the systemic exposure of PI3K or AKT inhibitors (and therefore increase the therapeutic window of these agents), it has been show that the tumor-specific delivery of PI3K inhibitors can be achieved using nanoparticles. The advantages of this method are the

reduction of secondary effects, such as hyperglycemia, and as a consequence the ability to combine other drugs that, in a systemic regime, would have severe adverse effects [132].

In the field of epigenetic inhibitors, two main targeting strategies have been explored in the context of PI3K therapy. For example, the use of histone deacetylase (HDAC) inhibitors has been demonstrated to be effective in preclinical models of medulloblastoma when combined with the PI3K inhibitor BKM120 [133]. Moreover, inhibitors of the bromodomain and extra terminal domain (BET) proteins also synergize with PI3K inhibitors when combined in breast cancer cells and transgenic mouse models [134]. Mechanistically, BRD4 appears to be involved in the transcriptional machinery required to upregulate RTKs upon PI3K/AKT inhibition, hence treatment with BRD4 inhibitors such as JQ1 would abrogate this effect.

Additional work will be required to identify the critical nodes of the epigenome that are required to target in order to modulate the response to PI3K and AKT inhibitors and pinpoint the specific tumor types that would benefit from such combinations.

6.5 Conclusions

The cumulative evidences regarding the role of the PI3K/AKT pathway in human cancers have prompted the development of inhibitors that specifically target this key signaling node. Despite the importance of PI3K and AKT in tumor biology, the clinical results have been less promising than initially anticipated. This is in part due to the multiple mechanisms of resistance that tumors exhibit to overcome these therapeutic agents. Both clinical and preclinical data suggest that pharmacologic combinations are required to increase the effectiveness of such compounds and, accordingly, clinical trials testing these combinations are undergoing. In general, it appears that the resistance to PI3K and AKT inhibitors is mediated by alternative kinase signaling that leads to the activation of downstream effectors, the most important of which is mTORC1. This signaling compensation stresses the importance of PI3K for the cells and could be explained from an evolutionary point of view as an attempt to maintain active a major pathway that regulates cell growth and survival. In fact, it is not surprising that many of the kinases involved in PI3K and AKT inhibitor resistance are part of the same family, the AGC kinases. Additional data has also revealed the importance of ER signaling in the resistance to these agents in breast cancer and clinical data could be supporting the combination with hormonal therapy soon. It will also be interesting to characterize novel mechanisms of resistance and targets such as epigenetic modulators and transcriptional regulators.

In summary, inhibitors of the PI3K/AKT pathway have a great potential in the clinical setting, but only when administered to the appropriate patients and in the right combination.

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