Resistance to Targeted Anti-Cancer Therapeutics 15
Series Editor: Benjamin Bonavida

Yosef Yarden
Moshe Elkabets *Editors*

Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways

Springer

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Volume 15

Series Editor:
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ISSN 2196-5501 ISSN 2196-551X (electronic)
Resistance to Targeted Anti-Cancer Therapeutics
ISBN 978-3-319-67930-3 ISBN 978-3-319-67932-7 (eBook)
https://doi.org/10.1007/978-3-319-67932-7

Library of Congress Control Number: 2017955951

Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG part of Springer Nature.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This volume is dedicated to the memory of the late Prof. Shraga Segal, our teacher, colleague, and dear friend.

Aims and Scope

For several decades, treatment of cancer consisted of chemotherapeutic drugs, radiation, and hormonal therapies. Those were not tumor specific and exhibited several toxicities. During the last several years, targeted cancer therapies (molecularly targeted drugs) have been developed and consist of immunotherapies (cell mediated and antibody), drugs, or biologicals that can block the growth and spread of cancer by interfering with surface receptors and with specific dysregulated gene products that control tumor cell growth and progression. These include several FDA-approved drugs/antibodies/inhibitors that interfere with cell growth signaling or tumor blood vessel development, promote the death of cancer cells, stimulate the immune system to destroy specific cancer cells, and deliver toxic drugs to cancer cells. Targeted cancer therapies are being used alone or in combination with conventional drugs and other targeted therapies.

One of the major problems that arise following treatment with both conventional therapies and targeted cancer therapies is the development of resistance, pre-existing in a subset of cancer cells or cancer stem cells and/or induced by the treatments. Tumor cell resistance to targeted therapies remains a major hurdle and, therefore, several strategies are being considered in delineating the underlining molecular mechanisms of resistance and the development of novel drugs to reverse both the innate and acquired resistance to various targeted therapeutic regimens.

The new series "*Resistance of Targeted Anti-Cancer Therapeutics*" was inaugurated and focuses on the clinical application of targeted cancer therapies (either approved by the FDA or in clinical trials) and the resistance observed by these therapies. Each book will consist of updated reviews on a specific target therapeutic and strategies to overcome resistance at the biochemical, molecular, and both genetic and epigenetic levels. This new series is timely and should be of significant interest to clinicians, scientists, trainees, students, and pharmaceutical companies.

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Preface

Unlike cytotoxic regimens, such as chemotherapy, which inhibit the ability of cells of all tumors to rapidly proliferate, molecular targeted therapies inhibit survival pathways or oncogenic mutations specific to each cancer's molecular subtype. The emerging ability to tailor a therapeutic drug to a patient is the outcome of deep knowledge of oncogenic circuitries, as well as detailed characterization of the specific repertoires of genetic aberrations driving individual tumors [1]. This new chapter of cancer pharmacology is attributable to two visionary concepts. The 1906 "Magic Bullet" concept of Paul Ehrlich, which was based on the use of histological dyes, predicted that it would be possible to target a pathological tissue while avoiding nearby healthy tissues [2]. In the same vein, the 2002 concept of Irwin Weinstein [3], called "Oncogene Addiction," argued that cancers harboring multiple genetic abnormalities are dependent on (or "addicted" to) one or only a few mutated genes. Hence, the reversal of just one or a few of these abnormalities might inhibit cancer cells. The excessive reliance of tumor cells on specific intracellular pathways is well exemplified by the dependency of chronic myeloid leukemia on BCR-ABL, an oncogenic fusion protein [4]. Yet another class of dependency is the so-called "non-oncogene addiction" [5], which is exemplified by the reliance of a fraction of colorectal tumors on autocrine loops involving the epidermal growth factor receptor (EGFR) and two of its ligand growth factors [6].

The harvest of the new era of molecular targeted therapy of cancer is truly impressive. This started in 1997, when three pioneer drugs were approved, namely imatinib, a kinase blocker specific to BCR-ABL, trastuzumab, a monoclonal antibody (mAb) to HER2 (a kin of EGFR), and rituximab, a mAb specific to CD20. Remarkably, the first wave of drugs was followed by an avalanche, such that the first two decades of the new millennium have witnessed the approval of more than 60 new cancer drugs, primarily protein kinase inhibitors (PKIs) and recombinant mAbs. This fruitage already dwarfs the preceding era of chemotherapeutic drugs and is bound to dominate, in the near future, some clinical indications of oncology.

In spite of the optimistic scenario, the wide occurrence of patient's resistance to the new drugs casts long shadows. In most cases, only a fraction of patients responds to molecular targeted therapies. This "primary" or "de novo" resistance laid the

foundation for a new discipline that searches for biological indicators, or biomarkers, potentially able to predict who are the patients that will benefit from a particular targeted therapy [7]. Similarly, "secondary" or "acquired" resistance characterizes patients who initially respond but become resistant while under treatment. Importantly, as more molecular targeted drugs are entering routine use in oncology wards around the world, we learn that resistance to the new drugs is one of the main barriers to further progress. From a biological perspective, acquired resistance may reflect, on the one hand, the remarkable robustness and adaptability shared by all living systems [8], and, on the other hand, the surprisingly large intra- and intertumoral genetic heterogeneity [9].

The objective of this volume, entitled *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways,* is to review the dynamic field of resistance to molecular targeted anti-cancer therapies. Because many of the new drugs block the action of trans-membrane receptors for growth factors, such as EGFR, or the signaling pathways such receptors commonly activate, for example the RAS-to-ERK pathway, we decided to focus on drugs targeting receptor tyrosine kinases (RTKs) and the oncogenic biochemical reactions they stimulate. In general, two classes of mechanisms might confer resistance. The first class refers to alterations occurring at the level of drug or host (patient), rather than within the malignant tissue. For example, pharmacokinetics effects that deplete a drug prevent uptake or metabolize it into less active fragments. The other class of mechanisms brings together all adaptive alterations taking place in the cancerous tissue. This group of molecular and cellular mechanisms represents tumor adaptation while under drug treatment [10, 11]. For example, Darwinian microevolution instigated by a molecular targeted drug may newly generate or expose pre-existing clones of cancer cells harboring novel, drug-resistant mutants [12], or amplify the target to quench the drug in question. Other mechanisms that enable evasion involve activation of alternative pathways of cell survival, which use either a parallel (bypass) track or an active target downstream of the blocked molecular target [11].

The first half of the volume concentrates on drugs targeting the RTK molecules themselves, whereas the other part deals with resistance to inhibitors of the corresponding downstream signaling pathways. A chapter contributed by **Livio Trusolino and Simonetta Leto** opens the volume and highlights the case of colorectal tumors treated with anti-EGFR mAbs, i.e., cetuximab or panitumumab. Importantly, *KRAS* point mutations and gene copy number gains are responsible not only for primary but also for acquired resistance in approximately 50% of patients who relapse while on mAb treatment. Other evasion mechanisms include activation of compensatory pathways (e.g., BRAF, PI3K, HER2, and MET), but mutations activating EGFR or preventing cetuximab binding appear to be quite rare. Notably, Trusolino and Leto conclude that the very same alterations that account for intrinsic refractoriness also foster progressively diminished response in the course of treatment. The second chapter, by **Maicol Mancini and Yosef Yarden**, deals with resistance of lung cancer to ATP-mimicking molecules, PKIs like erlotinib and crizotinib, which are respectively specific to mutant forms of EGFR or ALK fusion proteins. Remarkably, resistance to these and other first-generation PKIs evolves in

patients within 10–24 months. Unlike resistance of colorectal cancer to anti-EGFR mAbs, the major mechanism conferring resistance of lung tumors to erlotinib entails second-site mutations. Other mechanisms of acquired resistance that have been confirmed in clinical specimens include increased expression of the compensatory RTKs MET and AXL, *EGFR* amplification, mutations in the *PIK3CA* gene, or a pronounced epithelial-to-mesenchymal transition. As exemplified by Mancini and Yarden, once resolved, mechanisms conferring resistance to PKIs may pave the way for next-generation drugs, or they may identify combination therapies simultaneously inhibiting the primary and alternative routes to oncogenesis.

The introduction and clinical approval of trastuzumab, a mAb specific to HER2, has significantly improved survival of patients with HER2-positive ($HER2^+$) metastatic breast cancer. However, as with other therapeutic antibodies, resistance to trastuzumab significantly shortens clinical application or necessitates alternative treatments, such as the addition of a second anti-HER2 antibody, called pertuzumab. **Jennifer Hsu and Mien-Chie Hung** discuss three molecular mechanisms potentially underlying resistance: (i) Upregulation of downstream signaling, such as the PI3K/AKT pathway, due to mutations in the gene encoding PI3K and/or inactivation or loss of PTEN, which antagonizes PI3K and negatively regulates AKT activities. (ii) Hindrance of trastuzumab binding to HER2 by means of either ectodomain shedding and generation of a constitutively active, truncated form of HER2, or through alternative translation initiation of *HER2* mRNA. An alternative mechanism involves binding of the cell surface glycoprotein mucin-4 (MUC4) to the extracellular domain of HER2, which can mask the trastuzumab-binding site on HER2. And (iii) Overexpression of other RTKs, such as EGFR, EPH-A2, the insulin-like growth factor receptor 1 (IGF-1R) and its ligands, or MET and its ligand, the hepatocyte growth factor (HGF). Resistance to experimental drugs targeting MET is described in a chapter contributed by **Simona Corso and Silvia Giordano.** HGF-MET signaling plays an important role in tumor progression, in particular during the late stages of invasive growth and metastasis. Nevertheless, Corso and Giordano critically discuss the status of MET targeting in highly metastatic tumors. Despite compelling evidence obtained in preclinical studies, which demonstrated that MET targeting in the respective MET-addicted tumors could be of therapeutic value, so far the results obtained in clinical trials employing MET-targeted drugs (e.g., PKIs and mAbs to MET or to HGF) have been disappointing. Among the reasons they list are questionable selection of patients, relatively rare and heterogeneous amplification of the *MET* gene, the need to differentiate between ligand-dependent and mutational activation of MET, as well as the choice of the type of PKIs and the frequent compensatory signaling by EGFR and the HER family members.

Following ligand binding and receptor dimerization, RTKs undergo catalytic activation that culminates in trans-phosphorylation of cytoplasmic proteins, as well as evokes transcriptional responses in the nucleus [13]. Simultaneous firing of several linear cascades is typical to active forms of RTKs, but mainly two such cascades are frequently activated in tumors due to mutations within critical components. These are the RAS-RAF-MEK-ERK cascade and the PI3K-AKT-mTOR

pathway. After reviewing the mutational status of the RAS-to-ERK pathway in tumors, **Galia Maik-Rachline, Izel Cohen, and Rony Seger** focus on clinically approved inhibitors of BRAF (e.g., vemurafenib) and MEK (e.g., dabrafenib), which are used to treat patients with metastatic melanoma. As with other PKIs, intrinsic resistance limits drug application, although the targeted mutation in BRAF is present. Interestingly, this may involve, among other factors, large secretome changes, which establish a tumor microenvironment that supports expansion of drug-resistant cancer cell clones but exhibits susceptibility to combination therapies. Resistance often emerges also in patients who initially respond to BRAF inhibitors. Mechanisms underlying the emergence of secondary resistance are surprisingly varied: expression of drug-resistant isoforms of the target, alterations of downstream components that reactivate the ERK cascade, and induction of upstream components (or other signaling pathways) that bypass the pharmacological effect. In analogy to the ERK pathway, hyperactivation of the PI3K cascade is frequent in human tumors, as reviewed by **Pau Castel and Maurizio Scaltriti**. Although this provided strong rationale to develop inhibitors targeting many different components of the pathway, the responses observed in patients treated with such inhibitors have been, in general, short lived and anecdotal. In the last few years, however, large clinical studies have demonstrated that specific compounds (e.g., AKT catalytic inhibitors and specific PI3Kalpha inhibitors) can elicit strong antitumor activities if administered to patients selected on the basis of specific activating mutations. Nevertheless, as described by Castel and Scaltriti, intrinsic and acquired resistance to inhibitors of the pathway currently limit the activity of these agents, but combinatorial strategies may delay emergence of drug resistance.

Inhibition of apoptosis is oncogenic and characterizes a broad range of tumor types, whereas promotion of cell cycle arrest is tumor suppressive [14]. Individual BCL-2 (B-cell lymphoma 2) family members couple apoptosis regulation and cell cycle control, while serving as a signaling nexus among kinase cascade-driven growth/survival signals. **Konstantinos Floros, Anthony Faber, and Hisashi Harada** open their chapter by reviewing genomic alterations, such as *BCL-2* translocations, which lead to a gain-of-function anti-apoptotic signal. They later describe venclexta (venetoclax), the first drug targeting BCL-2, which has been approved for the treatment of patients with chronic lymphocytic leukemia (CLL). Two other, yet experimental drugs, are also described and the authors provide a critical discussion of mechanisms of resistance, as well as pharmacological strategies that overcome resistance. For example, a combination of venetoclax and ibrutinib, a Bruton Tyrosine Kinase (BTK) inhibitor, which is also approved for CLL. Like the BCL-2 inhibitor's field, the arena of drugs able to halt the cell cycles is relatively young and only a few drugs have been approved. Normally, cell cycle entry and progression are tightly regulated by growth factors, but deregulation of the cell cycle is a common characteristic of cancer cells. Progression through the cell cycle is critically controlled by multiple signaling pathways (e.g., ERK, PI3K, and integrin signaling), which activate enzymes known as cyclin-dependent kinases (CDKs); hence small molecule CDK inhibitors are being developed as potential cancer therapeutics. The first pharmacological CDK4/6 inhibitors, Palbociclib and Ribociclib, have been approved for the treatment of women with hormone receptor

positive, HER2-negative advanced breast cancer. **Wolf Ruprecht Wiedemeyer** describes frequent genomic alterations targeting the CDK-cyclin-RB-E2F axis in cancer and later critically reviews emerging mechanisms of response and resistance to CDK4/6 inhibitors. In addition, Wiedemeyer discusses drug combinations, such as palbociclib and inhibitors of the RTK/PI3K/AKT/mTOR pathway, and the yet unknown clinical potential of CDK1/2 inhibitors.

The last chapter, by **Nili Dahan, Ksenia Magidey, and Yuval Shaked**, deals with resistance to clinically approved anti-angiogenic agents, such as bevacizumab, an anti-VEGF (vascular endothelial growth factor) antibody, and PKIs able to inhibit angiogenesis (e.g., sorafenib and sunitinib). The authors refer to the unexpected complexity of clinical application of such agents and the frequently observed resistance to therapy, thought to underlay the generally modest gains in long-term survival. Given that anti-angiogenic agents target the tumor-supporting vascular system, rather than the malignant tissue, and the cellular heterogeneity of the microenvironment, resistance likely reflects both tumor- and host-mediated mechanisms. Because the tumor microenvironment is genetically stable and highly dynamic, resistance to anti-angiogenesis agents involves no overt genetic aberrations but rather activation of alternative mechanisms that sustain tumor vascularization, including a plethora of cytokines and growth factors secreted by bone marrow-derived cells and tumor-associated macrophages. Understanding these mechanisms is key to developing strategies able to overcome therapy resistance and improve clinical outcome of patients treated with anti-angiogenesis drugs.

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Beer-Sheeva, Israel — Moshe Elkabets

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Contents

About the Series Editor

Benjamin Bonavida, Ph.D. is currently Distinguished Research Professor at the University of California, Los Angeles (UCLA). His research career, thus far, has focused on basic immunochemistry and cancer immunobiology. His research investigations have ranged from the mechanisms of cell-mediated killing, sensitization of resistant tumor cells to chemo-/immunotherapy, characterization of resistant factors in cancer cells, cell-signaling pathways mediated by therapeutic anticancer antibodies, and characterization of a dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop in many cancers that regulates cell survival, proliferation, invasion, metastasis, and resistance. He has also investigated the role of nitric oxide in cancer and its potential antitumor activity. Many of the above studies are centered on the clinical challenging features of cancer patients' failure to respond to both conventional and targeted therapies. The development and activity of various targeting agents, their modes of action, and resistance are highlighted in many refereed publications.

Acknowledgements

The Series Editor acknowledges the various assistants that have diligently worked in both the editing and formatting of the various manuscripts in each volume. They are Leah Moyal, Kevin Li, and Arah Cho.

About the Guest Editors

Moshe Elkabets received a B.Sc. in medical laboratory, and both a Master and a Ph.D. in tumor immunology from Ben-Gurion University of the Negev, Beer-Sheva, Israel. For postdoctoral training, he first studied at the laboratory of Dr. Sandra McAllister (Brigham's and Women Hospital, Harvard Medical School) and later at the laboratory of Dr. Jose Baselga (Massachusetts General Hospital and Memorial Sloan Kettering Cancer Center). Since 2015, Dr. Elkabets leads a research laboratory in the Department of Microbiology, Immunology and Genetics at Ben-Gurion University of the Negev. His laboratory is interested in cancer therapeutics, primarily resistance to molecular targeted therapies (e.g., PI3K and EGFR inhibitors) and immunotherapies. Dr. Elkabets' research is currently supported by the Israel Science Foundation (ISF), Israel Cancer Association (ICA), Israel National Center for Personalized Medicine (INCPM), an Alon Fellowship, and the Teva Founders Prize.

Yosef Yarden is the Harold and Zelda Goldenberg Professor of Molecular Cell Biology. He currently serves as Director of the Dwek Institute for Cancer Therapy Research and Head of the Department of Biological Regulation of the Weizmann Institute of Science. He received a B.Sc. in biological and geological sciences from the Hebrew University of Jerusalem (1980), and a Ph.D. in molecular biology from the Weizmann Institute of Science (1985). His postdoctoral training was undertaken at Genentech, Inc., in San Francisco and at the Massachusetts Institute of Technology. In 1988, he joined the Weizmann Institute of Science's faculty. At the Institute, he has served as Dean of the Faculty of

Biology (1997–1999), Vice President for Academic Affairs (1999–2001), Director of the MD Moross Institute for Cancer Research (1999–2001), and Dean of the Feinberg Graduate School (2001–2007). Dr. Yarden's research attempts to resolve the transcriptional and other molecular mechanisms underlying growth factor's action, as well as the pharmacological opportunities offered by such understanding.

Chapter 1
Resistance of Colorectal Tumors to Anti-EGFR Antibodies

Livio Trusolino and Simonetta M. Leto

Abstract Only a small fraction (10%) of genetically unselected patients with chemorefractory metastatic colorectal cancer benefits from the anti-EGFR antibodies cetuximab or panitumumab ('primary' or 'de novo' resistance). Further, almost all patients who initially respond become resistant over the course of treatment ('secondary' or 'acquired' resistance). Studies in cell lines, patient-derived tumorgrafts, and archival surgical specimens have identified many biomarkers of both primary and acquired resistance to anti-EGFR antibodies, and it is now evident that resistance mechanisms revolve around common genetic lesions and share analogous signaling traits. Here we discuss how resistance to the EGFR blockade is attained in colorectal cancer and elaborate on alternative therapeutic strategies that are now under development to improve response and contrast relapse.

Keywords Colorectal cancer • Epidermal growth factor receptor • Mitogen-activated protein kinase kinase • Drug resistance

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_1

Abbreviations

BRAF	v-Raf murine sarcoma viral oncogene homolog B1
CRC	Colorectal cancer
ctDNA	Circulating tumor DNA
EGFR/ErbB1/HER1	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
HER2/neu/ERBB2	V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
mCRC	Metastatic colorectal cancer
MEK	Mitogen-activated protein kinase kinase
moAbs	Monoclonal Antibodies
NRAS	Neuroblastoma RAS viral oncogene homolog
PIK3CA	Phosphatidylinositol 3-kinase, catalytic, alpha
PTEN	Phosphatase and tensin homolog
RR	Response rate
RTKs	Receptor Tyrosine kinases

1.1 Introduction

Colorectal cancer (CRC) is the third commonest cancer worldwide, with approximately 20% of newly-diagnosed patients already presenting with metastatic disease and 50% of patients developing metastasis in subsequent months or years. The median overall survival (OS) is around 20 months [1–5].

The outlook of patients with metastatic colorectal cancer (mCRC) has been advanced by the introduction in the clinical practice of cetuximab and panitumumab, two monoclonal antibodies (moAbs) that inhibit the epidermal growth factor receptor (EGFR/ErbB1/HER1). These agents are typically administered in combination with chemotherapy in the second- or third-line treatment of individuals who have become resistant to previous rounds of cytotoxic chemotherapy [6–8]; in this chemorefractory setting, patients achieve an objective response and disease stabilization rates of approximately 10% and 20%, respectively [7–9]. Different from other tumor types, such as non-small cell lung cancers (NSCLCs) or melanomas, in which actionable targets such as EGFR or BRAF are constitutively hyperactive as a consequence of underlying genetic alterations [10, 11], mutational abnormalities in the *EGFR* gene are extremely infrequent in colorectal tumors (see below).

The 70% of CRC tumors that are intrinsically refractory to EGFR blockade display primary (also known as innate) resistance. Acquired (or secondary) resistance refers to disease progression in the face of an ongoing treatment that was initially effective. In both primary and secondary resistance, lack of response can be

explained by compensatory signaling activities driven by mutational events or adaptive mechanisms such as biochemical feedbacks or gene expression changes [12, 13]. In the case of colorectal cancer, acquired resistance typically occurs within 3–18 months after treatment initiation [7, 8]. Starting with seminal observations in 2006–2007 [14, 15], several biomarkers of primary resistance to anti-EGFR moAbs in mCRC patients have been progressively identified and biologically validated, and some of them are now routinely used to exclude a number of molecularly defined nonresponders from unnecessary treatment [16, 17]. The topic of acquired resistance has received preclinical and clinical focus more recently, with the emergence of new critical information only in the last 5 years.

Here, we will survey the current state of the art on primary and acquired resistance to anti-EGFR moAbs in mCRC, from early mechanistic investigations to clinical applications, and will discuss fresh knowledge on how to improve the response and delay the relapse in mCRC patients. This chapter is inspired, with relevant updates, to a review article that we have recently authored [18].

1.2 The Genomic Landscape of Resistance to Anti-EGFR Antibodies in Patients with Metastatic Colorectal Cancer

EGFR is a member of the ErbB family of receptor tyrosine kinases (RTKs), which also includes HER2/neu (ERBB2), HER3 (ErbB3) and HER4 (ErbB4) [19]. Following homo- and hetero-dimerization of EGFR with itself or other ErbB members, induced by EGF or other EGF-like ligands, several downstream signal transduction pathways are activated, including the RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR axes, but also SRC-like family kinases, PLCγ-PKC, and STATs [19, 20]. Such activation stimulates key processes involved in tumor growth and progression, including proliferation, survival, angiogenesis, invasion, and metastasis [21] (Fig. 1.1).

Of note, the *EGFR* gene is very rarely mutated or amplified in CRC. Because 'addiction' to the EGFR pathway does not have genetic underpinnings, this dependency may represent an aberrant declination of para-physiological traits typical of normal colonic tissues. In the adult intestine, mucosal renewal after tissue damage is prompted by increased EGFR signaling (through transcriptional induction of the receptor and autocrine production of the cognate ligands) [22–24], and is impaired by EGFR inhibition [22]. Importantly, EGFR neutralization curbs the propensity of epithelial cells to undergo neoplastic transformation promoted by inflammatory stimuli [25]. Altogether, these observations suggest that persistent upregulation of EGFR activity during chronic intestinal inflammation—a condition that typically predisposes to colorectal cancer—may act as a pro-tumorigenic cue. This stimulation would positively select for cancer cells relying on EGFR-driven signals for their growth, explaining why a fraction of CRCs are strictly dependent on EGFR activity even in the absence of underlying genetic alterations. On this ground, it

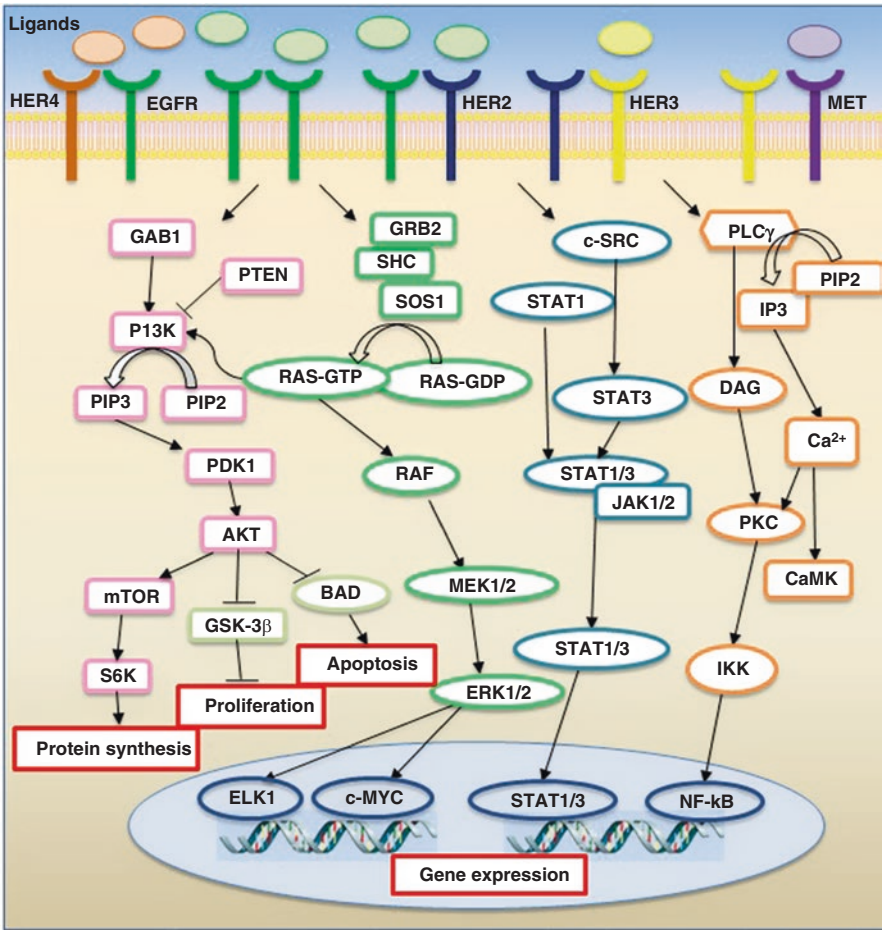

Fig. 1.1 EGFR signaling pathways. (**a**) Following ligand binding and the ensuing receptor homo- and hetero-dimerization, ErbB family members trigger several signaling pathways, including the RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR axes, the SRC family kinases, PLCγ-PKC, and STATs. All these signals stimulate cell proliferation and/or survival

comes with no surprise that the increased expression of EGFR and EGFR ligands not only encourages intestinal regeneration during inflammation, but also characterizes 'EGFR-addicted' tumors with marked sensitivity to EGFR inhibition [26–28].

In the absence of genetic alterations correlating with sensitivity to anti-EGFR antibodies, patient stratification is only applied by subtraction: in general terms, the commonest mechanisms of innate resistance involve genomic alterations affecting EGFR downstream effectors, such as *KRAS/NRAS* and *PIK3CA* mutations, with consequent constitutive pathway hyperactivation. The RAS and PI3K signaling

cascades can also be triggered by upstream RTKs other than EGFR [29], leading to an oncogenic shift [30]. In this situation, the primary drug target remains unaltered and continues to be inhibited while an alternative signal transducer becomes activated, circumventing the effects of EGFR inhibition [13, 31] (Fig. 1.2a–c).

It is becoming increasingly clear that tumors can contain a high degree of mutational heterogeneity within the same lesion [32]. Thus, secondary resistance can arise not only through stochastic acquisition of *de novo* genetic lesions along treatment, but also through therapy-induced selection of intrinsically resistant minor subclones already present in the original tumor [33]. If secondary resistance can be re-interpreted as the emergence, under drug pressure, of rare tumor subpopulations featuring primary resistance, then the molecular mechanisms of primary and acquired resistance are expected to be the same. Accordingly, hereinafter we will delineate resistance predictors as absolute traits, specifying, for each determinant, how it contributes to primary or secondary resistance. We will also concentrate on current research efforts that have put forward alternative strategies to bypass such resistances in patients with no other therapeutic options. Table 1.1 summarizes the main predictors of primary and acquired resistance observed in mCRC patients and describes potential approaches for tackling them therapeutically.

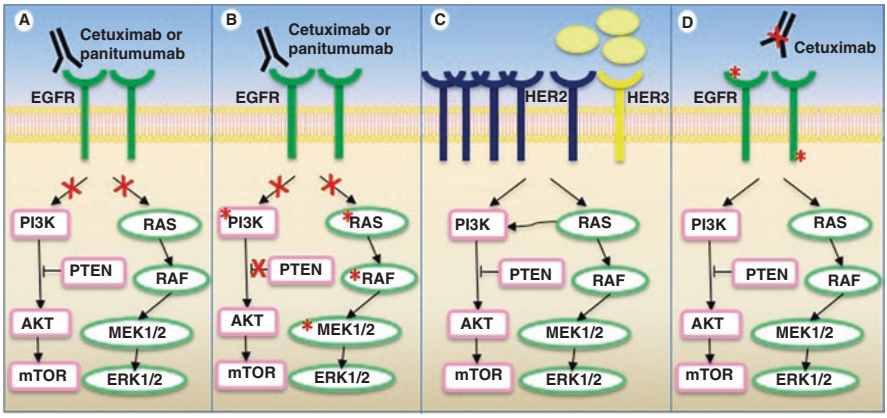

Fig. 1.2 Mechanisms of resistance to anti-EGFR moAbs in mCRC. (**a**) By binding the extracellular domain of EGFR, both cetuximab and panitumumab prevent ligand-induced activation of downstream signaling. (**b**) Activating mutations of genes encoding EGFR transducers such as *KRAS* (by either point mutations or gene amplification), *BRAF*, *PIK3CA* and *MAP2K1* (MEK1), or *PTEN* loss of function, cause relentless activation of downstream signaling that circumvent EGFR inhibition. (**c**) Excessive activation (by either receptor gene amplification or high ligand expression) of alternative receptors, such as HER2 or MET (not shown), can substitute for EGFR inhibition and activate downstream pathways. (**d**) Additional genetic alterations within the target receptor may abolish antibody binding (EGFR extra-cellular domain mutations) or mediate EGFR activation even in the presence of the drug (kinase domain mutations)

Table 1.1 Mechanisms of primary and acquired resistance to anti-EGFR moAbs in mCRC patients and alternative therapeutic approaches

Biomarker	Scientific approach	Alternative strategies proposed	References
Primary resistance			
KRAS mutations	*KRAS* mutant cell lines *in vitro* and *in vivo*	Combination of EGFR and MEK inhibitors was more effective than either agent alone in reducing cell viability *in vitro*	[15]
		Combination of dasatinib (Src kinase inhibitor) with cetuximab induced tumor growth delay but not regression *in vivo*	[60]
	Synthetic lethal interactions in *KRAS* mutant cell lines	Combined IGF-IR and MEK inhibition induced partial tumor regression *in vivo*	[58]
		TAK1 inhibition promoted apoptosis in KRAS-dependent APC-mutant CRC cells and tumor regression *in vivo*	[57]
		Proteasome and topoisomerase inhibitors selectively impaired cell viability (GATA2 and CDC6 could be potential new targets)	[53]
		Combined BCL-XL and MEK inhibition promoted tumor regression *in vivo*	[56]
	Patient-derived xenografts of *RAS* mutant CRCs	Inhibition of MEK and PI3K/mTOR induced tumor growth delay but not regression. This strategy may retard progression in patients	[52]
BRAF mutations	*KRAS* or *BRAF* mutant cells, mouse xenografts and GEMMs.	Combined targeting of BCL-2/BCL-XL and TORC1/2 induced selective apoptosis *in vitro* and tumor regression *in vivo*	[59]
	BRAF V600E CRC models	Combined BRAF and EGFR inhibition was synergistic *in vitro* and *in vivo*	[62, 68, 69]
		Calfizomib (proteasome inhibitor) reduced cell viability *in vitro* and suppressed tumor growth *in vivo*	[74]
	Cell lines with concurrent *PIK3CA* mutations or PTEN loss/BRAF V600E GEMMs	Combination therapy with BRAF and PI3K inhibitors induced apoptosis in vitro, delayed tumor growth *in vivo* and caused tumor regression in GEMMs	[70, 72, 73]
	"BRAF-like" CRC cell lines	"BRAF-like" CRC cell lines were selectively sensitive to the microtubule poison vinorelbine both *in vitro* and *in vivo*	[76]
PIK3CA mutations or PTEN loss	Cells carrying *PIK3CA* mutations or *PTEN* loss but not *BRAF/KRAS* mutations	Everolimus (mTOR inhibitor) slowed cell growth *in vitro* and resulted in long term-tumor growth arrest *in vivo*	[92]
	Analysis of NHS and HPFS studies and VICTOR trial	Adjuvant low-dose aspirin in *PIK3CA*-mutant patients improved survival. Further prospective studies are required	[98, 99]

HER2 amplification	*HER2*-amplified patient-derived xenografts	Combination of cetuximab/pertuzumab with lapatinib induced overt long-lasting tumor regression	[105]
	HERACLES clinical trial	Combination of trastuzumab and lapatinib was active in heavily pretreated mCRC patients	[110]
HER2 mutations	CRC cell lines and patient-derived xenografts with *HER2* mutations	Dual HER2-targeted therapy with trastuzumab and small-molecule inhibitors such as lapatinib or neratinib produced durable tumor regression	[108]
MET activation	HGF-overexpressing cells	Co-treatment with cetuximab and MET inhibitors induced marked tumor regression	[124]
	MET amplified patient-derived xenografts	MET inhibition achieved long-lasting abolition of tumor growth *in vivo*	[119]
EGFR mutation	Patient-derived xenografts with the *EGFR* kinase domain mutation V843I	Combination of cetuximab and afatinib induced marked and long-lasting inhibition of tumour growth	[128]
FGFR1 amplification	Patient-derived xenografts with *FGFR1* amplification	Combination of cetuximab with the selective FGFR kinase inhibitor BGJ398 durably suppressed tumor growth	[128]
PDGFRA mutation	Patient-derived xenografts with the *PDGFRA* R981H mutation	Combination of cetuximab with the PDGFR inhibitor imatinib exerted strong, but short lived, anti-tumor activity	[128]
MAP2K1 (MEK1) mutation	Patient-derived xenografts with the *MAP2K1* K57N mutation	Vertical blockade of MEK and ERK resulted in strong inhibition of tumor growth	[128]
Acquired resistance			
RAS/BRAF activation	CRC cell lines with acquired *KRAS/BRAF* point mutations and/or KRAS amplification and one patient-derived xenograft	Combinations of cetuximab with pimasertib (MEK inhibitor) induced moderate tumor shrinkage *in vivo*	[48]
	Cetuximab sensitive CRC cell lines and patient-derived xenografts	Dual blockade of EGFR and MEK delays the onset of acquired resistance driven by the RAS/MAPK pathway	[61]
HER2 activation	Cells with high heregulin levels or *HER2* amplification	Pertuzumab/lapatinib restored sensitivity to cetuximab *in vitro*	[106]
MET activation	*MET* amplified patient-derived xenografts	Combined inhibition of MET and EGFR induced long-lasting disease stabilization *in vivo*.	[119]
EGFR mutations	Mutations in the *EGFR* ectodomain (S492R, G465E and G465R) found in patients.	Panitumumab remained active in a patient with S492R mutation, which abrogated cetuximab binding. Anti-EGFR monoclonal antibody mixtures or oligoclonal antibodies displayed strong antitumour activity in patient-derived cell cultures and xenografts with *EGFR* mutations in the G465 residue	[126, 128, 129]

1.2.1 RAS

The RAS family includes three small G proteins (KRAS, NRAS, and HRAS) that couple EGFR to downstream activation of the RAF-MEK-ERK pathway [30]. Several retrospective trials have linked *KRAS* mutations in exon 2 (codons 12 and 13), which are found in approximately 40–45% of CRCs [17, 34], to primary resistance to cetuximab or panitumumab [14, 35–37]. The robust predictive significance of such correlations was sufficient for the regulatory approval of companion diagnostics for routine assessment of *KRAS* exon 2 mutations, and now the clinical use of anti-EGFR moAbs is limited to the subset of patients with *KRAS*-wild-type colorectal cancers [34, 38–42].

Although the exclusion of patients with *KRAS* (exon 2)-mutant tumors from anti-EGFR therapy has increased the percentage of responders from 10% to 13–17%, most *KRAS* (exon 2) wild-type tumors remain insensitive to anti-EGFR moAbs [34, 40]. Rare mutations of *KRAS* in codons other than 12 and 13, as well as mutations of *NRAS*, have been found to correlate with therapeutic refractoriness. The relatively high cumulative frequency of such additional mutations, and their successful validation as resistance biomarkers in prospective trials, strongly call for systematic evaluation of these genotypes in clinical practice to enlarge the fraction of patients to be spared anti-EGFR therapy [43]. A very low frequency of *KRAS* amplification (0.7%) has also been reported and demonstrated to correlate with primary resistance [44].

RAS activating mutations and gene copy number gains are responsible not only for primary resistance but also for acquired resistance in 40–60% of patients who progress on cetuximab or panitumumab [45–47]. As mentioned above, such mutations are either pre-existing in minor tumor subclones before treatment initiation [45, 46] or arise as *de novo* alterations under drug pressure [46, 47]. *KRAS* mutations could be detected non-invasively 5–10 months before radiographic evidence of disease progression by analyzing cell-free circulating tumor DNA (ctDNA) [45, 46]. Using this methodology, two recent studies have documented the emergence of several independent clones displaying heterogeneous patterns of *KRAS* and *NRAS* mutations in concomitance with progressive desensitization to EGFR blockade [48, 49].

At present, patients with *KRAS*-mutant mCRC are treated with chemotherapy (with or without anti-angiogenic therapy) and, in the chemorefractory setting, with the multi-target inhibitor regorafenib [50, 51]. To date, direct pharmacologic blockade of the mutant KRAS protein has been unsuccessful; therefore, preclinical studies have concentrated on approaches as different as targeting downstream effectors such as MEK and PI3K [52], leveraging synthetic lethal interactions [53–58], or deploying high-throughput drug screens [59]. Most of these attempts showed that the combinatorial inhibition of two different pathways induces some anti-cancer effects in *KRAS* mutant CRC mouse models, albeit seldom with manifest tumor shrinkages [60] (see Table 1.1). Some of these preclinical strategies have been translated in recently completed phase I/II clinical trials (NCT01085331; NCT01390818;

NCT02039336), for which the results are eagerly awaited. In the case of acquired resistance due to *RAS* mutations, preclinical evidence suggests that combination therapies *ab initio* with EGFR and MEK inhibitors could delay or reverse the emergence of resistance [48, 61].

1.2.2 BRAF

Point mutations of *BRAF*, which encodes a serine/threonine kinase directly activated by RAS and impinging on the downstream effector MEK, are found in 4–13% of advanced CRCs and are typically mutually exclusive with *KRAS* mutations [17, 62].

The *BRAF* V600E mutation has been described as a determinant of poor response to cetuximab and panitumumab [15, 17, 62, 63]. However, the negative predictive power of *BRAF* mutations is undermined by their low frequency and is further biased by the pervasive role of mutant *BRAF* as a negative prognostic biomarker [41, 62–64]. Overall, the predictive impact of this alteration remains to be established and requires further prospective evaluation before clinical applicability [17, 41, 62, 65].

Unlike RAS, BRAF can be efficiently blocked by clinically approved compounds; BRAF small-molecule inhibitors are extensively and successfully used in *BRAF*-mutant melanoma, for example, with response rates (RRs) ranging between 48% and 67% [10, 66]. However, selective BRAF inhibitors such as vemurafenib have failed in *BRAF*-mutant CRCs (RR of 5%) [67]; this lack of efficacy has been ascribed to rapid feedback activation of EGFR following BRAF inactivation, resulting in constitutive signaling through the MAPK–ERK pathway and continued tumor cell proliferation [68, 69]. Accordingly, preclinical studies have demonstrated that BRAF blockade can resensitize to anti-EGFR antibodies [62, 68–70]. At the clinical level, interim reports from an ongoing clinical trial have shown 22% RRs in patients with *BRAF*-mutant mCRC treated with a combination of cetuximab and encorafenib, an investigational BRAF inhibitor [71]. The trial has now entered a phase II expansion cohort (NCT01719380). Investigators are also collecting tumor and blood samples from patients before and after treatment to analyze the drugs' pharmacodynamic consequences, while a broad genomic survey is planned to identify predictive biomarkers [71]. Other combinatorial approaches under preclinical or clinical evaluation [59, 72–74] are listed in Table 1.1.

Intriguingly, some *BRAF* wild-type CRCs display a gene expression signature and a clinical behavior (poor prognosis) that are very similar to those typifying *BRAF*-mutant tumors [75]. By applying a loss-of-function genetic screen, cell lines from this specific tumor subtype were shown to have defects in microtubule formation, unveiling a potential vulnerability to microtubule-disrupting agents [76].

BRAF mutations could be also captured non-invasively by ctDNA analysis, together with concomitant *KRAS* and *NRAS* mutations [48, 49], in patients who had responded to anti-EGFR antibodies and then progressed. Hence, the emergence of *BRAF* mutant subclones may also sustain acquired resistance.

1.2.3 PI3K-AKT-PTEN Pathway

PI3Ks include different classes of lipid kinases; in particular, activation of class IA PI3Ks can be triggered by upstream stimulation from RTKs [77], but also through RAS intermediation [78] or signaling from G protein-coupled receptors [19].

Class IA PI3Ks are heterodimeric proteins composed of a regulatory (p85) and a catalytic (p110) subunit [79]. Activating mutations of *PIK3CA* (encoding p110α) have been detected in 10–20% of CRCs [17, 80–82]; most of them occur in exons 9 and 20, respectively, in the helical and kinase domain [80, 83]. In a retrospective analysis of 110 mCRC patients treated with cetuximab or panitumumab, a statistically significant association between primary resistance to EGFR inhibition and *PIK3CA* mutations (11 in exon 20 and 4 in exon 9, all in *KRAS* wild-type tumors) was reported [84]. Another study, conducted in a patient cohort with a higher prevalence of exon 9 mutations, did not confirm such a correlation [82]. These discrepant data were then reconciled by a retrospective consortium analysis on a larger collection of 1022 tumor samples; the consensus is now that, in the *KRAS* wild-type subpopulation, only the *PIK3CA* exon 20 mutations may be predictive of lack of response to anti-EGFR moAbs [17]. This study also highlighted a strong association between *PIK3CA* exon 9 (but not exon 20) mutations and *KRAS* mutations, reinforcing the notion that *PIK3CA* exon 9 mutations do not have an independent predictive value for anti-EGFR antibody efficacy.

Loss of function of PTEN, a phosphatase that contrasts PI3K activity, occurs in 30% of CRCs through various mechanisms including gene deletion, frameshift or nonsense mutations, and promoter methylation [85, 86]. PTEN inactivation (usually evaluated as lack of protein expression) has been associated with poor sensitivity to anti-EGFR moAbs in mCRC patients in several studies [16, 85, 87, 88], whereas others have only put forward a prognostic role [63]. All in all, both *PIK3CA* exon 20 mutations and PTEN inactivation are promising predictors of reduced responsiveness to anti-EGFR therapies. However, due to the low incidence of exon 20 mutations (2–5%) [89] and lack of an established method for assessment of PTEN inactivation [17, 85, 88, 90, 91], further prospective trials and methodological efforts are necessary to validate the clinical utility of PI3K pathway activation as a negative response determinant.

In principle, patients with tumors exhibiting *PIK3CA* mutations or PTEN loss of function, without concomitant *KRAS/BRAF* mutations, may respond to therapies targeting PI3K or PI3K-downstream transducers, such as mTOR or AKT [92]; however, clinical data have demonstrated only minimal single-agent activity of such therapies at tolerated doses [93–95]. Since the PI3K/AKT inhibition is commonly counteracted by feedback activation of tyrosine kinase receptors [96], it is expected that blockade of the PI3K pathway will provide greater benefit when combined with RTK inhibitors [97]. Phase I/II studies testing mTOR inhibitors, such as everolimus or temsirolimus, in combination with RTK inhibitors or anti-EGF moAbs (in some cases, in the presence of a chemotherapy backbone) are presently being conducted or have been recently completed in mCRC patients (NCT01154335; NCT01139138; NCT01387880; NCT00827684).

Finally, prevention studies have shown improved survival by low-dose aspirin in patients with *PIK3CA*-mutant CRC [98–100]; this observation, which demands further prospective evaluation, could be at least partially related to the fact that the PI3K-AKT axis induces NF-κB-dependent transcriptional upregulation of COX2, which has been demonstrated to exert pro-survival signals in CRC cells [100–102]. Therefore, a *PIK3CA*-mutant makeup may render CRC cells vulnerable to apoptosis by aspirin-mediated COX2 inhibition.

Recently, the presence of *PIK3CA* mutations has been also detected in tissue samples from mCRC patients treated with cetuximab who relapsed while on treatment. Of note, such mutations coexisted with other acquired mutations (in *KRAS, NRAS* or *BRAF* genes) within the same sample [103].

1.2.4 HER2

When considering the cumulative frequency of *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* alterations, approximately 60–65% of anti-EGFR resistant cases can be ascribed to the presence of such mutations [16]; in the remaining 30% of 'quadruple negative' cases, still-unidentified features sustain lack of response.

HER2 is the only member of the ErbB family that is not bound by growth factor ligands; it is activated through hetero-dimerization with other ligand-stimulated receptors [20], with the most powerful growth-promoting cues generated by HER2-HER3 heterodimers; *HER2* overexpression, usually caused by gene amplification, enables HER2 constitutive signaling regardless of the activation state of the other partners [104].

Several preclinical and clinical studies have shown that *HER2* amplification is a predictor of poor sensitivity to anti-EGFR antibodies [105, 106]. Based on genotype-response correlations in a platform of patient-derived mCRC tumorgrafts, *HER2* amplification was found to be significantly associated with resistance to cetuximab and specifically enriched in the quadruple negative population [91]. Aberrant HER2 signaling (by either *HER2* amplification or overproduction of the HER3 ligand heregulin) was confirmed as a mediator of lack of response in an independent report [106]. In retrospective clinical studies, patients with colorectal tumors displaying *HER2* amplification or heregulin overexpression and treated with cetuximab or panitumumab had shorter progression-free and overall survival compared with patients with *HER2* wild-type tumors [105–107]. Notably, in patients with acquired resistance, *HER2* amplification was detected in a small fraction (14%) of pretreatment tumor cells and in a much larger proportion of cells (71%) in samples biopsied after anti-EGFR therapy. Similarly, heregulin levels, as assessed in both plasma and tumor specimens, were found to be significantly higher in patients who had relapsed on anti-EGFR therapy with respect to responders [106]. Hence, increased HER2 signaling drives both primary and acquired resistance.

Besides *HER2* amplification, also *HER2* activating point mutations can confer resistance to EGFR blockade in CRC cell lines and patient-derived tumorgrafts [108]. In both instances (amplification and mutations), monotherapy with either anti-HER2

antibodies or HER2 small-molecule inhibitors was not sufficient to induce regression of patient-derived tumorgrafts in mice, and only a combination of antibodies and chemical inhibitors led to massive tumor shrinkage [108, 109]. At least for *HER2* amplification in CRC, trastuzumab (the prototypical anti-HER2 antibody) alone was found to be mainly active against HER3, with minor inhibitory effects on HER2 and EGFR. In contrast, the reversible HER2 small-molecule inhibitor lapatinib prompted rapid and drastic dephosphorylation of all ErbB receptors, but also led to delayed reactivation of HER3 as a compensatory mechanism. Indeed, the stronger effect of the antibody-small molecule combination was attributed to the ability of trastuzumab, through preferential targeting of HER3, to prevent lapatinib-induced HER3 rephosphorylation [109].

These preclinical findings encouraged the design and execution of HERACLES, a clinical trial that assessed the efficacy of the trastuzumab-lapatinib combination in mCRC patients with *KRAS* wild-type, *HER2*-amplified, cetuximab-resistant tumors. Eight (30%) patients achieved objective responses, and 12 (44%) had stable disease [110]. Because this patient subpopulation was heavily pretreated and resistant to both conventional chemotherapy and anti-EGFR antibodies, the outcome data are particularly compelling and testify to the potential of HER2 as a viable target in the treatment of colorectal cancer.

Active HER2 also exacerbates the oncogenic properties of *HER3* mutations, which have been recently described in about 11% of colon cancers [111]. One could envision a ‘dosage effect’ whereby low-grade *HER2* amplification or low levels of heregulin, which alone would not be enough to foster therapeutic resistance, might in fact attenuate sensitivity to EGFR inhibition by cooperating with co-existing *HER3* mutations. Investigational anti-HER3 antibodies and small molecules have been shown to productively contrast HER3-mediated signals and tumor progression in preclinical studies *in vivo* [111] and are now being tested clinically. Therefore, *HER3* mutations in CRC merit investigation as new potential biomarkers of resistance to anti-EGFR treatment as well as new predictors of response to other therapeutic options.

1.2.5 MET

Similar to EGFR family members, the MET tyrosine kinase receptor for hepatocyte growth factor (HGF) can activate growth, survival and motility pathways through the RAS- ERK cascade, the PI3K-AKT axis, and stimulation of SRC and STAT [112–114]. Excessive MET signaling may occur by several mechanisms, including genetic abnormalities such as *MET* amplification and exon 14 skipping mutations (splicing variants that result in the deletion of a negative regulatory domain of the MET kinase), but also as a consequence of increased HGF expression/activity [96]. When genetically altered, *MET* can act both as a primary oncogenic driver and as a determinant of resistance to EGFR tyrosine kinase inhibitors, in particular in NSCLCs harboring *EGFR* mutations [115–117]. *MET* amplification also sustains tumorigenesis and correlates with response to MET small-molecule inhibitors in gastroesophageal cancer [118].

In CRC, *MET* amplification has been documented as a mechanism of primary and acquired resistance to cetuximab and panitumumab [119]. In retrospective analyses, *MET* amplification was detected in around 1% of mCRC samples, in line with previous findings [120]. However, this frequency increased to 12.5% in a subgroup of cetuximab-resistant patient-derived tumorgrafts with wild-type forms of *KRAS, NRAS, BRAF, PIK3CA* and *HER2*. Notably, MET-mediated resistance appears to be driven by a dosage effect: only focal, high-grade amplification of the *MET* locus correlated with overt therapeutic refractoriness, whilst tumors with modest gene copy number gains or polysomy of chromosome 7, where the *MET* gene is located, were still susceptible to cetuximab [120]. Preclinical trials in MET-positive xenografts from CRC cell lines and patient-derived materials revealed that MET inhibition, with or without concurrent interception of EGFR, led to long-lasting abolition of tumor growth [119, 121]. In this vein, a phase II clinical trial aimed to assess the efficacy and safety of the dual MET-ALK inhibitor crizotinib in patients with solid tumors (including CRCs) harboring *MET* genetic alterations has been designed and is currently recruiting participants (NCT02034981).

MET amplification was also found in the tumors of three out of seven patients who had developed a form of acquired resistance to the anti-EGFR antibodies that could not be ascribed to the emergence of secondary *KRAS* mutations. Importantly, the *MET* amplicon was detected in circulating, cell-free DNA as early as 3 months after treatment initiation, well before relapse was observed radiologically. Similar to *HER2* amplification and *KRAS* mutations, rare *MET*-amplified cells could be identified in pre-treatment tumor material from one out of three patients with MET-dependent acquired resistance, suggesting that pre-existing subclones were positively selected under the pressure of anti-EGFR therapy [119].

A recent case report suggests that *MET* amplification in CRC not only precludes sensitivity to upstream EGFR blockade, but also prevents responsiveness to agents targeting the downstream RAS pathway. A patient with a *BRAF*-mutant mCRC who had initially responded to combined EGFR and BRAF inhibition progressively developed resistance. Genetic analysis of matched biopsies before and after therapy revealed a higher representation of *MET*-amplified cancer cells in the post-treatment tissue, and dual blockade of both BRAF and MET proved to be clinically effective [122]. Again, these results point to MET hyperactive signaling as a pervasive resistance trait in mCRC, and highlight the value of MET therapeutic targeting to oppose disease progression.

MET activation can attenuate sensitivity to cetuximab also as a consequence of paracrine HGF stimulation, as observed in CRC cell lines [119, 123] or, more recently, in CRC spheroids enriched in cancer stem cells [124]. In these studies, only concomitant inhibition of both MET and EGFR substantially regressed tumors *in vivo*. This experimental evidence might have clinical relevance, as HGF overexpression correlates with reduced sensitivity to cetuximab in patients [124]. However, the definition of cut-offs to dichotomize HGF-positive versus HGF-negative tumors in the clinic is not trivial, which undermines the portability of assessing HGF levels for patient stratification.

1.2.6 EGFR

Additional genetic alterations within the target oncoprotein, which affect drug binding thus preventing kinase inhibition, are frequently responsible for both primary and acquired resistance in cancer; an emblematic example is represented by the T790M 'gatekeeper' secondary mutation in the *EGFR* gene, which drives resistance to first-generation EGFR small-molecule inhibitors in *EGFR*-mutant NSCLC [125]. In colorectal cancer, different mutations in the extracellular domain of EGFR have been recently described as a typical mechanism of acquired resistance, namely, S492R, G465E and G465R mutations [126–128] (Fig. 1.2d). Structural analyses indicate that while S492 selectively lies in the cetuximab binding site, G465 is located in the center of the region in which the epitopes of both cetuximab and panitumumab overlap. Accordingly, S492R abrogates cetuximab binding but retains panitumumab interaction, whereas G465E and G465R prevent binding of both antibodies. Studies in patient-derived tumorgrafts [128] and cell cultures [129] harboring mutations in the G465 residue have shown that new-generation anti-EGFR antibodies that bind EGFR epitopes different from those recognized by cetuximab and panitumumab are very effective in opposing the growth of these tumors.

Resistance may be also driven by mutations in the EGFR kinase domain: two alterations have been identified as circulating mutations by cell-free DNA analysis [49], and one has been detected in cetuximab-resistant patient-derived tumorgrafts [128]. Treatment of such tumorgrafts with an EGFR small-molecule inhibitor or cetuximab alone was not effective, but the combination resulted in substantial and durable inhibition of tumor growth [128].

1.3 Newly Emerging Biomarkers of Drug Resistance and Sensitivity

A recent systematic survey of molecularly annotated patient-derived tumorgrafts has functionally linked therapeutic responses to EGFR inhibitors with complete exome sequence and copy number analyses as a way to identify new resistance traits and, potentially, new druggable targets. By doing so, in addition to the genetic abnormalities described above, new alterations have been found, including mutations/amplification in *FGFR1*, *PDGFRA* and *MAP2K1* [128] and outlier overexpression of *IGF2* [28]. All these tumorgrafts proved to be susceptible to therapies targeting the resistance-conferring genetic alterations. Another actionable lesion in CRC that has recently received clinical attention is the *NTRK1* chromosomal rearrangement, which leads to the synthesis of a highly expressed fusion protein with constitutive NTRK kinase activity. A case report has described a patient with metastatic colorectal cancer harboring an *LMNA–NTRK1* rearrangement who achieved a remarkable clinical and radiographic response to entrectinib (RXDX-101), a multikinase inhibitor targeting TRK, ALK, and ROS1, which was followed by the

emergence of resistance [130]. Longitudinal monitoring of the *LMNA–NTRK1* status by ctDNA analysis revealed the acquisition of two novel NTRK1 kinase domain mutations (G595R and G667C) that were absent from ctDNA collected at the time of treatment initiation. According to structural studies, such mutations are expected to abrogate or reduce entrectinib binding to the catalytic pocket, rendering tumors less vulnerable to this specific inhibitor [131].

While the quest for resistance biomarkers has yielded considerable results in the past years, data remain immature as far as the identification of positive determinants of responsiveness to EGFR blockade is concerned. As noted above, *EGFR* is very rarely mutated or amplified in CRC, and the only known means to achieve EGFR hyperactivation seems to be increased paracrine/autocrine expression of some EGFR ligands, in particular amphiregulin and epiregulin. Accordingly, high levels of amphiregulin and epiregulin correlate with a better response to anti-EGFR moAbs [26, 27, 29, 132, 133]. However, as already discussed for HGF, the clinical application of this information is hindered by the difficulty in setting thresholds to distinguish ligand-positive versus ligand-negative tumors. Intriguingly, responsive cases appear to be enriched for genetic lesions (mutations or amplification) of *IRS2*, a cytoplasmic adaptor protein that relays signals from tyrosine kinase receptors to downstream effectors [128]. In functional assays, RNA interference-mediated silencing of IRS2 was accompanied by attenuated sensitivity to cetuximab and reduced activation of EGFR-dependent pathways, in line with the role of IRS2 as an amplifier of tyrosine kinase signals. The clinical applicability of this information for optimized selection of responsive patients remains to be determined.

1.4 Outlook

Although many genetic determinants of resistance to anti-EGFR antibodies have been recently documented, and some of them have been validated as alternative pharmacologic targets, there is still space for the identification of additional druggable alterations and the deployment of further therapeutic strategies. Genome-scale analyses of CRC tumor collections are expected to provide a fresh catalog of new mutations, rearrangements, and copy-number alterations with therapeutically actionable potential [134, 135] and will receive further momentum by proteogenomics data [136]. Moreover, promising results are being offered by treatments that disrupt immune evasion strategies. To stimulate immune suppression, tumor cells often engage immune checkpoint molecules, such as CTLA-4 and PD1, which quench cytotoxic T-cell activation. Antibodies against CTLA-4 (e.g., ipilimumab) or PD1 (e.g., nivolumab, pembrolizumab) have been shown to induce durable tumor regressions [137, 138] in mismatch repair–deficient colorectal cancer, likely because the large number of somatic mutations present in these hypermutated tumors increase the presentation of non-self immunogenic neo-antigens and, hence, sensitize to immune checkpoint blockade [139].

Although several resistance mechanisms have been documented so far, mutant *RAS* is the only clinically validated biomarker for selection of mCRC patients eligible to treatment with anti-EGFR antibodies. This attrition between experimental discovery and clinical implementation advocates the introduction of new clinical trial designs that capitalize on reliable preclinical findings. In this regard, a successful story is our experience with mCRC cases harboring *HER2* amplification: from retrospective identification of this alteration in archival patient material, and after establishing a statistically robust correlation between the occurrence of *HER2* amplification and primary resistance to EGFR inhibition, we moved to testing different therapeutic options in HER2-positive patient-derived tumorgrafts and found one treatment that resulted in overt and long-lasting tumor regression [105, 109]. The very same regimen was then applied to patients with *HER2*-amplified tumors with positive results [110]. In this case, reliable tumor models, stringent endpoint criteria for animal studies, and accurate genetic selection were the ingredients that made this translational effort a winning opportunity.

Future clinical trials will be informed by real-time monitoring of tumor evolution along treatment so as to adjust therapies (likely, combination therapies) to the continuing mutability of cancer. While multi-dimensional analysis of serial biopsies is, in principle, the most informative approach, it should also be considered that an individual tumor biopsy may not be representative of overall intratumor heterogeneity, and post-treatment tumor tissue is difficult to obtain. Such limitations can be overcome by less invasive analyses on ctDNA, which can offer a high degree of sensitivity and specificity to detect the surfacing of resistance-conferring mutations over the course of therapy [49, 140]. The mechanism by which ctDNA is released into the bloodstream and whether multiple metastases, or different regions within the same tumor, shed ctDNA homogeneously are still unclear; however, the proof-of-concept that such an approach is viable and its merit in raising an early warning of acquired resistance are now consolidated [46, 49, 141, 142]. Inevitably, to gather a more comprehensive picture of tumor adaptation to targeted treatment and to more effectively tackle the ever-evolving resistant phenotype at the therapeutic level, mutational analysis needs to be integrated by other molecular approaches that detect changes in gene expression, proteins, and protein activities. While this is feasible, at present, only in bioptic material—with all the hurdles and challenges related to repeated biopsies discussed above—hints are emerging whereby non-invasive techniques may prove useful also to measure RNA and protein/phosphoprotein levels in blood, for example by isolating circulating exosomes [143].

If appropriately dosed in quantity and scheduled in time, new investigational therapies could also leverage tumor heterogeneity to their own advantage: creating a “balance” between drug activity and graded responsiveness of different clones to drug pressure might be useful to retard the onset of resistance and, ideally, to turn cancer into a chronic disease. Intriguingly, the prevalence of *KRAS* mutant subclones that become detectable in the blood of mCRC patients on anti-EGFR therapy has been demonstrated to decline after treatment withdrawal, leaving space to *KRAS* wild-type populations that regain drug sensitivity [142]. This could explain why some mCRC patients benefit from multiple challenges with anti-EGFR antibodies.

More than a decade after the introduction of cetuximab in the treatment of metastatic colorectal cancer, much is known about the genetic determinants of primary and acquired resistance to anti-EGFR moAbs in CRC. What is now becoming increasingly clear is that therapeutic resistance is not a fixed, irreversible state, but rather the expression of a resilient phenotype that reacts to drug pressure through manifold sophisticated elusion strategies. The time is ripe to move from a static vision of the disease to a more flexible appraisal of tumor evolution, adaptation and dynamic instability.

Acknowledgments Work in the author's laboratory is supported by AIRC, Associazione Italiana per la Ricerca sul Cancro—2010 Special Program Molecular Clinical Oncology 5 × 1000, project 9970— and AIRC Investigator Grant, project 18532; FPRC, Fondazione Piemontese per la Ricerca sul Cancro, Ministero della Salute 2011; ERA-NET TRANSCAN, project TACTIC.Clinical trials available at:NCT01085331: http://clinicaltrials.gov/ct2/show/NCT01085331?term=NCT01085331&rank=1NCT01390818: http://clinicaltrials.gov/ct2/results?term=NCT01390818&Search=SearchNCT02039336: http://clinicaltrials.gov/ct2/show/NCT02039336?term=NCT02039336&rank=1NCT01154335: http://clinicaltrials.gov/ct2/show/NCT01154335?term=colorectal+cancer&rank=33NCT01139138: http://clinicaltrials.gov/ct2/show/NCT01139138?term=colorectal+cancer&rank=67NCT01387880: http://clinicaltrials.gov/ct2/show/NCT01387880?term=everolimus+AND+colorectal+cancer&rank=2NCT00827684: http://clinicaltrials.gov/ct2/show/NCT00827684?term=everolimus+AND+colorectal+cancer&rank=9NCT02034981: http://clinicaltrials.gov/ct2/results?term=NCT02034981&Search=Search

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 2
Resistance of Lung Cancer to Kinase Inhibitors Specific to EGFR or ALK

Maicol Mancini and Yosef Yarden

Abstract Lung cancer is by far the major cause of cancer-related death. The identification of oncogenic mutations in the genes encoding for the epidermal growth factor receptor (EGFR) and the anaplastic lymphoma kinase (ALK) opened the way for development of relatively effective tyrosine kinase inhibitory (TKI) drugs, such as erlotinib and crizotinib, respectively. Unfortunately, resistance to these and other first-generation TKIs evolves in patients within a year or two. Several mechanisms underlie acquired resistance and they include second-site mutations, compensatory signaling pathways and phenotype alterations. Once resolved, mechanisms conferring resistance to TKIs may pave the way for next-generation TKIs, or they may identify combination therapies simultaneously inhibiting the primary and alternative routes to oncogenesis. Herein, we review the first-, second- and third-generation inhibitors of EGFR and ALK, along with the many ways permitting lung cancer cells to evade pharmacological interceptors in experimental systems and in clinical settings.

Keywords Tumor heterogeneity • Kinase domain mutation • Acquired resistance • Signal transduction • Compensatory pathway

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_2

Abbreviations

ALK	Anaplastic lymphoma kinase
CRC	Colorectal cancer
EGFR	Epidermal growth factor receptor
EMT	Epithelia-to-mesenchymal transition
ERK	Extracellular regulated kinase
HER2	Human EGF receptor 2
HGF	Hepatocyte growth factor
mAb	Monoclonal antibody
NSCLC	Non-small cell lung cancer
PI3K	Phosphatidylinositol 3-kinase
RTK	Receptor tyrosine kinase

2.1 Introduction to Lung Cancer

Despite advances in diagnosis and therapy, lung cancer remains the major cause of cancer-related mortality [1]. Tobacco smoking is by far the major source of lung cancer, worldwide. Remarkably, in many western countries lung cancer accounts for more cancer-related deaths than the next three most common cancer types combined [2]. This miserable statistics is due not only to high incidence, which has decreased slightly in recent years, but also to late diagnosis: at presentation, the majority of patients with lung cancer have locally advanced or metastatic disease. As a consequence, the estimated overall 5-year survival rate for patients with lung cancer is only 17%. Hence, developing novel lung cancer prevention, diagnosis, and treatment strategies remains a high priority challenge.

Approximately 85% of lung cancer patients are diagnosed with non-small cell lung cancer (NSCLC). There are three major histological subtypes of NSCLC: adenocarcinoma, the largest one, large cell carcinoma and squamous cell carcinoma. Figure 2.1 presents the classification of NSCLC according to histology and according to identity of the driver mutation. Surgery is the recommended treatment for NSCLC patients with stage I–II disease, but high-dose radiation therapy may be offered to patients with clinical stage I NSCLC who have medical contraindications to surgical resection. NSCLC patients presenting more advanced disease usually receive a platinum-based combination chemotherapy, which offers only modest prolongation in survival [3]. The discovery in 2004 of mutant forms of the epidermal growth factor receptor (EGFR) in NSCLC identified groups of patients who are sensitive to tyrosine kinase inhibitors (TKIs) like gefitinib and erlotinib [4–6]. Similarly, the discovery, 3 years later, of the first genomic rearrangements within the gene encoding for the anaplastic lymphoma kinase, *ALK* [7, 8], provided a

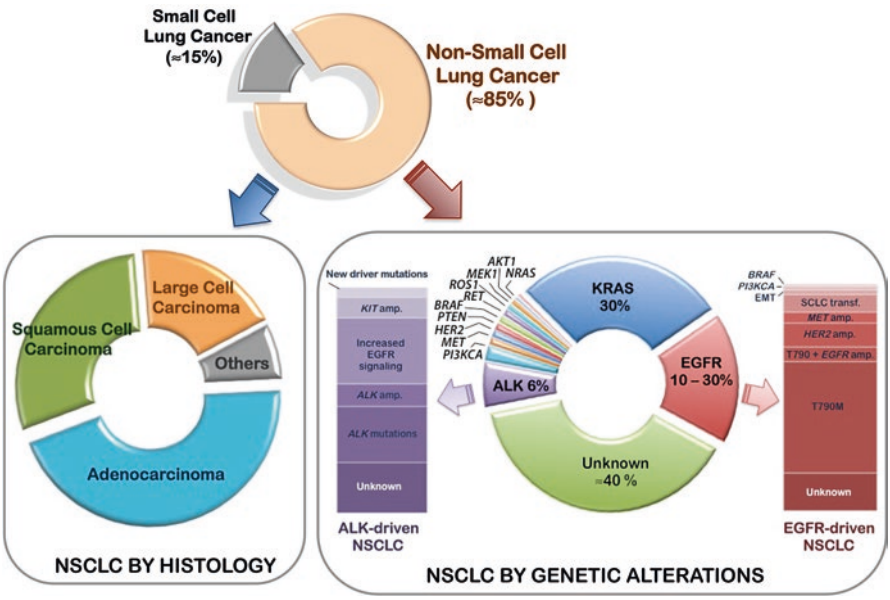

Fig. 2.1 Stratification of lung cancer patients by histology and by genetic aberrations. The different histological types of lung cancer are indicated. In addition, mechanisms underlying acquired resistance to the first generation EGFR and ALK kinase inhibitors are indicated (see the *bottom right panel*). Note that the fraction of patients carrying EGFR mutations varies according to ethnic origin. The characteristic driver mutations of never-smokers are EGFR, ALK, ROS1, ERBB2, RET, BRAF (V600E), and NTRK1 mutations, while KRAS mutations characterize smokers

second biomarker linked to an approved use of a targeted agent, a TKI called crizotinib. When applied in early clinical trials in patients with *ALK* rearrangements, crizotinib achieved remarkable clinical effects [9, 10], which led to the 2011 accelerated approval of the drug for treatment of *ALK*-positive advanced NSCLC. Table 2.1 lists the major small molecule inhibitors already approved in oncology. Currently, large clinical studies are investigating the role of EGFR-specific TKIs in the *EGFR*-mutant population, ALK inhibitors in the *ALK*-positive population, and immunotherapy in the non-biomarker-selected population [11]. Moreover, it was estimated that up to 69% of patients with advanced NSCLC could have a potentially actionable molecular target, analogous to EGFR mutations and ALK rearrangements [12]. Thus, the ability to select patients on the basis of genomic changes has introduced lung cancer to the era of targeted therapy, and shifted treatment of many patients from chemotherapy to molecularly tailored drugs. For patients with advanced NSCLC who do not fit an approved molecular targeted therapy, the standard first-line treatment remains platinum-based therapy, with or without bevacizumab, an antibody targeting the vascular endothelial growth factor.

Table 2.1 Kinase and other small molecule inhibitors approved in clinical oncology

Name (trade name)	Year	Known target	Indication
Rucaparib (Rubraca)	2016	PARP1/2	Ovarian cancer in women with BRCA1/2 mutations
Venetoclax (Venclexta)	2016	Bcl-2	CLL with 17p deletion
Alectinib (Alecensa)	2015	ALK, RET	ALK-positive NSCLC
Cobimetinib (Cotellic)	2015	A/B/C-Raf and BRaf (V600E or V600 K)	Melanoma
Lenvatinib (Lenvima)	2015	VEGFRs/FGFRs/PDGFR/Kit/RET	Tyroid cancer, Advancer RCC
Palbociclib (Ibrance)	2015	CDK4/6	Breast cancer
Ixazomib (Ninlaro)	2015	Proteosome inhibitor	Multiple myeloma
Sonidegib (Odomzo)	2015	Smoothened	Basal cell carcinoma
Osimertinib (Tagrisso)	2015	EGFR	EGFR-mutant NSCLC
Ceritibib (Zykadia)	2014	ALK, IGF-1R, InsR, ROS1	ALK-postive NSCLC after crizotinib resistance
Nintedanib (Ofev)	2014	VEGFR, FGFR, PDGFR	Idiopathic pulmonary fibrosis
Olaparib (Lynparza)	2014	PARP	Ovarian cancer BRCA1/2 mutation
Idelalisib (Zydelig)	2014	PI3Kδ	CLL
Afatinib (Gilotrif)	2013	EGFR	NSCLC
Dabrafenib (Tafinlar)	2013	B-Raf	Melanoma
Ibrutinib (Imbruvica)	2013	Bruton's kinase	Mantle cell lymphoma, CLL, Waldenstrom's macroglobulinemia
Trametinib (Mekinist)	2013	MEK1/2	Melanoma
Axitinib (Inlyta)	2012	VEGFR1/2/3	RCC
Bosutinib (Bosulif)	2012	BRC-Abl, Src, Lyn and Hck	CML
Cabozantinib (Cabometyx)	2012	VEGFR2, Met, Axl, RET, Kit, TrkB, Flt3, Tie2	Advanced RCC, Metastatic medullary thyroid cancer
Tofacitinib (Xeljanz)	2012	JAK3	Rheumatoid arthritis
Ponatinib (Iclusig)	2012	BCR-Abl, BCR-Abl T315I, VEGFR, PDGFR, FGFR, Eph, Src family kinases, Kit, RET, Tie2 and Flt3	CML, Ph chromosome positive ALL
Regorafenib (Stivarga)	2012	VEGFR1/2/3, BCR-Abl, B-Raf, B-Raf (V600E), Kit, PDGFRα/β, RET, FGFR1/2, Tie2 and Eph2A	CRC

Crizotinib (Xalkori)	2011	ALK, c-Met and Ros	ALK-postive NSCLC
Ruxolitinib (Jakafi)	2011	JAK1/2	Myelofibrosis and PV
Vandetanib (Caprelsa)	2011	EGFRs, VEGFRs, RET, Brk, Tie2, EphRs and Src family kinases	Medullary thyroid cancer
Vemurafenib (Zelboraf)	2011	A/B/C-Raf and B-Raf (V600E)	Melanoma BRAFV600E mutation
Everolimus (Afinitor)	2009	FKBP12/mTOR	Progressive neuroendocrine tumor of pancreatic origin, RCC, subependymal giant cell astrocytoma, breast cancer
Pazopanib (Votrient)	2009	VEGFR1/2/3, PDGFRα/β, FGFR1/3, Kit, Lck, Fms and Itk	RCC, soft tissue sarcoma
Lapatinib (Tykerb)	2007	EGFR and ErbB2	Breast cancer
Nilotinib (Tasigna)	2007	BCR-Abl, PDGFR	CML
Temsirolimus (Torisel)	2007	FKBP12/mTOR	RCC
Dasatinib (Sprycel)	2006	BRC-Abl, Src, Lck, Yes, Fyn, Kit, EphA2 and PDGFRβ	CML, ALL
Sunitinib (Sutent)	2006	PDGFRα/β, VEGFR1/2/3, Kit, Flt3, CSF-1R and RET	RCC, GIST, pancreatic neuroendocrine tumors
Erlotinib (Tarceva)	2004	EGFR	NSCLC and Pancreatic cancer
Sorafenib (Nexavar)	2005	C-Raf, B-Raf, B-Raf (V600E), Kit, Flt3, RET, VEGFR1/2/3 and PDGFRα/β	Hepatocellular carcinoma, RCC, DTC
Gefitinib (Iressa)	2003	EGFR	NSCLC
Imatinib (Gleevec)	2001	BCR-Abl, Kit and PDGFR	CML, ALL aggressive systemic mastocytosis, GIST
Sirolimus (Rapamune)	1999	FKBP12/mTOR	Renal transplant

2.2 Oncogenic Drivers of Lung Tumors

Analysis of 27 cancer types revealed that the median frequency of non-synonymous mutations greatly varied across cancer types and among patients with the same type of disease [13]. Thus the frequency varies by more than 1000-fold across cancer types. The tissue type of origin explains approximately half of the variation: at the extreme side, lung cancer (and melanoma) exceeds 100 mutations per one megabase (Mb), while pediatric cancers show frequencies as low as 0.1/Mb (i.e., one change across the entire exome). Similarly, mutation frequencies vary dramatically across patients within a cancer type, such that in lung cancer the frequency ranges across four orders of magnitude, from 0.1 to 100/Mb [13]. Because of a large burden of passenger events per tumor genome, the high rates of somatic mutations and genomic rearrangements in lung cancer challenge the identification of the most frequent driver gene alterations. Genetic heterogeneity extends to the spectrum and grouping of mutations: Lung cancers, for example, share a mutational spectrum dominated by C → A mutations, consistent with exposure to the polycyclic aromatic hydrocarbons in tobacco smoke [14]. In line with the C-to-A transversion, a mean somatic mutation rate of 8–10 mutations per megabase (1 million base pairs) is found in specimens of adenocarcinomas from the lungs of individuals who smoke, but the rate is approximately tenfold lower in specimens from patients who have never smoked (0.8 to 1 mutation per megabase) [15].

The most commonly mutated oncogenes in lung adenocarcinoma are *KRAS* (33% of tumors), *EGFR* (14%), *BRAF* (10%), *PIK3CA* (7%), and *MET* (in 7%; see Fig. 2.1). Mutations involving tumor suppressors include *TP53* (in 46% of tumors), *STK11* (in 17%), *KEAP* (in 17%), *NF1* (in 11%), *RB1* (in 4%) and *CDKN2A* (in 4%). In addition, approximately 10% of specimens from lung adenocarcinomas involve mutations in chromatin-modifying genes (e.g., *SETD2* and *ARID1A*) and RNA-splicing genes (e.g., *RBM10*). Interestingly, squamous-cell carcinomas are characterized by fewer mutations in genes encoding RTKs, and a greater frequency of loss of tumor-suppressor functions (e.g., *PTEN, NOTCH1,* and *RB1*). Patients with NSCLC who never smoked show higher prevalence for EGFR mutations, while specimens from groups enriched with present or past smokers frequently show mutations in *TP53, KRAS* and *NF1* [15]. Two mutations, the L858R point mutation and the exon 19 deletion (del746-750), represent the vast majority (close to 90%) of the activating EGFR mutations in NSCLC. However, several rare mutations, such as exon 20 insertions, have been reported [16]. EGFR normally functions as a receptor for seven different growth factors. Once bound by a growth factor, EGFR undergoes transient auto-phosphorylation, by means of receptor dimerization and trans-phosphorylation, followed by rapid inactivation [17]. However, the oncogenic mutations stably activate auto-phosphorylation and activation of downstream signaling pathways. Similarly, ALK functions as the putative receptor for the neurotrophic factors called midkines. Upon ligand binding, ALK undergoes transient phosphorylation, but rearrangements of the *ALK* gene promote constitutive phosphorylation of the fusion protein. The first identified fused gene, *EML4* (echinoderm microtubule-associated protein-like 4)-*ALK* [7], is the predominant *ALK* fusion in NSCLC, but additional fusion proteins exist [7, 8].

2.3 Clonal Dynamism of Lung Cancer

Identifying the cell of origin of lung cancer is hampered by the wide cellular heterogeneity of the airway epithelium, as well as by the large variation of lung cancer phenotypes. The proximal airways are populated by basal, ciliated, neuroendocrine, goblet and other cell types, whereas alveoli comprise type I and type II pneumocytes (also called AT1 and AT2 cells). Studies in mice indicated that, during development, squamous alveolar type (AT) 1 cells and surfactant-secreting AT2 cells arise directly from a bipotent progenitor, whereas after birth new AT1 cells derive from rare, self-renewing AT2 cells [18]. This function is activated by AT1 injury, while AT2 self-renewal is likely induced by EGFR ligands and oncogenic KRAS to generate clonal adenomas. Thus, there is a switch after birth, when AT2 cells function as stem cells that contribute to alveolar renewal and cancer. In a similar way, the combined loss of p53 and RB1 in a mouse model could efficiently transform neuroendocrine cells, thereby leading to small cell lung cancer (SCLC) [19].

Using mice, Berns and colleagues found that SCLC tumors were often composed of phenotypically different cells, either a neuroendocrine or a mesenchymal profile [20]. Furthermore, ectopic mutant RAS switched the neuroendocrine into the mesenchymal phenotype. When engrafted as a mixed population, the mesenchymal cells endowed the neuroendocrine cells with metastatic capacity, illustrating potential relevance of cancer cell heterogeneity in dictating tumor properties. Yet another line of animal experimentation supported the possibility that tumor heterogeneity can enhance the metastatic potential of SCLC: Genome sequencing demonstrated polyclonal seeding of metastases from a primary tumor and a linear spread of one metastatic lesion to another [21].

Similar to animal models, genomic analyses of human lung cancers are increasingly revealing evidence of clonal dynamism within primary and secondary tumors [22]. In other words, tumors consist of multiple distinct subclones that share a common ancestor, but they differ in terms of genomic alterations occurring later in the evolution of the primary tumor or the respective metastases. For example, sequencing 25 spatially distinct regions from seven operable NSCLCs found evidence of branched evolution, with driver mutations arising before and after subclonal diversification [23]. Copy number alterations, translocations and mutations indicated pronounced intratumor heterogeneity. Wide intratumor heterogeneity emerged also from a study that applied multiregion whole-exome sequencing on 11 localized lung adenocarcinomas [24]. Interestingly, 20 out of 21 known cancer gene mutations were identified in all regions of individual tumors, indicating a common origin. Moreover, all three patients with relapses after surgery (<21 months) had significantly larger fractions of subclonal mutations in their primary tumors than patients without relapse. These data indicate that a larger subclonal mutation fraction may be associated with increased likelihood of postsurgical relapse in patients with localized lung adenocarcinomas. Clearly, additional studies and genome sequencing of more lung tumors will likely uncover mutagenic processes that drive the acquisition of new mutations and propel branched evolution.

2.4 The Armamentarium of Targeted Drugs and Classes of Patient Resistance

Most clinically approved anti-cancer drugs that intercept well-defined molecular targets are either protein kinase inhibitors, especially TKIs designed to inhibit single enzymes or several different protein kinases [25], or monoclonal antibodies (mAbs) [26]. Importantly, these two classes of drugs remarkably differ in terms of molecular size, cellular site of action, route of delivery and production costs (see Fig. 2.2). Notably, while mAbs are highly specific to an antigen, target selectivity of TKIs varies according to drug's target and concentration [27]. As we discuss below, another difference between mAbs and TKIs entails mechanisms that confer drug resistance. Secondary mutations confined to the target kinase domain often confer resistance to TKIs, such as the secondary alterations reported in patients with chronic myeloid leukemia treated with imatinib [28], and the secondary replacement of threonine 790 of EGFR by a methionine (T790M), which emerges in most NSCLC patients treated with EGFR-specific TKIs [29–31]. By contrast, resistance to mAbs frequently involves compensatory signaling mechanisms rather than secondary mutations [32]. One notable exception is an acquired EGFR ectodomain mutation (S492R) that prevents binding of anti-EGFR mAbs and confers resistance to mAbs [33].

The concept called 'oncogene addiction' helps explain how some cancers that contain multiple genetic and epigenetic abnormalities are dependent on (or 'addicted' to) just one or a few genes for maintenance of the malignant phenotype

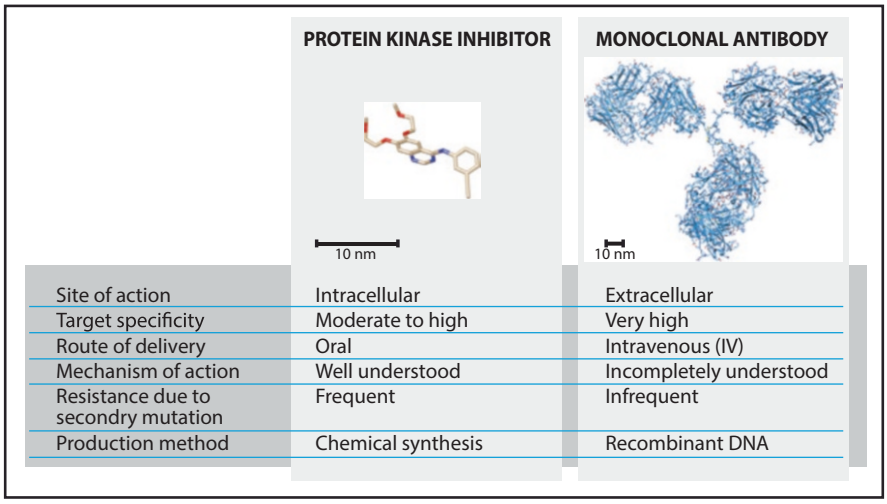

Fig. 2.2 Comparison of structural and other features of a typical protein kinase inhibitor (erlotinib) and a human immunoglobulin G antibody, such as panitumumab. Note the different scales and molecular complexities. Listed are some of the main differences between the two major classes of these anti-cancer drugs

[34]. Accordingly, reversal of only one or a few of these abnormalities can inhibit cancer cell growth, and in some cases translate to improved survival rates. Unfortunately, although several TKIs often achieve substantial prolongation of patient overall survival time, a common hurdle is the aforementioned drug resistance. Thus, emergence of resistance remains a major limitation to the successful management of advanced cancer. Two types of patient resistance limit clinical applications of TKIs and dictate treatment regimens (Fig. 2.3): Primary resistance (also called *de novo* resistance) is defined as the lack of treatment response, and it may be broadly attributed to tumor intrinsic factors or to patient/drug-specific factors. The other type, acquired resistance, which is the focus of this review, refers to disease progression after an initial response to a TKI.

As noted, primary resistance refers to a broad spectrum of mechanisms that prevent a TKI from reaching its intracellular target. This may include pharmacokinetics effects that deplete a drug, prevent uptake or metabolize the drug into less active fragments. Similarly, drug-drug interactions and patient-specific variables that inhibit drug delivery to cancer cells might underlay lack of response. For example, a study that compared current smokers and non-smokers found that smokers

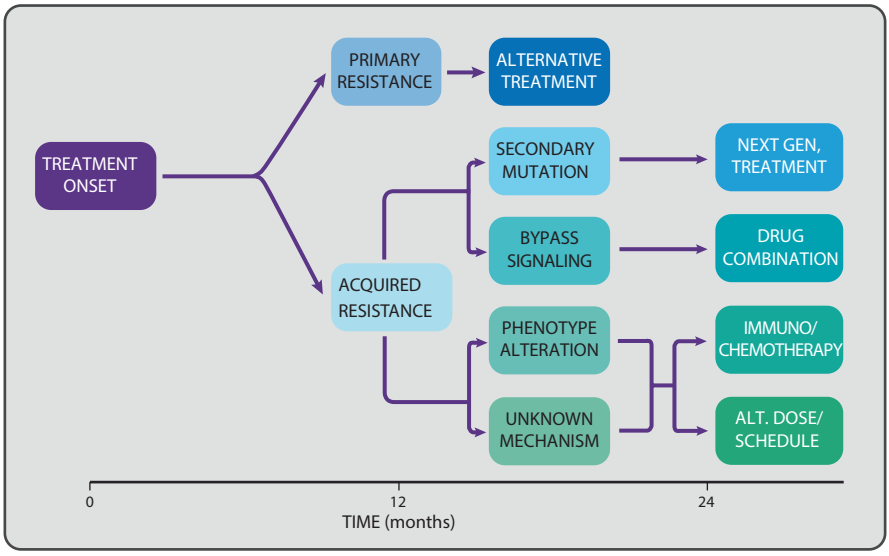

Fig. 2.3 Schematic treatment scenarios aimed at overcoming patient resistance to molecular targeted cancer drugs, including TKIs. Primary resistance, which is defined as a de novo lack of treatment response, requires treatments using alternative drugs. By contrast, acquired resistance to a drug refers to slow or rapid onset of disease progression after an initial response. Listed are four common mechanisms of acquired resistance, which might develop while a patient is still receiving the targeted therapy. Secondary mutations evolving in the molecular target, amplification of the corresponding gene or emergence of new splice variants may dictate using next generation drugs. Likewise, the emergence of bypass signaling tracks can be overcome by adding a second drug, which targets the "evasion" pathway. Alternative drug doses or schedules may overcome phenotypic transformation of cancer cells undergoing drug treatment

achieved significantly lower exposure to erlotinib following a single dose of the drug, consistent with accelerated metabolic clearance in current smokers [35].

2.5 General Mechanisms of Acquired Patient Resistance to Kinase Inhibitors

According to the acquired resistance scenario, while patients are still receiving the targeted drug their tumors develop an "escape" mechanism to evade continuous blockade [36, 37]. Because the acquired resistance profoundly limits the clinical application of TKIs, molecular machineries enabling tolerance to drugs have lately been under intense investigation. In general, tumor adaptations while under treatment may be due to alterations taking place in the cancerous tissue or to alterations occurring at the level of the drug or the host (patient). Whether or not adaptation occurs at a single cell level (de novo alterations) or at the population level (pre-existing clonal variation) is currently debated and might differ among the general mechanisms we review below. Figure 2.4 schematically presents the major mechanisms of acquired resistance to first-generation EGFR kinase inhibitors. Similar mechanisms are involved in acquired resistance to ALK-specific TKIs.

(a) Secondary mutations: Genetic alterations in the targeted oncoprotein include second-site mutations, gene amplification and splice variants. This type of escape characterizes resistance to many TKIs and it often involves a "gate keeper" mutation, so called because the size of the amino acid side chain at this position determines the relative accessibility of a hydrophobic pocket located adjacent to the ATP-binding site of the target kinase. For example, the T790M mutation of EGFR modifies a threonine residue located in the ATP-binding cleft of the kinase domain and confers drug resistance by increasing affinity to ATP [38]. Analogous mutations have been detected in patient samples at the time of resistance to imatinib and crizotinib (ABL T315I and ALK L1196M, respectively). It is conceivable that target amplification, for example overexpression of the BCR-ABL oncoprotein in AML [39], mediates patient resistance by means of drug "out-competition".

(b) Bypass signaling: Both genomic and non-genomic mechanisms recover signaling, despite TKI-mediated arrest of the oncogenic target protein kinase [40]. Because tyrosine-specific and other kinases share common downstream signaling routes, parallel compensatory pathways may be evoked and propel resistance to drugs. Yet another frequent mechanism of resistance places a constitutively active effector downstream of the blocked kinase; for example, a mutant form of the *PIK3CA* gene, which is placed downstream of EGFR in lung cancer [41]. In analogy to TKIs, a mutant form of RAS can confer resistance of CRC to an anti-EGFR antibody [42–44], and both activating *PIK3CA* mutations and *PTEN* loss may mediate resistance of breast cancer to trastuzumab, an anti-HER2 antibody [45, 46]. Additional components of the EGFR pathway, such as BRAF [41, 47] and MAPK1 [48], might also compensate for an inactive form of EGFR. Similar "bypass tracks"

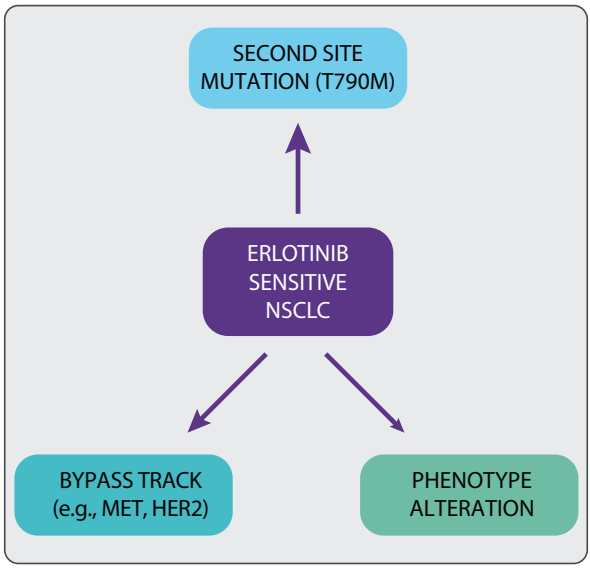

Fig. 2.4 Mechanisms underlying acquired resistance to first-generation EGFR inhibitors, such as elrotinib. Three mechanisms are presented and their relative frequencies are shown by the thickness of the connecting arrows. Target alterations represent the major mode of secondary resistance and they refer to mutations within the extinguished kinase, which weaken inhibition by the inhibitor. The most frequent one is a replacement of the gatekeeper threonine 790 of EGFR with a bulky methionine. Bypass tracks refer to pathway alterations permitting signal transfer in a mode independent from the extinguished kinase. Amplification of the gene encoding EGFR might also confer resistance. The last mechanism, namely phenotypic alterations, involves gross phenotypic alterations of TKI-treated NSCLC cells

have been extensively characterized in the context of resistance to the BRAF inhibitor called vemurafenib in BRAF-mutant melanoma. For example, increased phosphorylation of the platelet-derived growth factor receptor (PDGFR) β was found in a fraction of post-vemurafenib biopsies [49].

(c) Phenotype alterations: Changes in tumor histology have been documented at the time of acquired resistance to EGFR-specific TKIs. A survey of 37 NSCLC patients identified five cases of morphological transition to SCLC, as well as two cases of EMT (epithelila-to-mesenchymal transition) [41]. Notably, all of the patients examined originally had adenocarcinoma histology, and all retained the original EGFR activating mutation. It is notable that EMT has been associated with the acquisition of stem/progenitor cellular characteristics [50], and it was shown that leukemic stem cells are resistant to imatinib [51, 52]. In conclusion, this pathway-independent mechanism of resistance to TKIs is both rare and poorly understood.

(d) Unknown mechanisms: Because phenotype alterations are relatively rare and many patients who develop resistance to TKIs show no secondary mutations, additional mechanisms of acquired resistance might exist. These non-genetic mech-

anisms may involve locally high concentrations of growth factors derived from tumors or from immune and stromal cells [53]. For example, MET activation through gene amplification [54] and increased production of the hepatocyte growth factor (HGF) [55] have been described as mechanisms of resistance to EGFR-specific TKIs. Similarly, activation of other RTKs, including the receptor for the insulin like growth factor 1, appears relevant to acquired resistance to inhibitors of both RTKs and BRAF, and resistance to HER2-targeted therapies may be mediated by compensatory activation of EGFR or its ligands [56].

2.6 Resistance to First-Generation Inhibitors of EGFR and ALK

Both EGFR and ALK are members of the RTK family. Like other RTKs, each has an extracellular domain, a transmembrane segment and a cytoplasmic tyrosine kinase domain. Unlike EGFR, which is functional in embryogenesis and in adulthood, for example in wound healing and in renewal of epithelial and neuronal stem cells [57], ALK is highly expressed during embryogenesis and thereafter becomes dormant [58]. In contrast to EGFR, which is mutated, overexpressed or carries internal deletions in tumors, most aberrations of the *ALK* gene involve fusion with another partner gene. In lung cancer, the major fusion partners of ALK are *EML4, KIF5B, KLC1, TFG, TPR, HIP1, STRN, DCTN1, SQSTM1 and BIRC6* [59]. EML4 replaces the extracellular domain of ALK, and fuses with the juxtamembranous segment to form a constitutively active tyrosine kinase.

Treatment of *ALK*-rearranged or *EGFR*-mutated cancers with the first-generation ALK inhibitors (crizotinib/Xalkori) or EGFR blockers (erlotinib/Tarceva and gefitinib/Iressa) is followed, after 10–24 months, by the development of drug resistance by the majority of patients. The emergence of secondary mutations of the EGFR and ALK genes is the major mechanism of resistance to treatment. In the case of *ALK*, second-site mutations and gene amplification drive approximately 30% of resistant cases. Most of the aberrations are in the form of point mutations; the first to be described were C1156Y and L1196M [60], and several other secondary point mutations have since been identified: G1269A, F1174L, 1151Tins, L1152R, S1206Y, I1171T, G1202, D1203N and V1180L. Notably, unlike the prevalence of the T790M gatekeeper mutation of EGFR, ALK is characterized by lower incidence of gatekeeper mutations with acquired resistance to crizotinib. The "*ALK* non-dominant resistance", meaning that the tyrosine kinase activity of ALK is unnecessary for tumor progression, involves emergence of bypass signaling, such as aberrant activation of other kinases (e.g., MET, amplification of KIT and increased autophosphorylation of EGFR) in drug-resistant tumors from patients [61]. *EGFR* and *KRAS* mutations, along with activation of the receptor for the insulin like growth factor 1 were also reported [62]. Furthermore, evidence of multiple resistance mechanisms developing simultaneously was found in some patients.

As previously indicated, the majority of patients who evolve resistance to the first-generation EGFR inhibitors develop a secondary mutation in *EGFR*, T790M [30, 31]. Interestingly, in vitro studies suggest that both emergence of pre-existing T790M clones and *de novo* acquisition of the T790M mutation within initially T790M-negative cells may occur [63]. Other mechanisms of acquired resistance that have been confirmed in clinical specimens entail amplification of the *MET* receptor tyrosine kinase [64, 65] and increased expression of the AXL receptor tyrosine kinase, either alone or in combination with its ligand, GAS6 [66]. Analysis of tumor biopsies from 37 patients with drug-resistant *EGFR* mutation positive tumors identified additional mechanisms, such as *EGFR* amplification and mutations in the *PIK3CA* gene, a pronounced epithelial-to-mesenchymal transition, as well as transformation from NSCLC into SCLC [41]. Interestingly, clinical experience has revealed that, after a drug-free interval, resistant cancers can regain response to EGFR-specific TKIs [67], but the underlying mechanisms remain unclear.

2.7 Resistance to Second-Generation Inhibitors of EGFR and ALK

Unlike crizotinib, which was originally developed as a MET inhibitor, the second-generation ALK-specific TKIs were specifically developed as ALK inhibitors. Hence, they act at lower doses and can overcome resistance that might result from sub-therapeutic exposure to crizotinib. For these reasons, the second-generation agents are being compared to crizotinib in first-line therapy for *ALK*-positive NSCLC. Each second-generation ALK inhibitor may associate with a distinct spectrum of resistance mutations, but the frequency of one ALK mutation, G1202R, increases significantly after treatment with second-generation agents [68]. As a result of promising clinical data, the second-generation inhibitor called ceritinib received “accelerated approval” in 2014. Promising anticancer activity has also been observed with other second-generation ALK inhibitors. For example, when administered to crizotinib-naïve patients alectinib achieved very high response rates [69]. Another promising second-generation ALK inhibitor, brigatinib, is a dual inhibitor of ALK and EGFR, including *ALK* L1196M and EGFR T790M mutants.

Several new drugs targeting EGFR-T790M were developed following the discovery that T790M is the main mechanism of resistance to the first-generation *EGFR* TKIs. They include neratinib, afatinib, and dacomitinib, compounds that exhibited promising anti-T790M activity in laboratory studies. However to date, none of the second-generation agents is considered an effective monotherapy in patients progressing on first-generation TKIs. This is due to inhibition of wild type EGFR and increased toxicity (e.g., skin and gastrointestinal tract), as well as low efficacy (response rates smaller than 10%), when tested on patients resistant to the first-generation drugs. Nevertheless, Pao and colleagues noted that the irreversible

second-generation inhibitor called afatinib was able to overcome the T790M mutation when tested in preclinical models in combination with an anti-EGFR antibody, cetuximab. This led to a clinical study combining afatinib and cetuximab, which enrolled patients who acquired resistance to erlotinib/gefitinib. Although the objective response rate exceeded 25%, this was comparable in T790M-positive and in T790M-negative tumors [70]. In addition, therapy-related grade 3 adverse events occurred in 44% of patients. A randomized phase II/III trial comparing afatinib plus cetuximab and afatinib alone is currently ongoing. It is notable that anti-EGFR mAbs downregulate EGFR-T790M but they simultaneously up-regulate both HER2 and HER3, leading to hyper-activation of the ERK pathway [71]. Accordingly, a triple mAb combination targeting all three receptors prevented the activation of ERK, accelerated degradation of all three receptors and markedly reduced growth of tumors in mice xenografted with cells that were resistant to erlotinib. In conclusion, because they recognize all mutated forms of EGFR, antibodies to EGFR-family members and to other receptors (e.g., MET) may offer an alternative strategy to overcoming acquired resistance to TKIs.

2.8 Resistance to Third-Generation Inhibitors of EGFR and ALK

Lorlatinib is a potent brain-penetrant ALK inhibitor, which displayed superior potency against all known clinically acquired ALK mutations, including the highly resistant G1202R mutant [72]. When tested in mice, the inhibitor led to regression of EML4-ALK-driven brain metastases and prolonged mouse survival. Currently, lorlatinib is being studied in a phase I clinical trial in patients who were refractory to crizotinib and ceritinib. Interestingly, in a patient who had metastatic ALK-rearranged lung cancer, the tumor did not respond to a second-generation ALK inhibitor, but it did respond to lorlatinib, and a new ALK L1198F mutation appeared to confer resistance to this drug [73].

Third-generation *EGFR* inhibitors, such as rociletinib (CO-1686) [74] and osimertinib (AZD9291) [75], exhibit increased specificity for T790M, as compared to WT-EGFR and, in general, they are well tolerated [76]. To date, only osimertinib (AZD9291) received clinical approval for metastatic T790M-positive NSCLC, which has progressed on or after *EGFR* TKI therapy. Osimertinib inhibits T790M-EGFR, while sparing wild type EGFR [77]. In a phase 1 trial, osimertinib demonstrated manageable tolerability and 51% response rate among T790M mutant tumors [78]. Nevertheless, patients treated with osimertinib acquire resistance, due to several mechanisms, including emergence of C797S mutations [79, 80]. Importantly, EGFR C797S arises in ~33% of patients after osimertinib treatment, but it occurs in <3% after rociletinib treatment. In addition, a novel tertiary *EGFR* mutation was observed in a single patient following rociletinib therapy [81], implying that the pattern of resistance mechanisms to rociletinib and osimertinib differ. In addition to the C797S muta-

tion, *EGFR*-independent mechanisms of resistance to osimertinib have been reported. *NRAS* mutations, including a novel E63K mutation, and amplifications of wild type *NRAS* or *KRAS* have been described as mechanisms of acquired resistance to osimertinib [80]. Another mechanism entails activation of MET and HER2 [82]. In contrast to osimertinib, analysis of circulating tumor DNA proposed that increased MET copy number might be a frequent rociletinib resistance mechanism [81]. In line with this, rociletinib-resistant tumors that develop MET amplification acquire sensitivity to treatment with the MET and ALK inhibitor crizotinib. Another major mechanism of resistance to rociletinib may involve reversal to T790 wild type EGFR [83]. Less prevalent mechanisms involve a transformation to small cell lung cancer or EGFR amplification [83]. In addition, overgrowth of cells harboring *HER2* amplification or *PIK3CA* mutations may also take place [76].

2.9 Perspectives and Future Therapies

Over the last decade, we have learned how immense is the challenge of lung cancer eradication. On the one hand, genomic studies have demonstrated the heterogeneous landscape in advanced lung cancer: multiple somatic mutations and copy number aberrations, spatial heterogeneity, and mutational patterns that can vary in response to cancer therapies have been newly discovered. On the other hand, we have learned that individual tumor cells are endowed with enormous biochemical plasticity, enabling switching among multiple signaling pathways to complement clonal diversity. Evidently, the combination of genetic diversity and functional plasticity underlies the remarkable ability of lung cancers to evolve resistance to specific kinase inhibitors. Resolving mechanisms of acquired resistance to targeted therapies already instructs treatment decisions, such as the design of effective drug combinations, changing drug dose and sequence, or applying immune checkpoint inhibitors. For example, our own animal study proposed that changing drug schedule from day to night treatment can enhance response to EGFR- and HER2-specific TKIs [84]. Because almost identical evasion mechanisms recur in the majority of patients treated with a particular drug, it is conceivable that the number of escape pathways available to lung tumors is finite. Hence, exhaustive mapping of clonal heterogeneity and functional plasticity of lung cancer hold great promise for the future of lung cancer treatments.

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 3
Mechanisms of Action and Resistance of Trastuzumab in Breast Cancer

Jennifer L. Hsu and Mien-Chie Hung

Abstract Breast cancer affects approximately 1 in 8 women. It is estimated that over 252,710 women in the United States will be diagnosed with breast cancer in 2017. Breast cancer-related deaths have declined over the last two decades as a result of early detection and improved treatment, particularly targeted therapies, such as trastuzumab that targets human epidermal growth factor receptor 2 (HER2), which is frequently overexpressed in breast cancer. However, resistance to trastuzumab, either *de novo* or acquired resistance, presents a major clinical challenge. Here, we summarize the mechanisms of action and resistance of trastuzumab in breast cancer and discuss potential strategies to overcome resistance.

Keywords Receptor tyrosine kinase • Drug resistance • Therapeutic antibodies • Small tyrosine kinase inhibitors • Breast cancer

Abbreviations

ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ADC	Antibody-drug conjugate
ADCC	Antibody-mediated cellular toxicity
AMPK	Adenosine monophosphate (AMP)-activated protein kinase
CTF	Carboxy terminal fragment
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
ECD	Extracellular domain

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_3

EGFR	Epidermal growth factor receptor
EphA2	Ephrin receptor A2
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
IGF-1	Insulin growth factor-1
IGF-1R	Insulin-like growth factor receptor 1
IGF-2	Insulin growth factor-2
IHC	Immunohistochemistry
JAK	Janus-activated kinase
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MUC4	Glycoprotein mucin-4
NK	Natural killer
NSCLC	Non-small cell lung cancer
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidyl-inositol-3,4,5-trisphosphate
PLCγ	Phospholipase Cγ
PTEN	Phosphatase and tensin homologue
STAT	Signal transducer and activator of transcription
T-DM1	Ado-trastuzumab emtansine
TKI	Tyrosine kinase inhibitor
TNFα	Tumor necrosis factor α

3.1 Introduction

The epidermal growth factor receptor (EGFR) family consists of four members, ERBB1 (EGFR/HER1), ERBB2 (HER2/neu), ERBB3 (HER3), and ERBB4 (HER4), which are cytoplasmic membrane-anchored receptor tyrosine kinases that regulate important biological processes, such as cell growth, differentiation, metabolism, and survival through activation of downstream signaling pathways [1–6]. All members of the EGFR family share sequence and structural similarities and contain an extracellular ligand-binding ectodomain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain [1, 7]. Following ligand binding, members of the ERBB family interact to form various combinations of homo- or heterodimers, which then induce autophosphorylation of the tyrosine residues within the kinase domain [1]. Recruitment of adaptor proteins at the phosphotyrosine residues subsequently initiates the downstream signaling cascades, such as phosphatidylinositol 3-kinase (PI3K), Ras, phospholipase Cγ (PLCγ), Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) [3, 8].

In contrast to EGFR, ERBB3, and ERBB4, which bind extracellular ligands to trigger downstream signaling, HER2 does not bind to any ligands directly. Rather, HER2 mediates downstream signaling in concert with a ligand-activated coreceptor, e.g., EGFR, ERBB3, or ERBB4 [1, 2, 9]. HER2 can also form homodimers and activate signaling cascades especially at higher concentrations as observed in cancers [2, 9]. The HER2/ERBB3 heterodimer is the most potent activator of two key pathways regulating cell survival and growth, the mitogen-activated protein kinase (MAPK) and the PI3K/Akt signaling cascades, and ERBB3 plays an essential role in HER2-mediated oncogenic signaling [10–12]. Activation of HER2 also decreases the protein levels of cell cycle negative regulator $p27^{Kip1}$ by promoting its mislocation through Jun activation domain-binding protein 1-mediated export into the cytoplasm, and subsequently its degradation via the ubiquitin-proteasome pathway [13].

HER2 is normally expressed at low levels on the cell surface, but in breast cancer, the number of HER2 receptors on the surface of each cell can reach up to 100 times more than a normal cell, which leads to aberrant activation of its downstream signaling cascades and uncontrollable cell growth [14, 15]. HER2 amplification/overexpression is observed in approximately 20% of breast cancer and is associated with poor clinical outcome and disease progression [16–18], and HER2 has proved to be one of the most successful targets in breast cancer. In this chapter, we briefly summarize the mechanisms of action and resistance of trastuzumab (Herceptin®; Genentech) as well as treatment strategies to overcome resistance in breast cancer.

3.2 Trastuzumab

3.2.1 Proposed Mechanism of Action

Several HER2-specific mAbs were developed and demonstrated to effectively inhibit tumor growth of HER2-overexpressing cell lines [19]. Among them, the murine mAb 4D5, which was later humanized to become trastuzumab, selectively targets the extracellular domain IV of HER2 with high affinity and prevents ligand-induced dimerization and subsequent activation of downstream pathways [20]. Following the clinical studies which demonstrated that the addition of trastuzumab to chemotherapy, compared with chemotherapy alone, increased the response rates, time to progression, and survival in patients with HER2-positive ($HER2^+$) metastatic breast cancer, trastuzumab received approval by the FDA in 1998 for the treatment of metastatic breast cancer with HER2 overexpression [21, 22].

Trastuzumab exerts its mechanism of action through several different approaches. First, it disrupts signal transduction pathways, most notably, MAPK and PI3K/Akt signaling, leading to apoptosis and arrest of proliferation [1, 23–25]. Trastuzumab produces cytostatic effects associated with downregulation of

AKT activity and results in increased G1 growth arrest via enhanced stability of the cell cycle inhibitor $p27^{Kip1}$ [23, 26, 27]. In addition, trastuzumab can block PI3K signaling by reducing tyrosine phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) and increasing its phosphatase activity and membrane localization [28]. Second, trastuzumab can block proteolytic cleavage of HER2 by the metalloprotease ADAM10 [29], which liberates its extracellular domain (ECD) and produces a truncated, membrane-bound, and kinase active carboxy terminal fragment (CTF), p95HER2 [30]. Interestingly, HER2 ECD can be detected in the serum of breast cancer patients, and results from clinical studies indicated that a decline in serum HER2 ECD following trastuzumab treatment could predict clinical benefit [31]. Third, trastuzumab exerts an antitumor effect through activation of the antibody-mediated cellular toxicity (ADCC) [32, 33]. Studies have demonstrated in cell lines and xenografts that this immunological effect of trastuzumab is mainly attributed to the binding of the Fc (fragment, crystallizable) region of the antibody to Fc gamma receptor present on natural killer cells [32, 34]. Immunohistochemistry (IHC) analysis of breast tissue samples from patients with HER2-overexpressing advanced breast cancer during a neoadjuvant treatment of trastuzumab and docetaxel in a clinical trial further validated the immune cell-modulated activity of trastuzumab via an increased number of natural killer (NK) cells and cytotoxic proteins, e.g., granzyme B, in tumor infiltrates after trastuzumab treatment [33].

3.2.2 Mechanism of Resistance

Although trastuzumab in combination with chemotherapy has significantly improved the outcome of breast cancer patients, *de novo* and acquired resistance to trastuzumab pose a major challenge in the clinic [35, 36]. A large proportion of patients with $HER2^+$ breast cancer do not respond to initial trastuzumab treatment (*de novo* resistance) and those who initially responded eventually experience disease progression (acquired resistance) [37–39]. The mechanisms of trastuzumab have been extensively studied and may involve the following: (1) upregulation of downstream signaling due to genetic alterations; (2) hindrance of trastuzumab binding to HER2; and (3) overexpression of ERBB receptors or other tyrosine kinase receptors. Each will be briefly described below.

3.2.3 Upregulation of Downstream Signaling

The constitutive activation of the downstream PI3K/Akt pathway due to mutations in the gene encoding PI3K and/or inactivation or loss of PTEN have been shown to contribute to trastuzumab resistance [28, 40]. PI3K catalyzes the lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to the phosphatidyl-inositol-3,4,

5-trisphosphate (PIP_3), which binds to the pleckstrin homology domain of the serine/threonine protein kinase Akt, resulting in the translocation of Akt to the membrane and its subsequent activation to promote cell survival and inhibition of apoptosis [41]. Activating mutations in the *PIK3CA* gene encoding the catalytic subunit (p110) of PI3K have been reported to induce constitutive activation of the PI3K/Akt pathway. The frequency of *PIK3CA* activating mutations in HER2+ breast cancers has been reported to be 23–33% [42]. PTEN antagonizes PI3K by dephosphorylating PIP_3 and negatively regulates AKT activities [43–46]. Hence, as PTEN normally blocks PI3K activation, the loss of *PTEN* results in constitutive activation of PI3K/Akt signaling and subsequently bypassing trastuzumab-mediated growth arrest [46, 47]. Breast cancer patients with *PTEN* deficiency demonstrated poorer response to trastuzumab compared with those with normal *PTEN* [28]. Zhang et al. reported that cytoplasmic tyrosine kinase SRC functions as a common mediator of multiple trastuzumab resistance pathways and is regulated via dephosphorylation by PTEN [48]. The increased activation of SRC was observed in both *de novo* and acquired trastuzumab resistant cells and correlated with trastuzumab resistance in patients [48]. A follow-up clinical trial indicated that patients with HER2-overexpressing metastatic breast cancer with *PTEN* loss and progressed on trastuzumab-based therapy had decreased overall survival compared with those with normal *PTEN* [49]. Moreover, studies reported that the combination of trastuzumab with everolimus, an inhibitor against AKT downstream molecule mTOR, provided an objective response rate of 15% and clinical benefit rate of 34% [49]. These findings further validated the role of *PTEN* deficiency in trastuzumab resistance. In addition, preclinical studies demonstrated that the combination of trastuzumab and the PIK3 inhibitor, GDC-0941, is highly effective against trastuzumab-resistant cells and tumors and can also overcome trastuzumab resistance caused by *PTEN* loss [24]. The PI3K inhibitors that are currently under clinical investigation for solid tumors harboring *PIK3CA* or *PTEN* mutations include buparlisib (BKM120), taselisib (GDC-0032), and GSK2636771 [50].

Akt has also been demonstrated to phosphorylate the tumor suppressor SIRT6 at Ser338, resulting in MDM2-mediated ubiquitination and subsequent degradation of SIRT6 [51]. The authors further reported a positive correlation between SIRT6 abundance and survival of breast cancer patients. Their findings suggested that stabilization of SIRT6 by preventing its degradation may be a potential therapeutic strategy to overcome trastuzumab resistance.

3.2.4 Epitope Masking

As indicated above, the ectodomain shedding of HER2 produces a truncated and constitutively active membrane-bound p95HER2 CTF of 95- to 100-kDa. In addition to the ectodomain shedding, the alternative translation initiation of *HER2* mRNA can give rise to two other p95HER2 fragments, a membrane-bound 611-CTF (100–115 kDa), which forms constitutively active homodimers, and a soluble

678-CTF (90–95 kDa), which is kinase inactive [52]. Both p95HER2 95–100 kDa and 110–115 kDa fragments lack the epitope for recognition by trastuzumab [53], and circulating HER2 ECD can compete with the full-length membrane-bound HER2 for binding to trastuzumab [54]. Pederson and coworkers found that the 611-CTF regulated genes linked to metastasis, and 611-CTF transgenic mice developed more aggressive and invasive mammary tumors compared with mice with full-length HER2 [52]. Up to 30% of HER2+ breast cancers express p95HER2 and are associated with metastasis and shorter disease-free survival [55, 56]. Retrospective studies indicated that the presence of p95HER2 fragments in tumors is associated with trastuzumab resistance [57, 58]. Interestingly, p95HER2 was shown to preferentially heterodimerize with HER3 to trigger pro-survival signaling [59]. Parra-Palau and coworkers reported that chemotherapy sensitizes p95HER2 (611CTF)-expressing patient derived xenograft from HER2+ breast cancers to trastuzumab [60].

Another mechanism contributing to trastuzumab resistance is the binding of cell surface glycoprotein mucin-4 (MUC4) to the extracellular domain of HER2, which can mask the trastuzumab-binding site on HER2 (epitope masking). Nagy et al. reported that MUC4 expression was correlated negatively with decreased trastuzumab binding, and that knocking down *MUC4* reversed trastuzumab resistance in a *de novo* trastuzumab-resistant JIMT-1 breast cancer cell line [61]. Hyperactivation of the signal transducer and activator of transcription-3 (STAT3) via a positive feedback loop was shown to upregulate MUC4 expression [62]. More recently, Mercogliano et al. reported that TNFα induces elevated expression levels of MUC4 and contributes to trastuzumab resistance in HER2+ breast cancer. The authors further identified MUC4 expression as an independent predictor of poor disease-free survival in HER2+ breast cancer patients and suggested the combination of TNFα-blocking antibodies as a therapeutic option to overcome trastuzumab resistance [63].

3.2.5 Expression of Other Receptor Tyrosine Kinases

HER2 can form heterodimers with other receptor tyrosine kinases to activate downstream signaling cascades to compensate for the inhibition of HER2 signaling by trastuzumab [10, 11, 64–67]. Ritter et al. demonstrated that trastuzumab-resistant cells exhibited higher levels of EGFR phosphorylation and EGFR/HER2 heterodimers, and the addition of EGFR TKIs, erlotinib, gefitinib, or lapatinib (a dual EGFR/HER2 inhibitor), induced apoptosis in those resistant cells [64]. The HER2-HER3 heterodimer potently activates the PI3K/Akt and MAPK pathways, and trastuzumab is unable to block the ligand-induced HER2/HER3 heterodimer [68]. The HER-2 targeting monoclonal antibody, pertuzumab (see later for more details), was developed to prevent HER2 dimerization with EGFR and HER3 [69].

The receptor tyrosine kinase Eph receptor A2 (EphA2) is overexpressed in many cancer cell lines and human tumor tissue specimens and can form a complex with

HER2 and activate signaling promoting cell proliferation and motility [70–72]. Eliminating EphA2 expression in ERBB2-driven murine mammary tumor models impaired tumor initiation and metastatic progression [72]. In addition, Zhuang and colleagues found that high levels of EphA2 expression in HER2+ breast cancer patients predict poor prognosis and identified a mechanism by which EphA2 contributes to trastuzumab via EphA2-mediated amplification of the PI3K/Akt and MAPK cascades [65]. Their findings suggested targeting EphA2 as a therapeutic strategy to overcome trastuzumab resistance. Amato et al. demonstrated that the EphA2 kinase inhibitor, ALW-II-41-27, inhibited cell viability of non-small cell lung cancer (NSCLC) cells *in vitro* and induced tumor regression in a NSCLC xenograft tumor model [73]. Targeting EphA2 was shown to overcome primary and acquired resistance to anti-EGFR therapy, cetuximab, in metastatic colorectal cancer [74]. Whether the addition of ALW-II-41-27 could overcome trastuzumab resistance in breast cancer remains unclear.

The overexpression of the insulin-like growth factor receptor 1 (IGF-1R) and its ligands, insulin growth factor-1 (IGF-1) and IGF-2, is often observed in breast tumors [75]. Activation of IGF-1R following ligand binding triggers cell survival signals, and overexpression of IGF-1R has been shown to confer resistance to trastuzumab via hyperactivation of SRC [48, 76]. Specifically, ectopic expression of IGF-1R in trastuzumab-sensitive breast cancer cells in the presence of IGF-1 ligand rendered trastuzumab ineffective in reducing cell proliferation, and the addition of IGF-binding protein-3, which suppresses IGF-1R signaling, reversed resistance [76]. The inhibition of SRC renders trastuzumab-resistant IGF-1R breast cancer cells sensitive to trastuzumab [48]. IGF-1R is also reported to form a heterodimeric complex with HER2 and HER3 in breast cancer cells resistant to trastuzumab through enhanced PI3K/Akt signaling and SRC activation [77]. Liu et al. reported that metformin, a type 2 diabetes drug with antitumor effects, reduces HER2 and IGF-1R interactions in trastuzumab-resistant breast cancer cells [78]. Metformin has been shown to activate the adenosine monophosphate (AMP)-activated protein kinase AMPK, which plays a critical role as a regulator of cellular energy homeostasis [79]. Interestingly, metformin inhibits the insulin/IGF signaling by decreasing insulin metabolism in the liver or by reducing IGFR expression. Whether AMPK regulates HER/IGF-1R interaction remains unclear.

The hepatocyte growth factor (HGF) receptor (also known as c-Met), which regulates important biological processes, including morphogenesis, cell proliferation, survival, differentiation, and anti-apoptosis, is also implicated in the progression and metastasis of many human cancers [80]. Overexpression of c-Met is observed in 20–30% of breast cancers and it has been reported to be an independent prognostic of poor prognosis for breast cancer patients [81–83]. Shattuck et al. reported the co-expression of c-Met and HER2 in HER2-overexpressing breast cancer cells and HER+ breast cancer tumor tissues [67]. Moreover, the inhibition of c-Met sensitized HER2-overexpressing breast cancer cells to trastuzumab, suggesting that c-Met contributes to trastuzumab resistance [67]. High risk of trastuzumab treatment failure in breast cancer patients has been reported to associate with high *MET* and *HGF* gene copy numbers [84].

3.3 Treatment Strategies to Overcome Resistance

Below we describe some strategies to overcome trastuzumab resistance.

3.3.1 Pertuzumab

The humanized monoclonal antibody pertuzumab (Perjeta®; Genentech) binds to domain II (trastuzumab binds to domain IV) of HER2 and blocks ligand-dependent HER2 heterodimerization with EGFR, HER3, or HER4, but most potently targets the heregulin-mediated HER2/HER3 signaling heterodimer [69, 85]. Inhibiting the formation of the HER2/HER3 heterodimer prevents the activation of downstream signaling, e.g., PI3K and MAPK, that regulate cell survival and growth [1, 11]. Similar to trastuzumab, pertuzumab also triggers ADCC [86]. Clinical studies of patients with advanced HER2+ breast cancer after trastuzumab treatment demonstrated that pertuzumab in combination with trastuzumab was more efficacious than pertuzumab alone [87]. In 2012, the FDA approved pertuzumab in combination with trastuzumab and docetaxel for the treatment of HER2+ metastatic breast cancer patients who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease based on a phase III multicenter randomized clinical trial [88]. More recently, follow-up data extended the results of previous analyses demonstrating the efficacy of the pertuzumab plus trastuzumab and docetaxel combination [89]). Pertuzumab was later approved in 2013 for use in combination with trastuzumab and docetaxel as neoadjuvant treatment of patients with HER2+, locally advanced, inflammatory, or early stage breast cancer [90]. Interestingly, tumor gene expression analyses indicated high expression of the programmed death ligand 1 (PD-L1), an immune checkpoint protein that facilitates cancer immunesurveillance escape, is associated with resistance after neoadjuvant treatment with regimens containing HER2-targeted treatments [91]. A phase I trial is currently underway to evaluate the safety and pharmacokinetics of the PD-L1 monoclonal antibody, atezolizumab, in combination with trastuzumab and pertuzumab in HER2+ breast cancer (NCT02605915).

3.3.2 Lapatinib

Lapatinib (Tykerb®; Novartis) is a reversible ATP-competitive small molecule tyrosine kinase inhibitor (TKI) which binds to the intracellular ATP binding domain of EGFR and HER2 and inhibits the activation of downstream signaling [92–94]. The combination of lapatinib and capecitabine in a randomized phase III trial was found to be superior than capecitabine alone in patients with metastatic breast cancer who progressed after treatment with regimens that included an anthracycline, a taxane,

and trastuzumab [95, 96]. On the basis of the phase III data, the FDA approved lapatinib in combination with capecitabine for the treatment of patients with advanced or metastatic HER2+ breast cancer and who have received prior therapy including an anthracycline, a taxane, and trastuzumab. Synergistic growth inhibition was observed in trastuzumab-treated HER2+ breast cancer cell lines for the lapatinib and trastuzumab combination [93]. Lapatinib combined with trastuzumab was later evaluated in a phase III clinical trial with results showing a significant overall survival advantage of the combination compared with lapatinib alone in HER2+ metastaic breast cancer patients whose disease progressed during trastuzumab treatment [97]. These findings further supported the dual blockage of HER2 as an approach to overcome resistance.

3.3.3 Neratinib

Neratinib (Puma Biotechnology) is an irreversible ATP-competitive small molecule TKI that blocks the intracellular ATP-binding site of EGFR, HER2, and HER4 [92–94]. The results from preclinical studies demonstrated that neratinib inhibits proliferation of HER2-overexpressing human breast cancer cell lines *in vitro* as well as an EGFR-overexpressing epidermal carcinoma cell line [92]. Phase II studies showed that neratinib was well tolerated among advanced HER2+ breast cancer patients with or without prior treatment with trastuzumab [98]. In a phase III (ExteNET) study, neratinib treatment significantly improved the 2-year disease-free survival of HER2-positive breast cancer patients after chemotherapy and trastuzumab-based adjuvant therapy [99]. Currently, neratinib is being evaluated in a number of clinical trials as a neoadjuvant therapy for patients with HER2+ breast cancer and as a treatment for patients with metastatic HER2+ breast cancer (clinicaltrials.gov). A new drug application for neratinib for extended adjuvant treatment of HER2+ early stage breast cancer has been accepted by the FDA and awaiting approval.

3.3.4 Ado-Trastuzumab Emtansine (T-DM1)

T-DM1 is an antibody-drug conjugate (ADC) containing trastuzumab covalently linked to the cytotoxic microtubule inhibitor, emtansine (DM1), via a thioester linker MCC (4-[N-maleimidomethyl] cyclohexane-1-carboxylate). T-DM1 contains about 3.5 molecules of DM1 per antibody and is internalized following binding of trastuzumab to HER2 on the cell surface [100, 101]. After binding of T-DM1 to HER2 on the cell surface, the HER2-T-DM1 complex is internalized via receptor-mediated endocytosis followed by lysosomal degradation, resulting in the release of intracellular DM-1-containing catabolites that bind to and inhibit microtubule polymerization, and subsequently induce cell cycle arrest and cell death [101]. In retaining the activity of trastuzumab, T-DM1 also disrupts the PI3K/Akt signaling

cascade, inhibits the HER2 ectodomain shedding, and induces ADCC [100, 101]. Preclinical studies of T-DM1 indicated greater activity compared with trastuzumab with retained selectivity toward HER2 [100]. Favorable results from clinical studies led to the approval of T-DM1 in second-line therapy by the FDA in 2013 for patients whose advanced HER2+ breast cancer progressed after trastuzumab treatment [102–104]. A phase III study (MARIANNE) evaluating T-DM1 for first-line treatment of HER2-positive, advanced breast cancer indicated that T-DM1 and T-DM1 plus pertuzumab did not achieve superiority compared with trastuzumab plus a taxane [105]. The acquired resistance to T-DM1 has been reported, and factors contributing to T-DM1 resistance include poor internalization and defective intracellular trafficking of the T-DM1-HER2 complexes, inefficient lysosomal degradation of T-DM1, expression of drug efflux proteins, and altered tubulins in addition to those mechanisms known to induce trastuzumab resistance [106]. To circumvent resistance, clinical studies to evaluate T-DM1 in combination with other targeted therapies, for example, immunotherapy (pembrolizumab or atezolizumab), HER/HER3 antibody (pertuzumab), TKIs (lapatinib or neratinib), and cyclin D kinase 4/6 inhibitor (palbociclib or ribociclib) as well as in triple combination (chemotherapy and TKI), for metastatic breast cancer are currently underway. Studies on T-DM1 combined with PI3K inhibitors (taselisib) are also ongoing (clinicaltrials.gov).

3.4 Conclusion

Trastuzumab has demonstrated remarkable clinical success and increased patient outcome. However, acquired and *de novo* resistance via multiple mechanisms remain a clinical challenge. Furthering our understanding of the resistance mechanisms has led to the development of therapeutic strategies to overcome this resistance and improve patient outcome. *HER2* somatic mutations have been reported in breast cancer, but these mutations occur almost always in the absence of *HER2* gene amplification [107, 108]. HER2 mutations were functionally characterized in breast cancer without *HER2* amplification [109]. While many of the identified mutations were found to be sensitive to HER2-targeted therapies in cell lines, those harboring the L755_T759 deletion mutation were resistant to lapatinib [109]. How this mutation affects patients treated with the combination therapy containing lapatinib should be further evaluated. Antibodies against immune checkpoints, e.g., PD-L1, PD-1, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), to unleash T cell-mediated anti-tumor activity have demonstrated success as a cancer treatment in recent years [110]. Preclinical studies indicated that PD-1 antibodies significantly improved the therapeutic activity of trastuzumab [111]. A phase Ib/II clinical trial is currently underway to evaluate the efficacy of PD-1 antibody (MK-3475) and trastuzumab in patients with trastuzumab-resistant, HER2+ metastatic breast cancers (NCT02129556). As more combination therapies are being evaluated, optimizing patient selection and predictive biomarkers are required to maximize clinical efficacy.

Acknowledgments The work was supported by the following grants: National Institutes of Health (CCSG CA016672); Cancer Prevention & Research Institutes of Texas (RP160710 and RP150245); National Breast Cancer Foundation, Inc.; Breast Cancer Research Foundation; Patel Memorial Breast Cancer Endowment Fund; The University of Texas MD Anderson-China Medical University and Hospital Sister Institution Fund; Ministry of Science and Technology, International Research-intensive Centers of Excellence in Taiwan (I-RiCE; MOST 106-2911-I-002-302); Ministry of Health and Welfare, China Medical University Hospital Cancer Research Center of Excellence (MOHW106-TDU-B-212-144003); and Center for Biological Pathways.

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Chapter 4
Mechanisms of Resistance to Molecular Therapies Targeting the HGF/MET Axis

Simona Corso and Silvia Giordano

Abstract Targeted therapies by means of compounds that inhibit a specific target molecule represent a new perspective in the treatment of cancer. In contrast to conventional chemotherapy which acts mainly on dividing cells, targeted drugs allow to hit, in a more specific manner, subpopulations of cells directly involved in tumor progression. The frequent alteration of receptor tyrosine kinases (RTKs) in human malignancies led them to be considered as targets for anti-neoplastic therapies; this resulted in the development of several inhibitors that showed a strong clinical activity. The concept of "oncogene addiction" has added a further rationale to the use of targeted therapies. In general, targeted therapies induce tumor regression in a good percentage of patients who are selected to express the target of the drug. However, almost invariably, responsive patients develop resistance to the treatment and undergo tumor relapse. A challenge associated with targeted therapies is, therefore, to predict mechanisms that could cause resistance to the treatment and to find ways to circumvent these hurdles.

The tyrosine kinase receptor for the Hepatocyte Growth Factor (HGF), encoded by the *MET* gene, has recently become a very interesting and studied target. This review will summarize the role of this oncogene in human tumor development, the strategies employed to achieve its inhibition and the mechanisms of resistance to MET-targeted therapies.

Keywords MET-targeted therapies • HGF/MET axis • Resistance

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_4

Abbreviations

EGFR	Epidermal growth factor receptor
FDA	Food and Drug Administration
FGFR2	Fibroblast growth factor receptor 2
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
mAb	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MNNG	Methylnitronitrosoguanidine
NSCLC	Non-small-cell lung carcinoma
PI3K	Phosphatidylinositide 3 kinase
RTK	Receptor tyrosine kinase
SF-RON	Short-form RON
TKI	Tyrosine kinase inhibitor

4.1 Introduction

The recent introduction of several selective tyrosine kinase inhibitors in cancer therapy has had a dramatic effect. However, after the excitement following the initial results, the clinicians had to face the problem of primary and secondary resistance to treatment. In fact, it has been noticed that a percentage of the patients expressing the target in their tumors do not respond to the treatment (primary or "de novo" resistance), while in most of the responders the treatment quite rapidly loses effectiveness (secondary or "acquired" resistance). Nowadays, the problem of acquired drug resistance has become more and more important and still represents a crucial limitation of treatment.

The use of *in vitro* and preclinical models, as well as the evaluation of clinical samples, allowed the identification of a number of molecular mechanisms responsible for *de novo* and acquired resistance to tyrosine kinase inhibitors (TKIs) [1]. The knowledge of these mechanisms of resistance is absolutely critical to select patients that can benefit from the treatment and to envisage new therapeutic strategies to bypass resistance.

4.2 The MET/HGF Axis

The *MET* proto-oncogene encodes the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF) [2, 3]. Ligand binding induces MET activation, which drives a complex biological program defined as "invasive growth", stemming from the stimulation of several biological activities such as cell proliferation, cell invasion, and protection from apoptosis. MET-induced invasive growth is a physiological

program occurring during the embryonic development and in adulthood, during tissue regeneration. However, its inappropriate activation impacts on several aspects of tumor progression. Several works have studied the MET-induced signaling pathways and the contribution of each of them to the different MET-induced biological activities (Fig. 4.1a; for a review see [4]).

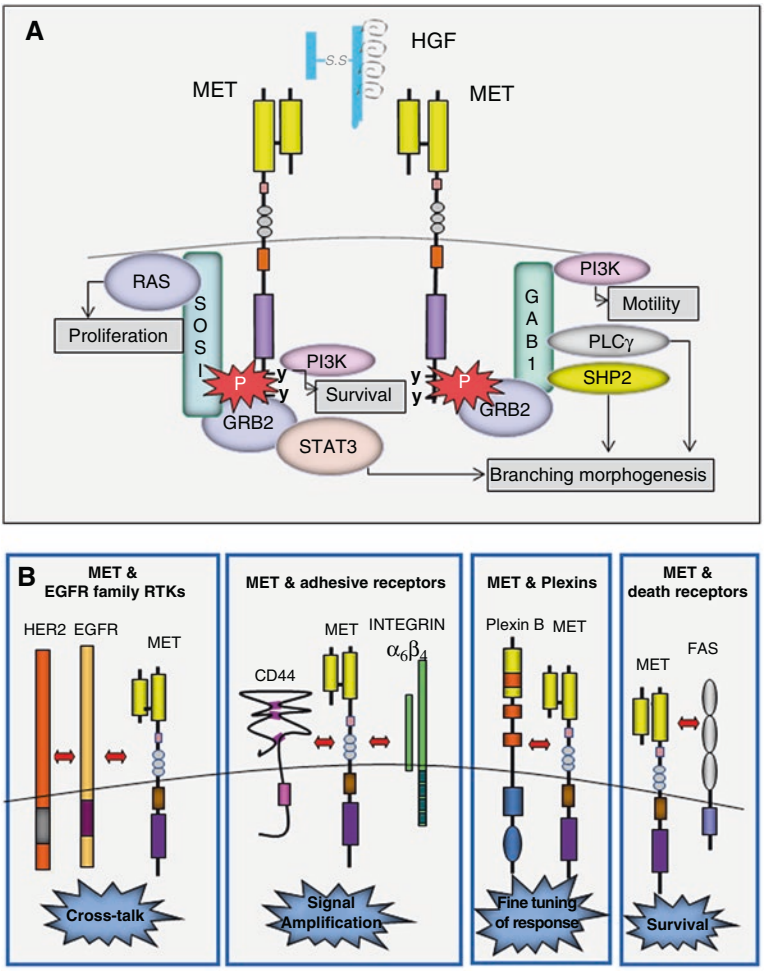

Fig. 4.1 Schematic representation of MET-driven signaling pathways. (**a**) After ligand-induced dimerization, the tyrosine kinase domain (*purple*) phosphorylates two key tyrosines (*red*) in the receptor cytoplasmic tail. These generate a docking site for several transducers (such as PI3K, PLCg, SHP2, STAT3) and signal amplifiers (such as GAB1 and GRB2). The involvement of the different transducers in the promoted biological activities is indicated (*grey boxes*). (**b**) Interactions between *MET* and other receptors. An illustration of MET cross-talk with different classes of membrane receptors: EGFR family RTKs, adhesive receptors, Plexins and death receptors. *Red arrows* indicate demonstrated physical interaction between the receptors. The roles taken on by these interactions are reported below the correspondent panel

As for other receptor tyrosine kinases (RTKs), MET can interact with other membrane receptors and these cross-talks lead to the activation of complex interacting networks. *In vitro* data suggest that these cross-talks are not mandatory for cell survival but they allow a better integration of the signals available in the extracellular environment. Even though in the physiological state these networks are likely redundant, in pathological conditions they cooperate in promoting tumorigenesis and/or metastasis and in inducing resistance to targeted drugs. The main receptors interacting with MET are (Fig. 4.1b): (1) tyrosine kinase receptors belonging to the Epidermal Growth Factor Receptor (EGFR) family. Reciprocal trans-phosphorylation between these receptors has been demonstrated in different systems as well as their ability to substitute for each other, mainly in tumor cells [5–7]; (2) adhesive receptors such as CD44 and the α6β4 integrin, both implicated in tumor progression and metastasis [8]. They behave as amplifying platforms as CD44 sustains activation of the Mitogen-activated protein kinase (MAPK) cascade [9, 10] while the α6β4 integrin acts as a supplementary docking platform for amplification of phosphatidylinositide 3-kinase (PI-3K), MAP kinase, and SRC-dependent pathways [11]; (3) B family plexins (the semaphorin receptors), which can trans-activate MET in the absence of HGF and promote pro-invasive signals [12]; and (4) the death receptor FAS, a critical modulator of apoptosis [13].

4.3 MET/HGF and Cancer

The activation of the invasive growth program is beneficial for cancer progression and metastasis. In fact, constitutive MET activation contributes to several aspects of tumor progression since it forces neoplastic cells to disaggregate from the tumor mass, erode basement membranes, infiltrate stromal matrices, and eventually colonize new territories to form metastases (for a review see [14]).

Indeed, the HGF-MET signaling plays an important role in development and tumor progression, in particular for tumor invasiveness and metastasis. Preclinical studies demonstrate that cells ectopically overexpressing MET or HGF are tumorigenic and metastatic in nude mice, while MET inhibition decreases these properties [14]. Moreover, cancer cell lines exhibiting *MET* gene amplification, leading to protein overexpression and constitutive activation, are "addicted" to MET; this means that they are dependent on this receptor for their growth and survival and thus MET inhibition is either cytostatic or cytocidal [15–17].

Deregulated MET activation in cancer can be due to different molecular alterations: (1) the unequivocal evidence linking *MET* and human cancer came from the identification of germline activating mutations in patients suffering from Hereditary Papillary Renal Carcinomas [18, 19]. Activating mutations in sporadically occurring tumors are relatively rare and have been mainly found in lung and kidney carcinomas as well as in hepatoblastomas (reviewed in [20]). These mutations are located either in the tyrosine kinase domain or in the juxtamembrane portion or in the extracellular Sema (semaphorin) domain. While overexpression

can render MET activation independent from HGF stimulation, for most mutated receptors the ligand is still required to elicit full activation [21]. Recently, mutations affecting receptor splicing have been identified in tumors such as lung, gastric and esophageal carcinomas [22–25]. The alternatively spliced receptor lacks sites of negative regulation and, thus, it is constitutively activated. (2) MET overexpression, in the absence of gene amplification, is likely the most frequent cause of constitutive MET activation in human tumors and it is often associated with poor prognosis. Overexpression can be due to factors such as hypoxia [26], activation of upstream oncogenes [27, 28], inactivation of tumor suppressor genes [29] or loss of microRNAs [30, 31]. (3) *MET* gene amplification, driving expression and constitutive receptor activation, has been described in gastro-esophageal, colorectal, endometrial and lung carcinomas, glioblastomas and medulloblastomas (reviewed in [20]). Frequently, *MET* amplification can be found in tumor cells which acquired resistance to molecular therapies targeting other kinases [32–34]. (4) Autocrine MET activation has been described in sarcoma, glioblastoma, breast carcinoma (reviewed in [20]) and, recently, in a high percentage of acute myelogenous leukemia [35] and (5) *MET* fusion genes. Originally, MET was identified as an oncogene following rearrangement with the TPR gene, in an osteosarcoma cell line treated with the carcinogen methylnitronitrosoguanidine (MNNG) [36]. However, gene fusions seem to be quite rare except in adult and pediatric glioblastomas [37, 38]. In these tumors the rearranged *MET* gene retains the carboxy-terminal kinase domain fused with different partners that drive constitutive dimerization.

4.4 MET Targeted Drugs

In light of the functional role played by the HGF/MET axis in different human tumors, over the last decade several strategies have been designed to inhibit the activation of the MET receptor, and clinical trials aimed at inhibiting MET by means of either TKIs or monoclonal antibodies (mAbs) have been performed (Fig. 4.2). At the moment, however, no drug has been approved for treatment of MET-driven human tumors, even though several drugs are under development.

Monoclonal antibodies have been widely used in the clinic and have shown promising results. The major advantage of these molecules is their high specificity. Disadvantages are: (1) the parenteral administration; and (2) the fact that they do not always efficiently target all the cells within the tumor, either because the increased interstitial pressure within the tumor impairs the proper distribution of the mAb, or because not all the cells express the antigen at the same levels (heterogeneous antigen distribution) or, finally, because some escape variants of cancer cells change the type of receptor expressed, or shed the extracellular portion of the targeted molecule as soluble forms, thus reducing the number of mAbs bound to the tumor cells. The most advanced mAbs targeting the HGF/MET axis are directed either against HGF or MET.

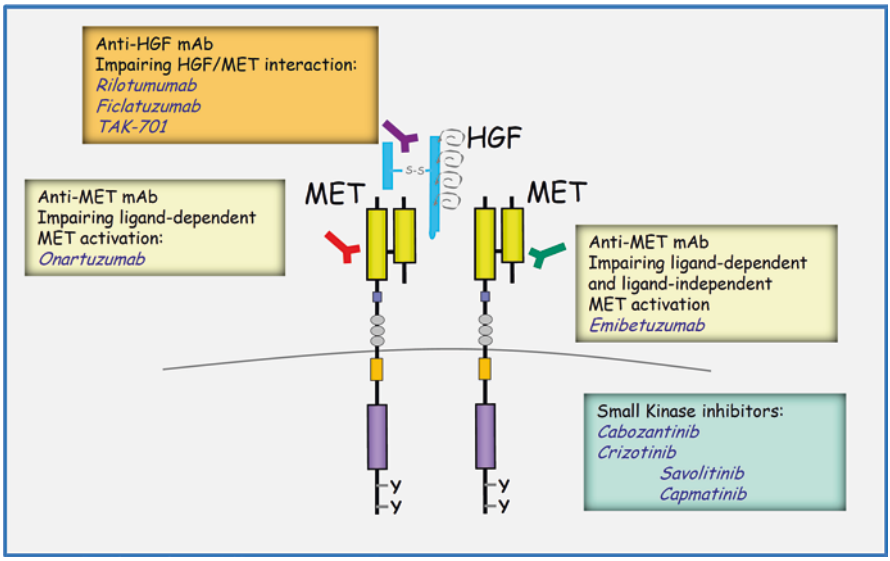

Fig. 4.2 MET-targeted drugs. Monoclonal antibodies targeting the HGF/MET axis can be broadly divided into those inhibiting the ligand HGF (*orange box*) and those inhibiting the receptor (*yellow*). The kinase inhibitors (*light blue box*) are further divided mechanistically into selective inhibitors (such as Savolitinib and Capmatinib) with minimal to no effects on a large panel of other kinases, or non-selective inhibitors (such as Cabozantinib and Crizotinib), which potently inhibit other kinases

4.4.1 Anti-HGF mAbs

Rilotumumab (AMG102, Amgen) is a human mAb that prevents HGF binding to MET [39]. In preclinical setting this mAb showed biological activity, being able to inhibit HGF/MET-driven cell growth [40]. Since in a Phase II study in gastric and esophagogastric junction cancers, rilotumumab in combination with epirubicin, cisplatin, and capecitabine improved both progression-free survival and overall survival in patients with tumors expressing high levels of MET [41], two phase III studies including patients with MET-positive gastroesophageal tumors were started. The RILOMET-1 study randomized patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma to epirubicin, cisplatin, and capecitabine with either rilotumumab or placebo. RILOMET-2 treated patients with the same characteristics with either cisplatin/capecitabine along with placebo or cisplatin/capecitabine with rilotumumab. Unfortunately, an interim safety review found an increased number of deaths as compared to chemotherapy alone and, thus, these studies were closed. Both these studies included patients with MET-positive tumors, though MET expression levels were not specified, according to ClinicalTrials.gov. Positive results were obtained in a Phase II trial performed in metastatic colorectal cancer patients with wild type KRAS [42]. Indeed, the combination therapy with the EGFR mAb panitumumab and rilotumumab resulted in a progression free survival of 5.2 months vs. 3.7 months with panitumumab alone.

Ficlatuzumab is an HGF neutralizing humanized mAb. Even though preclinical studies [43] and a Phase I trial in combination with Gefitinib performed in NSCLC patients demonstrated its activity [44], a Phase II, placebo-controlled study was prematurely stopped as a blinded analysis found that patients positive for both vascular endothelial growth factor signaling pathway and EGFR mutations experienced higher discontinuation rates. This observation significantly compromised the feasibility of the trial.

TAK-701, a HGF neutralizing humanized mAb which was shown to be able to reverse HGF-dependent resistance to EGFR inhibitors in non-small-cell lung carcinoma (NSCLC) patients [45] is now evaluated as a single agent in a phase I study in patients with advanced solid tumors.

4.4.2 Anti-MET mAbs

Onartuzumab. Onartuzumab is a recombinant humanized monovalent monoclonal anti-c-MET antibody that binds the extracellular domain of c-MET, blocking HGF ligand binding and inhibiting subsequent receptor activation. In a randomized placebo-controlled phase II study in recurrent NSCLC, onartuzumab plus erlotinib significantly improved progression-free survival and overall survival as compared to erlotinib plus placebo in MET-positive patients [46]. However, in the METLung double-blind randomised placebo-controlled phase III study in recurrent NSCLC, onartuzumab plus erlotinib did not significantly improve progression-free or overall survival over erlotinib alone [47]. Negative results were also obtained in the METGastric Phase III trials where the addition of onartuzumab to first-line mFOLFOX6 did not significantly improve clinical benefits in advanced gastroesophageal HER2-negative, MET 2+/3+ patients [48]. Similarly, there was no evidence of further clinical benefit with the use of onartuzumab in other Phase II trials in different tumors such as lung, gastric, breast cancers and recurrent glioblastomas [49–53].

4.4.3 Emibetuzumab

This humanized, bivalent anti-MET antibody inhibits both ligand-dependent and ligand-independent MET activation [54]. In fact, similarly to onartuzumab, it inhibits HGF binding to MET and thus receptor activation. Moreover, it also inhibits HGF-independent MET activation (often due to *MET* gene amplification) by promoting receptor internalization and degradation [54, 55]. In a Phase I study, treatment with emibetuzumab alone or in combination with erlotinib resulted in a durable partial response in NSCLC [56]. The combination of emibetuzumab and ramucirumab demonstrated early evidence of antitumor activity in a phase II trial in patients with advanced hepatocellular carcinoma (HCC) [56].

4.4.4 Small Kinase Inhibitors

Most of the MET tyrosine kinase inhibitors (TKIs) developed so far are ATP competitors. They interact with the ATP binding site of the kinase catalytic domain, mimicking the hydrogen bonds normally formed by the adenine ring of ATP. Type 1 inhibitors, such as crizotinib, bind to the active conformation, while type 2 inhibitors, like cabozantinib, recognize the inactive form of the enzyme. Covalent inhibitors are considered more potent than competitive inhibitors. Even if they are also ATP mimetics, they form irreversible covalent bonds with the kinase active site. Since protein kinases share high levels of homology in the ATP binding site, both the reversible and non-reversible inhibitors are often not specific for a single kinase and show cross-reactivity with other enzymes. MET inhibitors can also be classified as selective and non-selective. The former includes AMG-208, ASLAN002, INC280, JNJ38877605, MK-2461, MK-8033, MSC2156119J, PF4217903, PHA665752, SGX126, capmatinib and suvolitinib; the latter includes ANG-797, cabozantinib, crizotinib, foretinib, golvatinib MGCD265, and MP470.

The most clinically advanced MET TKI is *Cabozantinib,* a non-selective oral multi-kinase inhibitor targeting c-MET, VEGFR2, KIT, RET, FLT3 and TIE-2, currently approved by the Food and Drug Administration (FDA) for metastatic medullary thyroid cancer and renal cell carcinoma. A phase II randomised discontinuation study evaluated cabozantinib in advanced solid tumours of nine different tumour types, including HCC [57–59]. Of note, this study did not evaluate MET expression as a predictor of response to cabozantinib, and given the broad spectrum of targets of cabozantinib, it is unclear how much of the activity is attributable to MET inhibition alone. Given the encouraging data from this study, a phase III randomised double-blind study is currently recruiting, to compare cabozantinib against placebo as second-line treatment for advanced HCC patients who have previously received sorafenib [60]. As in the cited phase II study, also in this trial patients have not been screened for MET expression in tumors. Discording results were observed in metastatic castration-resistant prostate cancer patients: while the Phase III COMET1 trial did not reveal any improvement of cabozantinib over prednisone [61], a phase II study showed clinical activity of cabozantinib, particularly in patients with bone disease [62]. Other studies have been performed in different tumors, such as soft tissue sarcomas [63], ovary [64], lung [65] and breast [66] cancers.

Crizotinib, Savolitinib and *Capmatinib* are other well studied MET TKIs. Crizotinib, a multikinase ALK, ROS1 and MET inhibitor, has been approved by the FDA for treatment of rearranged ALK or ROS1 lung tumors. Savolitinib is a highly-selective and potent small-molecule MET inhibitor which has shown activity in preclinical models of gastric and papillary renal cell cancers and it is currently undergoing Phase I/II clinical testing. Capmatinib is another highly selective MET inhibitor, currently in phase I trials as a therapeutic in multiple cancer types.

4.5 Mechanisms of Resistance to MET Inhibitors

As previously mentioned, targeted therapies induce tumor regression in a variable percentage of patients, selected to express the target of the drug. However, almost invariably, responsive patients develop resistance to the treatment and undergo tumor relapse. A challenge associated to targeted therapies is, therefore, to predict the mechanisms that could cause resistance to the treatment and to find ways to circumvent these hurdles. In general, resistance to TKIs can be due to few general mechanisms: (1) mutations in the target that impair the interaction with the drug; (2) amplification of the target gene, resulting in overexpression of the encoded protein that renders the concentration of the inhibitor insufficient to block it; (3) activation (due to mutation, amplification or rearrangement) of downstream transducers, which convey a constitutive signal independent from the inhibited TKI; and (4) activation of parallel compensatory pathways, promoting a signal able to compensate the drug-inhibited one.

As for the MET tyrosine kinase, several *in vitro* models helped to identify molecular mechanisms sustaining resistance to MET inhibitors which fall in the different above cited groups.

4.5.1 MET Mutations that Impair the Interaction with the Drug (Fig. 4.3a)

Many mutations have been identified in the intracellular domain of MET and for some of them an activating role has been demonstrated [67]. Biochemical and crystallization studies have shown that some mutations can modify the structure of the kinase domain, thus impairing the ability of the TKI to interact with it. MET TKIs can be functionally classified into two main categories: (1) type I inhibitors that typically interact with the ATP-binding site of the active form of the kinase; and (2) type II inhibitors that display only partial interaction with the ATP-binding cleft and extend into an adjacent allosteric pocket that is exposed exclusively in the inactive kinase conformation. Thus, mutations located in different amino acids of the intracellular domain can have a diverse effect on sensitivity to TKIs of the two types. To investigate this important point, Tiedt et al. performed a mutagenesis screening to identify mutations that can interfere with the inhibitory activity of TKIs [68]. They found that indeed some mutants were not able to interact with the Type I kinase inhibitor NVP-BVU972 MET; however, a different spectrum of mutations resistant to a Type II inhibitor (AMG 458) were observed. These data suggest that mutations in the MET kinase domain can be responsible for primary or acquired resistance to TKIs, whose activity can be influenced by the binding modality of the drug. The work of Bahcall confirmed these observations in patients [69]. A patient with lung adenocarcinoma harboring both a mutation in EGFR and an amplification of MET, became resistant to combined MET/EGFR inhibition with the MET inhibitor savolitinib and the EGFR TKI osimertinib. When resistance appeared,

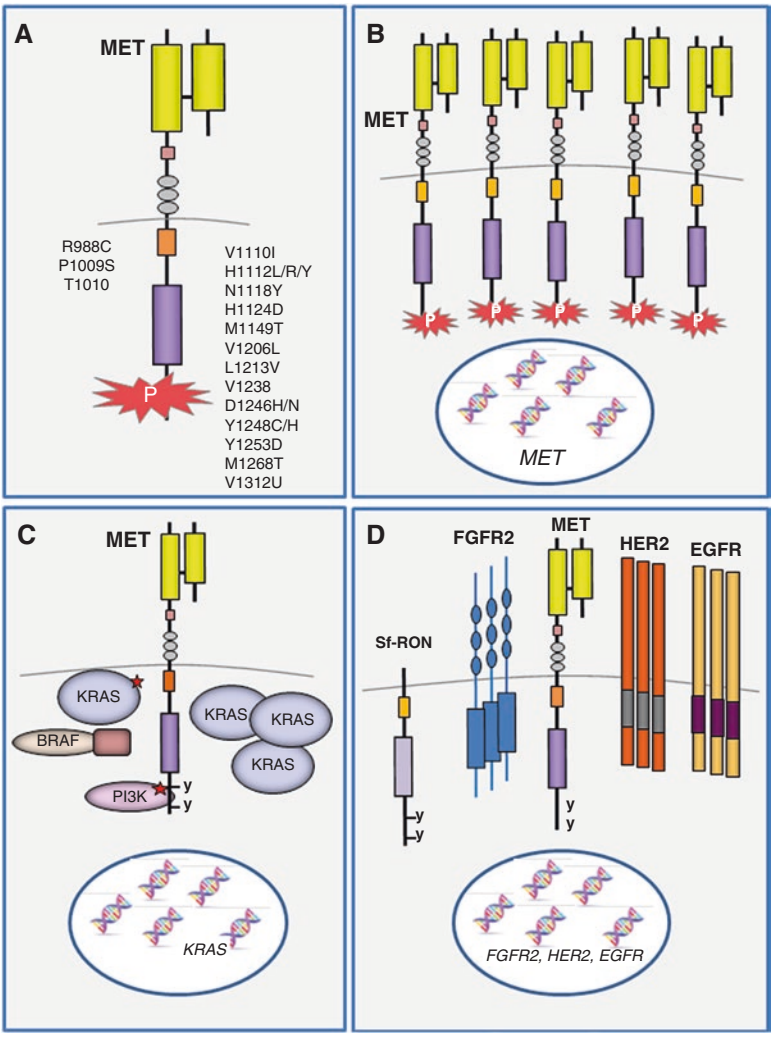

Fig. 4.3 Mechanisms of resistance to MET-targeted drugs. (**a**) Mutations in MET that impair the interaction with the drug. Several point mutations have been identified in the intracellular portion of the receptor; their presence can impair the response to KIs; (**b**) amplification of the MET gene, resulting in overexpression and constitutive activation of MET that renders the concentration of the inhibitor insufficient to block it; (**c**) activation due to KRAS mutation/amplification, PI3K mutation or BRAF gene rearrangement, leading to MET-independent activation of the signaling pathway; (**d**) activation of parallel compensatory pathways, promoting a signal able to compensate the drug-inhibited one. Activation can be due either to gene amplification or to receptor truncation

a new MET kinase domain mutation, D1228V, was detected. As MET^{D1228V} induces resistance to type I MET TKIs by impairing drug binding, the patient was treated with erlotinib combined with cabozantinib, a type II MET inhibitor, and exhibited a striking response. Interestingly, the same mutation was identified in a patient with NSCLC

displaying a MET-activating exon skipping variant and acquired resistance to the type I MET inhibitor crizotinib [70]. The take home message of all these findings is that, in the presence of mutations in the MET receptor, the choice of the TKI to be used is critical to avoid resistance onset, as type I and II MET TKIs are available and they can have a different spectrum of sensitivity versus the mutant receptors.

Differently from what has been observed in the case of the EGFR (where the S492R mutation impairs EGFR/Cetuximab interaction [71], no mutation in the extracellular domain of MET impairing mAb binding has been identified up to now.

4.5.2 *Amplification of the MET Gene (Fig. 4.3b)*

MET amplification is one of the main mechanisms of addiction to this oncogene as it renders tumor cells sensitive to the activity of MET inhibitors [15–17]. Interestingly, the *MET* gene is located within a known chromosomal common fragile site, FRA7G [72] and it has been shown that conditions interfering with DNA replication (called "replication stress") induce a perturbation of chromatin organization of this region, predisposing it to breakage [73]. This replication stress is believed to exert a selective pressure for gene amplification that disappears once the oncogene has attained sufficient amplification. The additional gene copy gain, induced or selected by drug treatment and counteracting the activity of the anti-MET drug, can rescue cell proliferation. Thus, MET amplification, depending on the number of gene copies, is both an oncogenic driver and a mechanism of resistance to treatment. Such a mechanism of resistance has indeed been shown *in vitro* in MET-addicted cells rendered resistant to either MET specific TKIs or MET antibodies [74, 75]. In both cases the amplified *MET* gene was not located on chromosome 7, as in normal cells, but in an episome. It is thus likely that during cell division the episomic copies do not partition symmetrically, leading to a different amount of gene copies in the daughter cells. Only those cells that display the appropriate amount of MET can survive in the presence of the inhibitor, resulting in an adaptive route.

4.5.3 *Activation of Downstream Transducers, Which Convey a Constitutive Signal Independent from the Inhibited MET (Fig. 4.3c)*

In several systems, the activation of the RAS family has been shown to be a mechanism of resistance to therapies targeting RTKs. The most evident example is colon cancer, where patients displaying activating mutations of KRAS do not respond to therapies targeting EGFR and, for this reason, are no longer treated with Cetuximab or Panitumumab (two EGFR targeted mAbs). Moreover, KRAS amplification or

mutation has been shown to be responsible for acquired resistance as well [76, 77]. Also in the case of gastric cancer preclinical studies have shown that KRAS amplification or activating mutation drives resistance to MET targeted therapies. Cepero et al. showed that cells rendered resistant to MET TKIs displayed MET gene amplification, leading to increased expression and constitutive MET phosphorylation, followed by subsequent amplification and overexpression of wild-type KRAS [74]. Cells harboring KRAS amplification progressively lost their MET dependence and acquired KRAS dependence. This phenomenon, known as "oncogene switch," has already been reported as a consequence of the inhibition of different tyrosine kinases, but it usually involves the activation of other kinases, driving parallel signaling pathways [78]. It is interesting to note that, as for *MET*, also the *KRAS* gene is located inside a chromosomal common fragile site, on chromosome 12 [79] and, thus, it is likely that the same mechanisms responsible for *MET* amplification can also account for this second event. Interestingly, resistance to treatment was reversible and the alterations leading to resistance were lost after drug withdrawal. One possible explanation for this finding relies on the fact that the amplified oncogenes *MET* and *KRAS* were extrachromosomal in the examined cells. The removal of the inhibitor results in an excess of signal transduction that may induce cellular stress, known to lead to the loss of extrachromosomal DNA [80, 81]. Cells undergoing loss of *MET* and *KRAS* extra copies, thus, have an advantage and become the prevalent population in the absence of MET inhibitor. These observations suggest that this mechanism of resistance may be less stable than others already described (such as the appearance of point mutations) and that, possibly, an intermittent therapy, favoring the loss of amplified copies, could give better results.

More recently, Leiser et al. showed that not only RAS amplification, but also activating KRAS and HRAS mutations confer resistance to MET targeting [82]. This observation has been proven not only in preclinical models but also in a gastroesophageal cancer patient who, after 2 years of response to MET inhibitors, developed resistance [83].

Also, genetic alterations in transducers downstream RAS have been shown to induce resistance to TKIs. Fujian et al. showed that PI3K p110a contributes to acquired resistance in a gastric cancer preclinical model [84], where only the combined inhibition of both MET and PI3K led to tumor shrinkage. Two different publications identified BRAF fusion proteins as mechanisms of resistance to MET TKIs and showed that the combined BRAF/MET inhibition effectively kills these cancer cells [85, 86].

4.5.4 Activation of Parallel Compensatory Pathways, Promoting a Signal Able to Compensate the Drug-Inhibited One (Fig. 4.3d)

A great deal of preclinical and clinical evidence has shown that in many cases resistance is sustained by the so called "kinase switch". This means that compensatory pathways become activated and drive a signal able to compensate the drug-inhibited

one. In the case of MET, experimental and clinical evidence has shown that a strong interplay takes place between MET and the EGFR family of receptors. The signaling pathways activated by these two families of receptors, in fact, share many transducers and can compensate for each other [87]. Indeed, MET activation can induce resistance to EGFR targeted therapies in around 20% of NSCLC and in experimental models of HER2-driven breast cancers (reviewed in [88]. The opposite is also true as EGFR activation (due to genetic lesions or to ligand stimulation) or HER2 activation have been shown to cause primary or acquired resistance to MET TKIs in MET-addicted cells [89–92] (and in gastroesophageal xenopatients [93]. Interestingly, combined MET/EGFR inhibition resulted not only in strong tumor reduction but also in a durable response, preventing the onset of resistance. The role of EGFR family in sustaining resistance to MET inhibitors has been validated also in gastroesophageal patients where co-amplification of MET and HER2 and/or EGFR have been identified.

RTKs other than those of the EGFR family have also been shown to drive resistance to MET-targeted therapies. This is the case of Fibroblast Growth Factor Receptor 2 (FGFR2) and RON. Liu and colleagues [94] generated a MET-amplified gastric cancer xenopatient which did not respond to MET targeted drugs. As FGFR2 amplification and overexpression where detected in this tumor, a combined MET/FGFR2 therapy was started which resulted in tumor growth inhibition. A role for FGFR2 in impairing the response to MET inhibitors in gastric cancer was confirmed also by Wu and coll. [95]. The same authors also found that the RTK RON, belonging to the same family as MET and aberrantly activated in various malignances, including gastric cancer, could induce resistance to MET inhibitors. Two transcripts of this gene, coding a full-length RON and a short-form RON (SF-RON), have been detected in gastric cancer tissues [96]. Wu et al. found that upregulation of sf-RON, but not stimulation of full length RON, conferred MET inhibitor resistance. As they found that sf-RON was up-regulated in MET+ gastric cancer, they propose MET/RON dual inhibition to prevent resistance onset.

4.6 Conclusions

Even though many preclinical studies have shown that MET targeting in MET-addicted tumors could be of great therapeutic value, so far the results obtained in clinical trials with MET-targeted drugs have been disappointing. This can be due to several factors. First of all, a major problem is the identification of patients which could benefit from such treatment. A platform that includes accurate, validated methods and reagents will help to improve MET-driven population selection. Preclinical studies have shown that the presence of at least 8 MET copies is a good criterium to identify MET addicted tumors [97]. Moreover, real gene amplification does not seem equivalent, in biological terms, to chromosomal amplification; thus, the two conditions should be differentiated. Many studies have selected patients by use of immunohistochemistry, but there

are no solid preclinical data showing that receptor overexpression is sufficient to drive addiction. Moreover, the level of overexpression required to induce MET-dependency has not been defined yet. It is also to be noted that different antibodies have been used to perform immunohistochemistry, directed either toward the intracellular or the extracellular portion, and an accurate comparison among them is missing. Not only the intensity of the signal, but also the percentage of cells positive for the signal still needs to be defined. Thus, at the moment, the most stringent criterium to identify patients likely sensitive to MET-targeted therapy seems to be the number of gene copies.

The second critical point is the choice of the drug to be used. MAbs targeting HGF or interfering with HGF binding to MET might be active only in tumors where MET activation is ligand-dependent. This is unlikely in the situation where the *MET* gene is amplified and strongly overexpressed as many studies have shown that in this condition MET activation is HGF-independent. Thus, drugs preventing receptor dimerization or inhibiting its kinase activity should be chosen. In the cases where point mutations are present in the MET kinase domain, the use of type I or II inhibitors should be carefully considered, as these inhibitors can be inactive in some mutant receptors. All these considerations suggest that once the patients have been selected on the base of target alteration, careful attention has to be paid to the choice of the drug.

The third important point is the decision to use MET drugs either in monotherapy or in combination with drugs targeting other RTKs. As discussed, preclinical and clinical studies have shown that other RTKs can confer resistance to MET drugs, in particular the members of the EGFR family. In some tumors, such as lung and gastroesophageal cancers, where EGFR family members are known to be frequently activated, an upfront treatment with a combination therapy targeting both the receptors should be considered. This could lead both to a stronger and long lasting response. The search for genetic alterations of downstream targets could also help in identifying those patients that, even though potentially eligible for treatment, are indeed resistant. Thus, the definition of positive and negative biomarkers of response is mandatory to identify MET drug-responsive patients and to avoid the loss of potentially useful drugs due to their use in an appropriate context.

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 5
RAF, MEK and ERK Inhibitors as Anti-Cancer Drugs: Intrinsic and Acquired Resistance as a Major Therapeutic Challenge

Galia Maik-Rachline, Izel Cohen, and Rony Seger

Abstract The ERK cascade regulates various cellular functions and is hyperactivated in more than 85% of cancers leading to dysregulated proliferation. This hyperactivation as well as oncogenic activating mutations in the different components of the cascade (mainly RAS, RAF and MEK) have inspired the development of several inhibitors targeting the different tiers of the cascade. As a result, clinically approved RAF and MEK inhibitors are used for targeted therapies of metastatic mutated BRAF melanoma. However, along with the impressive clinical results observed with many of these patients upon initial treatment, other patients do not respond to the drugs, and development of resistance in the sensitive group is unavoidable. Deciphering the molecular mechanisms underlying this intrinsic or acquired resistance is a necessity in order to enhance the treatment efficacy of the drugs used for ERK-addicted cancers. Several resistance mechanisms have been proposed up to date, which result from either preexisting mechanisms in some or all cells within the tumors or due to drug-induced mechanisms. These include (a) expression of drug-resistant RAF isoforms (b) molecular or genetic alterations of downstream components that reactivate the ERK cascade and (c) induction of upstream components and other signaling pathways that bypass the drug blockage. All these mechanisms eventually result in inducing reactivation of ERK or other survival-related pathways. Here we review the mechanisms underlying drug resistance and future efforts to develop activity-independent, resistance-escaped, more efficacious anti-tumor drugs.

Keywords Acquired resistance • Intrinsic resistance • BRAF-mutated melanoma • Cancer • ERK cascade • Kinase specific inhibitors

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_5

Abbreviations

EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinase
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK/ERK kinase
RTK	Receptor Tyrosine Kinase

5.1 Introduction

The ERK cascade (RAF-MEK-ERK) is a central signaling pathway that plays an integral role in the initiation and regulation of most stimulated cellular processes such as proliferation, survival and differentiation. The cascade is activated upon stimulation of upstream cell surface receptors that further transmit their signals to RAF mainly through Grb2 and SOS that switch on the small inactive GTPase RAS. This activation of RAS enables the recruitment of RAF (mostly B and CRAFs) to the plasma membrane, promoting their homo- or hetero-dimerization and subsequently their activation [1, 2]. Activated RAFs, in turn, phosphorylate and activate MEK1 and MEK2 (MEK), which further phosphorylate and activate ERK1/2 (ERK). Once activated, ERK phosphorylates many downstream targets in the cytoplasm and in the nucleus. Essentially, hundreds of proteins (more than 300) have been identified as ERK cytosolic and nuclear substrates, as well as ERK interacting proteins [3]. Having a crucial regulatory role in cell function, ERK signaling must be precisely regulated and capable of adapting to dynamic environmental changes. The magnitude of the signal, its duration, the different locations of the cascade's components and their interaction with other pathways and scaffold proteins further govern the outcome that culminates in the desired biological effects [4]. Another important part of this regulation is the induction of negative feedback loops containing phosphatases and other regulators that result in ERK-mediated control of its own activity [5].

Being such a central regulatory component, dysregulation of the ERK cascade results in various human diseases including cancer. Indeed, hyperactivation or other divergent properties of the ERK cascade are reported in more than 85% of all cancers [6]. The different components of the ERK cascade as well as the upstream RAS are frequently mutated in human cancers. The RAS is the most frequently mutated oncogene in human malignancies, as about 30% of all human cancers present activating mutations in these family genes. It is mostly common in pancreatic cancer where it is responsible for 90% of the cases as well as in colon cancer (50%). Mutations in RAFs have been reported in about 7% of all cancers. Among the three RAF family members, BRAF is by far the most frequently mutated isoform with the highest frequency observed in melanoma (~50%). Other oncogenes in the MAP3K level of the cascade are *COT* and *MOS*, but they are much less prevalent than the

BRAF mutation. Various oncogenic MEK mutations exist as well, but those appear in only ~1% of all cancers [7], while oncogenic ERK mutations are very rare, and usually detected in RAF or MEK inhibitors-treated tumors [8, 9]. Obviously, the expression of each of the *RAS/RAF/MEK/ERK* oncogenes in human cancers, which covers ~40% of all cancers results in activating ERK and their downstream targets. However, the cascade is also active in more than 45% of all cancers that are driven by oncogenes that are not related to the ERK cascade (e.g. PI3K/AKT). The activation of ERK in these cancers is indirect, but is important for the enhanced proliferation that accompanies the cellular transformation [6].

Since the ERK cascade is often hyperactive in a large number of cancers, leading to their enhanced proliferation, a major effort has been geared towards the development of inhibitors targeting the different components of the pathway. The initial, first-generation inhibitors were not efficient enough mainly because they were aimed to target a broad-spectrum of RAS or RAF activities. However, progress has been made with the development of second-generation inhibitors, selective against the mutated form of RAF, or the active form of MEK, and these drugs are already in clinical use. Such is the case with the successful introduction of the two BRAF inhibitors; Vemurafenib (Zelboraf) and Dabrafenib (Tafinlar), which have remarkable efficacy when administered as monotherapies of mutated BRAF V600E/K metastatic melanoma (mutated BRAF melanoma) patients [10, 11]. The efficacy is even increased when these drugs are used in combination with the MEK inhibitor, Trametinib (Mekinist), which has dramatically improved the median overall survival of mutated BRAF melanoma patients [12, 13]. However, although these drugs may have a strong initial response, their effect is limited, because of intrinsic or acquired resistances to the drugs. The intrinsic (innate) resistance results in a lack of response even if the tumors present BRAF mutations, while even the patients who initially respond, relapse due to acquired (adaptive) resistance within a year of disease treatment [14]. In addition, the use of the drugs may result in some severe side effects such as the paradoxical development of cutaneous squamous-cell carcinomas and keratoacanthomas [15]. However, despite these therapies' drawbacks, blocking the ERK cascade is still considered a prime target for the treatment of many cancers. Much effort is being invested in understanding the downsides of current treatments in order to develop better inhibitors. Indeed, a large number of next-generation RAF and MEK inhibitors, along with new ERK-specific inhibitors, are currently under investigation in various trials. Moreover, recent advances and novel approaches in drug discovery have renewed the challenge of targeting the undruggable RAS to directly inhibit the RAS protein (mainly KRAS). In this chapter, we review the various inhibitors of the ERK cascade available today and thoroughly discuss the molecular mechanisms of intrinsic or acquired resistance to these drugs, which are often attained by similar or even identical mechanisms. We examine future novel approaches, mainly in the form of selective small molecule inhibitors, which have the potential to better inhibit the RAS-ERK pathway, avoiding the side effects that are currently seen with the available drugs used today.

5.1.1 The ERK Cascade Inhibitors

The ERK cascade plays a major regulatory role in the induction of various cellular processes. Importantly, the cascade is hyperactivated in most cancers, mediating the unregulated enhanced proliferation of the transformed cells. Therefore, this cascade is considered as a favorable potential candidate for targeted cancer therapy. Indeed, various inhibitors targeting the different tiers of the cascade have been successfully developed. Some of them are already clinically approved, while some are still undergoing preclinical and clinical evaluations. The development of inhibitors against the upstream component, *RAS*, the most frequently mutated oncogene in human cancer, has proven to be far more challenging. Three decades of unsuccessful attempts to target this protein have so far yielded no approved directed therapies. We will therefore focus mainly on RAF and MEK inhibitors targeting this cascade, and specifically on the ones that have already been approved for clinical use. We will also touch on recent advances in the development of ERK inhibitors.

5.1.1.1 RAF Inhibitors

The RAF family of protein kinases is composed of three isoforms, ARAF, BRAF and CRAF (Raf-1), which share a high-sequence homology [16]. Initial attempts to inhibit RAF activity were dedicated to *CRAF*, as it was the first potential oncogene identified. Indeed, the first RAF inhibitor to gain regulatory approval was Sorafenib (Nexavar; Bayer/Onyx Pharmaceuticals), which was developed as an inhibitor of the catalytic activity of CRAF (CRAF inhibitor). However, it turned out that this drug also inhibits several other kinases, not specifically CRAF [17]. Although this drug has promising clinical effects for a broad range of tumor types, and was approved for clinical use, it exhibits a very limited efficacy in treating mutated BRAF melanomas [18]. Later on, it became clear that CRAF mutations in human cancer are rare, appearing in only 1% of all cancer cases and ARAF mutations are even more random [19]. Therefore, it is possible that the effect of this drug is not due to RAF inhibition, but rather due to its effect on other protein kinases. The impetus for second-generation RAF inhibitors came from the identification that oncogenic mutations of RAF are mainly in BRAF and are responsible for driving ~7% of all cancers. Of these, 50% were identified in melanomas including more than 40 distinct BRAF mutations with V600E being the most frequent mutation [20]. Specific inhibitors selectively targeting the V600E BRAF mutation were vigorously developed leading to the next approved drug for mutated BRAF melanomas, Vemurafenib (ZELBORAF™; Roche-Genentech/Plexxikon). Initially developed as the small molecule PLX4072 [21], this compound showed selective inhibition of mutated BRAF in several models, while blocking ERK activity. Further improvement of this compound demonstrated its favorable safety and efficacy in a phase I trial in 2010, showing specificity mainly to mutated BRAF melanomas [22]. Significant improvements in disease-free progression were further demonstrated in

phase II [23] and phase III clinical trials [10]. Along with the development of vemurafenib, another ATP competitive inhibitor, Dabrafenib (TAFINLAR^{r};GSK), that demonstrated selective inhibition of mutated BRAF melanomas, entered clinical trials [24] and was eventually approved. The response rate to this drug was ~60% in mutated BRAF melanoma patients including 7% with complete responses, which was comparable to the efficacy results observed with vemurafenib [11].

Some novel RAF inhibitors are currently under development in order to produce better efficacy drugs, overcoming resistance with fewer side effects. These include: LGX818 (Novartis), a highly potent BRAF V600E selective inhibitor, along with TAK-632 and MLN2480 (Takeda), selective pan-RAF inhibitors. Importantly, the latter suppresses RAF activity in mutated NRAS-transformed cells that are not affected by the former drugs, as well as in mutated BRAF melanomas with acquired drug resistance [25]. Other compounds, ARQ736, PLX3603 and LY3009120 [26] are additional new pan-RAF inhibitors that prevent ERK activation in BRAF mutated cancer cells. Recent trials with these inhibitors have been comprehensively summarized [27].

5.1.1.2 MEK Inhibitors

The next tier of the ERK cascade is composed of the MAPKK isoforms MEK1 and MEK2 [28]. Several activating mutations of MEK1 have been identified thus far, and some of them serve as driving oncogenes in various cancers [29]. However, their prevalence is much lower than that of RAS and RAF, as they appear in 3.5% of epithelial cancers [29] and were reported as the driving oncogenes in a small percentage (~1%) of melanoma, ovarian, colon and lung cancers [7, 30]. Although the prevalence of MEK oncogenes is relatively low, the activity of MEK is elevated either directly by oncogenic RAS, RAF, COT and MOS, or indirectly in 85% of cancers. All these turn MEK into an attractive protein for the inhibition of RAS and RAF mutated tumors as well as ERK activity.

The first generation of MEK inhibitors, PD98059 and U0126, were not suitable for in vivo use. The following ones that entered clinical trials, CI-1040 (PD184352) [31] and its improved analogue PD0325901 [32–34], were discontinued following phase I trials, and their development was abandoned. However, continued interest in the field led to the development of third-generation MEK inhibitors, among which is Selumetinib (AZD6244; AstraZeneca/Array BioPharma), a highly selective allosteric inhibitor whose efficacy is still being evaluated in several clinical trials, either as a single agent or in combination with other chemotherapeutic agents [35, 36]. Trametinib (MEKINIST; GSK1120212, GSK) is the only MEK inhibitor currently approved by the FDA for monotherapy or combined therapy with dabrafenib for the treatment of mutated BRAF melanomas. It is an allosteric MEK inhibitor that inhibits not only MEK activity, but also MEK activation by RAF, producing prolonged ERK inhibition [37]. Its phase III trial [12] on mutated BRAF melanoma patients at an advance stage, who were not previously treated with RAF inhibitors, successfully improved rates of progression-free and overall survival as compared to

chemotherapy treatment. Interestingly, trametinib treatment can be beneficial even for mutated BRAF melanoma patients who had been previously treated with a BRAF inhibitor, as compared to patients who were previously treated with chemotherapy and/or immunotherapy [38]. These trials initiated a new therapeutic strategy of combining RAF and MEK inhibitors. Indeed, the combination treatment regime of trametinib and dabrafenib showed improved pharmacokinetics, safety and antitumor activities [13]. Other successful combinations include the MEK inhibitor, cobimetanib, and the RAF inhibitor, vemurafenib, as well as MEK162 and LG818 [27]. Several current clinical trials for dual inhibition of RAF and MEK were comprehensively summarized recently [27].

Several other MEK inhibitors are currently under development and in different stages of clinical trials or approval. These inhibitors aim at retaining efficient inhibition of MEK, while avoiding some of the side effects observed with trametinib (e.g. drug accumulation in the brain). These inhibitors include Cobimetinib, a structural analog of CI-1040, (GDC-0973, XL-518, RG7421; Genentech/Exelixis [39, 40], Rafametinib (BAY 86–9766; Bayer/Ardea Biosciences [41], Pimaserib (AS703026; Merck KGaA), Binimetinib (MEK162) and AZD8330 (Novartis/Array Biopharma), RO5126766 (the first dual RAF and MEK inhibitor) and RO4987655 (Roche), G-573 and GDC-0623 (Genentech) as well as TAK-733 (Takeda) [35].

5.1.1.3 ERK Inhibitors

Cellular responses to external stimuli are integrated into ERK, which coordinate the overall signaling activity of the cell. Two kinases comprise this last tier of the cascade, ERK1 and ERK2, which share 85% amino acid sequence identity and are activated in >85% of cancers. Other alternatively spliced isoforms such as ERK1c exist as well [42], but are probably not involved in tumorigenesis. Oncogenic ERK mutations are very rare [8, 9] and no approved specific ERK activity inhibitors are currently available, but their development is gaining renewed interest. Located at the bottom of the cascade and integrating signaling from various upstream components, the development of ERK inhibitors is still an attractive therapeutic approach. Indeed, some recently developed ERK inhibitors are currently under investigation and some are already in clinical trials. One such an inhibitor is SCH772984 [43], which was identified by an affinity-based screen of compounds that selectively bind to the unphosphorylated form of ERK2. It is an ATP competitive inhibitor of ERK with nanomolar cellular potency in tumor cells. Interestingly, upon binding to ERK, it also prevents ERK phosphorylation by MEK due to allosteric changes. Importantly, SCH772984 effectively inhibited ERK signaling and cell proliferation in BRAF and MEK inhibitor-resistant models [43]. Several other ERK inhibitors under development are: SCH900353, which is an improved clinical version of SCH772984, as well as BVD-523 (Biomed Valley Discoveries) and RG842 (GDC0994; Genentech/Roche), but their data is not yet available [44]. Finally, substantial effort is being invested towards the development of drugs to inhibit kinases downstream of ERK such as RSK [45, 46]. However, at this stage, none of these compounds have entered advanced stage clinical trials.

5.2 Mechanisms of Resistance to the ERK Cascade Inhibitors

Several inhibitors of different components of the ERK cascade have been approved up to date. All are well tolerated, with relatively mild toxicity and manageable side effects. Two major downsides, however, are that these drugs affect almost exclusively mutated BRAF melanomas and that they may induce other cancers, mainly skin ones, due to "paradoxical activation" of ERK signaling by RAF inhibitors [47, 15]. Another important disadvantage of the drugs is the relatively short duration of response due to development of drug resistance. After an initial phase of very impressive responses to these inhibitors, the majority of the patients acquire resistance to the drugs, and relapse within 6–8 months [48]. The emergence of resistance is due to either reactivation of ERK signaling, failure of the inhibitors to shut down ERK activation, or activation of alternative pathways that overcome the inhibition of ERK. Very good model systems for the study of the mechanism of resistance are the mutated BRAF melanomas, which are usually entirely dependent (addicted) on ERK signaling for their survival. Although other cancers do show increased/deregulated ERK activation as well, they may depend on other pathways for their survival (e.g. AKT) and, therefore, are less sensitive to the ERK-related drugs. It is possible that resistance mechanisms are involved in cancer cells with elevated ERK activity that are transformed by other oncogenes. But because those cells are not "addicted" to the ERK cascade, the effects of the drugs are not significant.

The resistance of the mutated BRAF transformed cells can be phenotypically classified into intrinsic (innate [14], or acquired (adaptive [49, 50]). In the intrinsic resistance, no clinical benefit is achieved at any stage of the treatment since the mutated BRAF-induced cancers (including some mutated BRAF melanomas) are not responsive to the drug due to inherent properties within the tumors [51, 52] or due to the interactions between the tumors and their microenvironments [53, 54]. However, in the acquired resistance, a progressive disease is observed following a clinical benefit phase as the initially inhibitor-sensitive cancers develop resistance during the treatment. Both types of resistance may be achieved by several mechanisms, some of which are similar between the two types. In principle, the mechanisms of resistance often result in reactivation of ERK signaling or activation of alternative signaling pathways. These are caused by variable distinct ways that can be divided into five categories which include: (I) activation of alternative pathways; (II) activation or overexpression of receptor tyrosine kinases (RTKs) and their ligands; (III) RAS activation; (IV) activation of MAP3Ks and (V) activating mutation of MEK or ERK (see Fig. 5.1 and Table 5.1). Some of these can be induced by the abolishment of negative feedback loops due to the drug-induced reduction in ERK activity [55]. Another mechanism of resistance that is distinct from those is "drug-induced resistance", which is described below.

The tumor's cells are not uniform and often contain resistance-related mutations or modifications formed by random mutagenesis or varying environmental changes [52] even before treatment's initiation, which are known as preexisting resistance mechanisms. The preexisting machinery may be present in most of the tumor cells making the

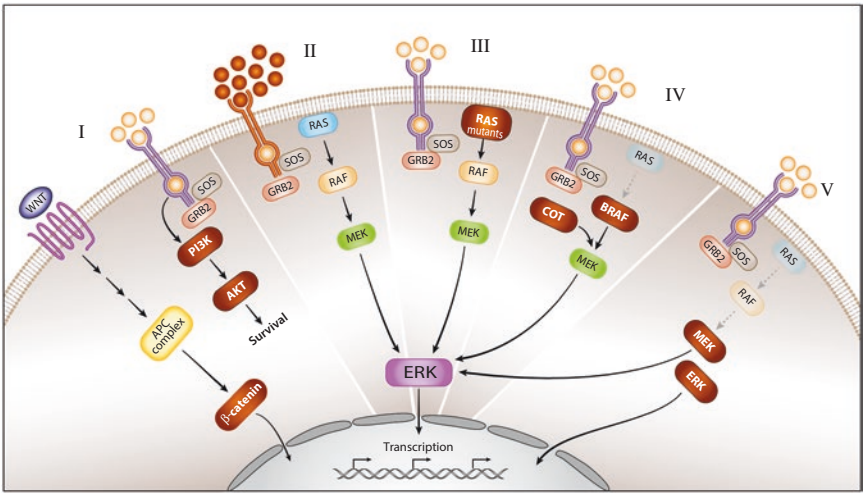

Fig. 5.1 Major resistance mechanisms to RAF and MEK inhibitors. Receptor tyrosine kinase activation (RTK) initiates signaling via the canonical RAS-ERK pathway. Resistance to inhibitors of various components of the pathway can develop due to: (I) activation of other pathways such as the PI3K-AKT or WNT survival/proliferation signaling pathways; (II) upregulation or hyperactivation of receptor tyrosine kinases (RTKs) and their ligands; (III) Accumulation of activating mutations of small GTPases (mainly NRAS) mutations: (VI) modifications of MAP3K, including accumulation of RAF activating mutations (e.g. V600E/K), expression of drug-insensitive splicing variants or BRAF, and amplification or activation of COT or other MAP3K; and (V) expression of activating MEK or ERK mutations. The modulated components are enlarged and colored in dark red and the down-regulated signaling components are in muted colors

tumor insensitive to the drugs, resulting in intrinsic resistance. In addition, the resistant cells can be the minority, although they still make up a substantial portion of the tumor. In this case there is an initial, small response to the drugs, but the growth of resistant cells is sufficient to restore the original size of the tumor very rapidly. Therefore, the initial response is not even detected in patients, giving rise to an intrinsic response-like phenotype [56]. Finally, the preexisting resistance may be present in a very small number of cells. In this case, there is a strong response of the tumor to the drug, due to the eradication of the sensitive cells, which leaves only the small number of resistant cells intact. Those then grow and reach the original tumor size within months, giving rise to the acquired-resistance phenotype. Thus, the preexisting resistance can lead either to an intrinsic or to an acquired resistance phenotype depending on the proportion of preexisting resistant cells in the tumor. As of today, a relatively large number of preexisting mechanisms of resistance have been described, which are interchangeable and have similar principal effects in all cases. Interestingly, the paradoxical effects of ERK activation by the drugs may participate in many of the mechanisms leading to both types of resistances [47, 15]. In addition to the preexisting resistance, tolerance to the drug can be gained due to drug-induced change mechanisms [57], which, by definition, lead to acquired resistance. Here, we describe the studies that led to the identification of intrinsic and acquired resistances as well as drug-induced mechanisms.

Table 5.1 Resistance mechanisms to RAF and MEK inhibitors

Resistance category	Mechanism of resistance	Cancer cells	Oncogene	Inhibitor	Reference
I. Alternative pathways	Activating PI3K/AKT mutations were detected in drug-resistant melanoma tumor samples	Melanoma	$AKT1^{Q79K}$ $AKT3^{E17K}$ $PI3KCA^{D350G}$	Vemurafenib Dabrafenib	[81]
	Suppression of the MED12 component of the MEDIATOR complex conferred resistance to vemurafenib via TGF-β signaling	Melanoma/colorectal cancer	$KRAS^{V12}$	Crizotinib Gefitinib Erlotinib Vemurafenib AZD6244 Sorafenib	[83]
	Relief of ERK negative-feedback loops induced ERK reactivation and hyperactivation of AKT	Melanoma/colorectal/thyroid cancers	$BRAF^{V600E}$	Vemurafenib Dabrafenib	[55]
	NF1 loss mediated resistance to RAF and MEK inhibitors through sustained MAPK pathway activation	Melanoma	$BRAF^{V600E}$	Vemurafenib AZD6244 VTX-11e	[80]
	WNT5A activated PI3K/AKT signaling, and promoted melanoma growth and survival via its receptors RYK and FZD7	Melanoma	$BRAF^{V600E/K}$	Vemurafenib	[67]
	The $AKT1^{Q79K}$ mutant displayed enhanced affinity to PIP3-amplified drug-induced upregulation of the PI3K-AKT pathway	Melanoma	$AKT1^{Q79K}$	Vemurafenib; AZD6244 GSK1120212 MK2206	[59]
	Upregulation of neurofibromin 2 (*NF2*), Cullin 3 E3 ligase (*CUL3*), and members of the STAGA histone acetyltransferase complex (*TADA1* and *TADA2B*)	Melanoma	$BRAF^{V600E}$	Vemurafenib	[85]
	An increase in the expression of the hippo pathway effector YAP promoted cell survival	Melanoma/colon/pancreas/thyroid/ non-small-cell lung cancers	$BRAF^{V600E}$	Vemurafenib Trametinib	[84]
	Downregulation of FRA1 (transcription factor, ERK substrate) led to hyperactivation of mainly the AKT signaling pathway. Combined BRAF and PI3K-AKT inhibitors inhibited resistance cell growth	Melanoma/lung adenocarcinoma	$BRAF^{V600E}$	Vemurafenib Dabrafenib	[54]

(continued)

Table 5.1 (continued)

Resistance category	Mechanism of resistance	Cancer cells	Oncogene	Inhibitor	Reference
II. RTK activation	Overexpression of ERBB3 or IGF1R induced AKT activity. Combined treatment with IGF-1R/PI3K and MEK inhibitors inhibited resistance cell growth	Melanoma	$BRAF^{V600E}$	SB590885 Vemurafenib GSK1120212 AZD6244 U0126 Tyrphostin; AG1024 GSK2126458	[77]
	Upregulation of PDGFRβ or NRAS mutations	Melanoma	$BRAF^{V600E}$	Vemurafenib AZD6244	[49]
	The increase of RTK ligands conferred resistance among which is hepatocyte growth factor (HGF)	Melanoma	$BRAF^{V600E}$	Vemurafenib	[76]
	Secretion of hepatocyte growth factor (HGF) by the tumor stromal cells that operates via the MET receptor reactivated the MAPK pathway	Melanoma/lung/adenocarcinoma	$BRAF^{V600E}$	Vemurafenib	[53]
	Rapid feedback activation of EGFR was caused by the BRAF mutant inhibition. Combined treatment with BRAF and EGFR inhibitors inhibited cell growth	Colorectal cancer	$BRAF^{V600E}$	Cetuximab Gefitinib Erlotinib Vemurafenib	[63]
	Upregulation of RTK (EGFR)	Colorectal cancer	$BRAF^{V600E}$	Vemurafenib Gefitinib Crizotinib Lapatinib	[62]
	Activation of EGFR–SFK–STAT3 signaling due to increased EGFR–SFK activity. Combined BRAF and Bcr-Abl inhibitors inhibited resistance cell growth	Melanoma	$BRAF^{V600E}$	Dasatinib Vemurafenib	[65]
	Upregulation of HER3 by autocrine secretion of neureguline-1 and CtBP1/2 transcriptional repressor activity	Thyroid cancer	$BRAF^{V600E}$	Vemurafenib Selumetinib Lapatinib AZD6244	[64]

Resistance category	Mechanism of resistance	Cancer cells	Oncogene	Inhibitor	Reference
III. Small GTPase activation	Activating RAS mutations were detected in drug-resistant melanoma tumor samples	Melanoma	$KRAS^{G12C}$ $KRAS^{G12R}$ $KRAS^{Q61H}$ $NRAS^{G12D/R}$ $NRAS^{G13R}$ $NRAS^{Q61K/R/L}$	Vemurafenib Dabrafenib	[81]
		Melanoma	$HRAS^{Q61L}$	Vemurafenib	[15]
	Reduced expression of RND3, an antagonist of RHOA activation, led to an elevation of RHOA-dependent signaling and increased cell migration	Melanoma	$BRAF^{V600E}$	Vemurafenib SB590885	[107]
	Elevated RAS-GTP levels and increased levels of AKT phosphorylation led to a drug-resistance effect that was abolished by the synergistic use of RAF/MEK/AKT inhibitors	Melanoma	$KRAS^{K117N}$	Vemurafenib RO5068760 MK-2206	[108]
IV. Activation of MAP3Ks	MAP3K8 (COT, TPL2) activated MEK independently of RAF signaling	Melanoma	$BRAF^{V600E}$	Vemurafenib	[50]
	Increased BRAF dimerization was caused by alternatively spliced 61 kDa isoform of BRAF(V600E) lacking RAS-binding domain (p61BRAF(V600E))	Melanoma	$BRAF^{V600E}$	Vemurafenib Dabrafenib	[71]
	ATP-competitive inhibitors mediated RAF dimerization by stabilizing a rigid closed conformation of the kinase domain	Colorectal cancer		Vemurafenib Sorafenib GDC-0879; AZ-628 SB inhibitors	[61]
	RAF isoforms existed predominantly as active monomers and did not require dimerization for their activation. Monomeric active form of mutated BRAF	Melanoma/lung cancers	$BRAF^{V600E/K}$		[51]
	Loss of full-length BRAF(V600E) coupled with expression of an aberrant form of $BRAF^{V600E}$ that retained RAF pathway dependence, or constitutive autocrine EGFR signaling driven by c-Jun-mediated EGFR ligand expression	Non-small-cell lung cancer	$BRAF^{V600E}$	Vemurafenib Dabrafenib	[56]
	P-loop mutations disrupted the autophosphorylation activity of RAF, relieving RAF autoinhibition	Melanoma			[90]

(continued)

Table 5.1 (continued)

Resistance category	Mechanism of resistance	Cancer cells	Oncogene	Inhibitor	Reference
V. MEK/ERK activating mutations	Activating MEK1 or MEK2 mutations caused an increase in the intrinsic kinase activity of MEK enhancing ERK phosphorylation	Melanoma	$MEK1^{Q56P}$ $MEK1^{P124L}$	AZD6244 Vemurafenib	[72]
		Colorectal cancer	$MEK1^{F129L}$	RO4927350 RO4987655	[74]
		Melanoma	$MEK1^{C121S}$	Vemurafenib AZD6244	[73]
		Melanoma	$MEK1^{Q60P}$	Dabrafenib Trametinib VRT-11E	[75]
		Melanoma	$MEK1^{V60E}$ $MEK1^{P124S}$ $MEK1^{G128V}$ $MEK1^{V154I}$ $MEK2^{V35M}$ $MEK2^{L46F}$ $MEK2^{N126D}$	Dabrafenib Trametinib VRT-11E GDC0941	[79]
		Melanoma	$MEK2^{C125S}$	Dabrafenib Trametinib	[109]
	Rare tumor-associated gain- and loss-of-function ERK mutations were discovered which induced variable responses to RAF and MEK inhibitors	Melanoma	Identified several ERK1 and ERK2 mutations	VX-11e SCH722984 Dabrafenib Trametinib	[9]
		Melanoma	$ERK2^{E81K}$ $ERK2^{S142L}$ $ERK2^{D321N/V}$ $ERK2^{E322K/V}$	VRT-11E SCH722984 Dabrafenib Trametinib	[8]

Mechanisms of resistance to RAF and MEK inhibitors are summarized in table 1 according to the different categories as in figure 1. These include: (I) activation of alternative pathways; (II) activation or overexpression of receptor tyrosine kinases (RTKs) and their ligands; (III) RAS activation; (IV) activation of MAP3Ks; and (V) activating mutation of MEK or ERK

5.2.1 Intrinsic Resistance

Although dysregulation of the ERK cascade is involved in the transformation of most cancers, the inhibitors that target the different components of the cascade are efficient almost exclusively in mutated BRAF cancers. Indeed, this is not the case when the driving oncogene is *RAS* instead of *BRAF*, in the rare occasion when mutated BRAF is combined with oncogenic RAS [58] or when cells are transformed by ERK-independent pathways [59]. Under these conditions, the addition of RAF inhibitor does not affect the activity of ERK or may even cause a deleterious effect as it can in fact lead to hyperactivation of ERK and enhanced cell proliferation through the paradoxical ERK activation. The molecular mechanism responsible for this effect lies in the dimerization of RAS-interacting RAFs. The activation of the catalytic activity of RAF is mediated by RAF homo- or hetero-dimerization upon RAS activation [60]. The RAF inhibitors target this dimer in a concentration-dependent manner. In wild type BRAF cells, transformed by oncogenic RAS or other oncogenes, ATP-competitive inhibitors actually promote RAF dimerization by stabilizing a rigid closed conformation of the kinase domain [61]. When applied at high-saturating concentrations, the inhibitors bind to both members of the dimer, blocking the kinase domains and effectively shutting down RAF signaling [47]. However, under non-saturating concentrations, inhibition is only partial since the inhibitors bind to only one member of the dimer, forming a new equilibrium. The RAF dimers are stabilized and RAF is transactivated, resulting in its strong hyper-activation that cannot be blocked by the various inhibitors. In cells expressing mutated BRAF, RAF inhibitors do not cause paradoxical activation, because all RAF isoforms exist predominantly as active monomers and do not require dimerization for their activation [51]. Thus, RAF inhibitors selectively bind and inhibit the monomeric active form of mutated BRAF, suppressing downstream ERK signaling.

As mentioned above, the RAF and MEK inhibitors do not affect cancers that are not "addicted" to the ERK cascade or contain WT BRAF. Additionally, some of the cancers, although bearing the BRAF mutation, are not sensitive to the drugs as well. Thus, despite their dependence on the ERK cascade for transformation, mutated BRAF lung and colon cancers as well as some of the mutated BRAF melanomas (~25%) show very little or no response to the RAF and MEK inhibitors [44, 62, 63]. This lack of effect of the drug on these ERK-addicted cancers is believed to be due to some sort of an escape mechanism from the inhibitor's blockade, which causes the intrinsic resistance. Interestingly, an expected resistance mechanism, which is a co-expression of downstream activating mutations (e.g. active MEK in the case of RAF inhibitors), was not identified in any of the studies. However, a large number of studies in the last five years demonstrated other mechanisms that induce this resistance.

One of the first mechanisms described for intrinsic resistance was the involvement of the tumor microenvironment that blocks the effect of vemurafenib on certain mutated BRAF melanomas [53, 54]. Using a co-culture system to systematically

assay the ability of stromal cells to influence the response of cancer cell lines to various anti-cancer drugs, it was shown that anti-cancer drugs that are capable of inducing apoptosis of tumor cells are frequently rendered ineffective when the tumor cells are cultured in the presence of stromal cells. Further proteomic analysis showed that it is mediated by the hepatocyte growth factor (HGF) secreted from the stromal cell, which operates via the MET receptor. This factor then induced sustained activation of the ERK and AKT pathways and immediate resistance to RAF inhibition. The HGF-induced resistance was greater under BRAF inhibition than MEK inhibition, and only the combination treatment with a MEK inhibitor and an AKT inhibitor suppressed the HGF-induced drug resistance to some extent. Dual inhibition of RAF and either HGF or MET resulted in the reversal of drug resistance, suggesting RAF plus HGF or MET inhibitory combination therapy as a potential therapeutic strategy for mutated BRAF melanomas [53]. This concept was recently expanded by demonstrating that targeted therapeutic inhibition of oncogenic drivers induces vast secretome changes which established a tumor microenvironment that supports the expansion of drug-resistant cancer cell clones, but is susceptible to combination therapy [54].

Apparently, HGF and MET are just one example, as there are other growth factors and receptors that may contribute to the intrinsic resistance as other hyperactivated receptors may induce this resistance mainly by stronger activation of the ERK cascade or by activating the parallel pathways. For example, the EGFR-mediated ERK cascade reactivation was significantly correlated with resistance to vemurafenib in BRAF-mutant colorectal cancers, and this resistance was overcome by combining RAF and EGFR inhibition that suppressed ERK activity [62]. Additionally, blockade of the EGFR showed strong synergy with RAF inhibition in melanoma as well [63]. Another study in that direction provided a rationale for combining ERK cascade antagonists with inhibitors of HER activity in BRAF-mutant thyroid cancer cells. Using screening approaches, it was shown that the increased expression and activation of HER2/HER3 signaling, due to increased ERBB3 transcription, induced intrinsic resistance to both RAF and MEK inhibitors [64]. Moreover, analysis of melanoma tumors from patients with intrinsic or acquired resistance to vemurafenib observed increased EGFR and SRC-family kinase (SFK) activity [65]. It was further shown that BRAF inhibitor-mediated activation of EGFR-SFK-STAT3 signaling is the mechanism that confers the resistance to the RAF and MEK inhibitors. Further progress of this idea has led to the development of two pan-RAF inhibitors that are capable of inhibiting SFKs as well. Importantly, it was shown that these drugs do not drive the paradoxical ERK activation and inhibit MEK and ERK activity in BRAF and NRAS mutant melanomas as well as in melanomas with resistance to BRAF or to combined BRAF/MEK inhibitors [66]. Taken together, overexpression of growth factors, their receptors and even PTKs such as the SFKs may evade the inhibition of ERK signaling by the cascade's inhibitors. This may be mediated either by stronger reactivation of the ERK cascade that can't be overcome by the RAF/MEK inhibitors or by inducing alternative pathways such as AKT.

An additional intrinsic mechanism involves the expression and activity of the WNT signaling in promoting the resistance of melanoma cells to BRAF inhibitors. It was found that the WNT5A protein and transcript levels were dramatically increased in BRAF inhibitor-resistant cell lines as well as in patient tumors. Moreover, WNT5A activated PI3K/AKT signaling and promoted melanoma growth and survival via its receptors RYK and FZD7 [67]. Two principal mechanisms of resistance were also discovered using mutated BRAF non-small cell lung cancers (NSCLCs; [56]). In this cancer, specifically the BRAF(V600E) mutation, but not other BRAF mutations, is initially sensitive to RAF-inhibitor treatment, but rapidly acquires resistance. This switch probably occurs due to changes of the expression of the full-length BRAF(V600E) to a shorter alternative spliced isoform of the protein that is much less sensitive to the drug. It was also shown that in some other lung cancer patients the resistance may occur due to constitutive autocrine EGF receptor (EGFR) signaling that overcomes the sensitivity to the drug and causes the hyper-phosphorylation of AKT.

5.2.2 Acquired Resistance

The observation that resistance is developed relatively quickly (usually within ~6 months) after the initiation of therapy with RAF and MEK inhibitors in mutated BRAF melanomas, has initiated many studies aiming at revealing the specific mechanism for the emerged acquired resistance. It is well accepted today that resistance is mediated by several mechanisms [14, 68–70], which can each operate either as a single cause, or in various combinations. As mentioned above, acquired mechanisms may be mediated by preexisting mechanisms, which exist in a fraction of the tumor cells that become predominant after the elimination of the drug sensitive tumor cells, by drug-induced reduction of negative feedback loops, or by drug-induced resistance that affects a large number of cells within the tumors. It is believed that most of the resistance mechanisms belongs to the preexisting mutations, and therefore, are similar in principle to the mechanisms described for the intrinsic resistance. However, not enough information is available on the direct effects of the drugs on the tumor. In this section below, we describe the studies that led to the identification of acquired resistance mechanisms.

One of the first studies in this direction showed that similarly to the intrinsic resistance, a subset of the resistant cells is expressing a spliced variant form of BRAF(V600E). Alternative splicing of exons 4–10 of this mutant generated a short form of a 61 kDa protein lacking the RAS-binding domain [71]. This p61BRAF(V600E) shows enhanced dimerization with low levels of RAS activation, which turns ERK signaling resistant to the RAF inhibitor. According to this study, splicing isoforms that dimerize in a RAS-independent manner can be responsible for the resistance of about 1/3 of the relapsed patients [71]. A functional genomic approach to study resistance mechanisms to RAF inhibitors identified COT (also known as MAP3K8 or Tpl2) that activates ERK primarily through MEK-

dependent mechanisms without the requirement of RAF signaling. Increased COT mRNA expression was observed in vemurafenib-resistant mutated BRAF melanoma patients, and depletion of COT expression in BRAF-mutated colon and melanoma cancer cell lines sensitized them to the RAF inhibitor PLX4720 treatment [50]. This finding actually provided a novel general mechanism for acquired resistance through direct activation of MEK by RAF-independent upstream components. Interestingly, this MEK activation can be achieved by other means, such as mutations within MEKs themselves. Although mutations within MEK are generally much less frequent as compared with those of RAS or RAF, activating mutations of MEK1 (Q56P, C121S, P124L, and F129 L) have been identified as responsible for the acquired resistance in mutated BRAF melanomas [72–74]. These different activating MEK1 mutations, which are similar to oncogenic MEK1 mutations, increased the intrinsic kinase activity of MEK, thereby enhancing ERK phosphorylation. A more recent study [75] used whole exome sequencing and whole transcriptome sequencing (RNA-seq) on drug-resistant tumors from five patients with acquired resistance to dabrafenib and trametinib treatments. This study identified a novel MEK2 activating mutation, MEK2Q60P, that may be responsible for the resistance, indicating for the first time that MEK2 can be involved in the resistance mechanism as much as MEK1.

Another mechanism that is involved in the induction of acquired resistance may arise from the amplifications of upstream components of the ERK cascade, similar to the mechanisms discussed above regarding the intrinsic resistance. These include elevation of expression or activation of growth factors, growth factor receptor, NRAS and even RAF itself. Using a whole genome sequencing of vemurafenib-resistant mutated BRAF melanomas, it was shown that acquired resistance is developed by mutually exclusive platelet derived growth factor receptor beta (PDGFRβ) upregulation or NRAS mutations. However, the induction of PDGFRβ RNA, protein expression and tyrosine phosphorylation of the receptor emerged as the main cause for the acquired vemurafenib resistance, as demonstrated in melanoma cell lines and patient-derived biopsies. PDGFRβ-upregulated tumor cells had low activated RAS levels and, when treated with PLX4032, did not reactivate the ERK cascade significantly. Additionally, other tumor cells with high levels of activated mutated NRAS, which were treated with PLX4032, significantly elevated ERK activity [49]. This mechanism was later supported by the demonstration of overexpression of other growth factor receptors, such as ERBB3 or insulin-like growth factor 1 receptor (IGF1R) that induce the activity of AKT and other ERK-independent pathways [76, 77]. A recent study analyzed melanoma tumors that developed resistance to BRAF or MEK inhibitors and revealed acquired EGFR expression in about 45% of the tumors [78]. The use of shRNA library revealed that suppression of the sex determining region Y-box 10 (SOX10) in melanoma causes activation of TGFβ signaling, leading to upregulation of EGFR and PDGFRβ, which confer resistance to BRAF and MEK inhibitors.

Expression alterations in other ERK cascade-related signaling molecules were reported as a cause for resistance as well. Thus, it was shown that an acquired elevated level of NRAS might lead to significant ERK reactivation and resistance [49].

Loss of expression of NF1 [79, 80], amplification of BRAF [81] and elevation of CRAF [82] or MEK1 activity [79] were also implicated in this process. Several other signaling components, which were identified by whole genome screens, mediate drug resistance as well. A large-scale RNAi screen identified the MED12 component of the transcriptional MEDIATOR complex as a critical determinant of drug response to tyrosine kinase inhibitors. MED12 suppression confers resistance to MEK and BRAF inhibitors via TGF-β signaling [83]. Another genome-wide RNAi screen implicated the loss of NF1 mentioned above in resistance to RAF inhibition through induction of the AKT pathway. A genetic screen in BRAF-mutant tumor cells showed that the hippo pathway effector YAP (encoded by YAP1) acts as a key mediator of resistance to RAF and MEK inhibitors by promoting cell survival [84]. Combined YAP and RAF or MEK inhibitions was synthetically lethal not only in several BRAF-mutant tumor types but also in RAS-mutant tumors. Finally, a genome-scale CRISPR-Cas9 knockout screen in human cells verified the involvement of NF1 and MED12 and also implicated the novel genes: *NF2, CUL3, TADA2B,* and *TADA1* in the resistance to RAF inhibitors, although their mechanisms of action are not fully understood [85]. These various resistance mechanisms were identified both in vitro, using specific drug resistance clones, as well as in various in vivo mouse models and specimens from patients who developed resistance to vemurafenib treatment.

ERK signaling and activation is an outcome of a complex network, which must be precisely controlled. One of the hallmarks of this regulation is the induction of negative feedback loops that result in ERK-mediated control of its own activity. Several resistance mechanisms have evolved due to relief of the ERK-induced feedback inhibition of mitogenic signaling by the RAF inhibitors [55]. Feedback loops can be totally dependent on a direct phosphorylation by ERK (e.g. inhibitory phosphorylation of SOS1, [86] or may be dependent on ERK-induced transcription (e.g. the dual specificity protein phosphatases (DUSPs) [87, 88] and the cytoplasmic regulator of the cascade, sprouty (SPRY) [89]). Almost every tier of the ERK cascade is targeted by negative feedback phosphorylation. Thus, under normal conditions, the extracellular stimulation of the ERK cascade initiates activation of immediate feedback loops that balance the activity of the cascade by reducing the expression or activity of upstream components. Elevated feedback regulation by overexpression of DUSPs have been reported in various cancers, resulting in moderating the intensity of ERK signaling in these cancers, thereby allowing their prolonged survival [88]. Modulations of these negative feedback loops in cancer are therefore expected to induce hyperactivation of the upstream components of the cascade. Thereby, this reduced negative activity induced alternative mitogenic/survival signaling that is not usually observed in non-treated BRAF mutated cancers. Indeed, it was reported that RAF inhibitors potently inhibit ERK signaling in mutated BRAF melanomas, causing a relief of ERK-dependent feedback and thereby reactivation of ERK activity as well as hyperactivation of AKT [55, 68]. Hence, the elimination of the negative feedback loops may serve as a mechanism of acquired resistance to the RAF/MEK drugs. Interestingly, it was also shown that RAF inhibitors can induce acquired resistance by relieving inhibitory autophos-

phorylation of the RAF themselves [90]. Thus, one way to solve this attenuated feedback inhibition was to combine RAF and MEK inhibitors that are supposed to overcome the lack of feedback at the RAF-MEK level. Indeed, this combination resulted in a prolonged duration of the combined drugs as the relapse for this combined regime occurred 10–12 months after initiation of the treatment [13].

The various resistance mechanisms to RAF inhibitors described up to now demonstrate high complexity levels. However, other inherent conditions may pose difficulties during treatment and resistance development. Melanoma genomic heterogeneity contributes significantly to acquired BRAF inhibitor resistance and therefore treatment failure [59]. By analyzing patients' tumor samples along with whole exome sequencing, 70% of disease progressive tissues demonstrated reactivation of ERK in mechanisms including NRAS mutations (18%), mutant BRAF amplification (19%), alternative BRAF splice variants (13%) and MAP2K1 mutations (3%). Additionally, they also detected PI3K-PTEN-AKT-upregulating genetic alterations among 22% of progressive resistant melanomas [91]. The complexity grows one step further as heterogeneity of resistance is present not only among different patients but rather within the same individual along the different stages of the treatment. A few reports have provided evidence that distinct molecular mechanisms of acquired resistance to RAF inhibition can be present within the same tumor or among multiple tumors from the same patient. Indeed, Shi et al., reported that nine different samples taken from the same patient progressing tumor over the period of BRAF inhibitor therapy demonstrated five distinct drivers of acquired BRAF inhibitors resistance, including KRAS mutation, BRAF alternative splicing and amplification [91]. Additional support for this concept came in another study demonstrating the coexistence within the same patient of different genetic alterations at metastatic sites leading to disease progression [92].

One question that has raised some interest in the past several years is whether the mechanisms of resistance to the RAF inhibitors are similar to those mechanisms that confer the resistance to MEK inhibitors. As described above, MEK inhibitors are as efficient and sometimes even demonstrate a better effect than the RAF inhibitors. Importantly, treatment with both types of drugs may result in acquired resistance usually after only several months of treatment. Most of the alterations in both cases converge into reactivation of the ERK cascade, specifically in tumors with strong dependency on ERK signaling for their growth. In many cases, the mechanism of resistance to MEK inhibitors is similar in principle to those of RAF inhibitors. These include the amplification of the *BRAF* gene and elevated BRAF expression with no evidence of acquired mutation in MEK1 or MEK2 [93, 94]. Other studies, previously mentioned above, show that the resistance is due to upregulation of MEK mutations such as MEK1F129L [74] and MEK2Q60P [75]. The resistance to MEK inhibitors is also proposed to occur due to amplification of the oncogenic proteins BRAFV600E or KRASG13D, which results in increased signaling through the ERK pathway [94]. Amplifications of these oncogenic proteins occur unrelated to MEK mutations and under certain conditions (such as up regulation of KRASG13D), can also activate other KRAS affected pathways such as the PI3K-PKB one [69]. Elevation of pAKT was also demonstrated in clinical mela-

noma biopsies from patients receiving BRAF inhibitor monotherapy or when combined with a MEK inhibitor [59].

Another mechanism of resistance was recently identified using single-cell analysis and molecular profiling [57]. This study demonstrated that the cells in each ERK cascade inhibitor-treated tumor exhibit a heterogeneous response. Where some of the cells are sensitive to the drug, others are less responsive to the treatment and some may adapt to the drug and survive. Interestingly, similarly to other stresses [52], the authors showed that the drug-induced resistance/adaptation of the cells results from up-regulation of several proteins including markers of the neural crest (e.g., NGFR) and the melanocyte precursor. This effect is transient, as it can revert to the original state within nine days of drug withdrawal. This transient expression seems to be regulated by the c-Jun/ECM/FAK/Src cascade, which is activated by the drugs in about one-third of the cell lines. Drugs targeting the components of this new cascade increase the maximum effect of RAF/MEK kinase inhibitors by promoting apoptosis. This study clearly shows a genuine mechanism of drug-induced resistance, which leads to acquired resistance that unlike many of the mechanisms discussed above is reversible upon drug withdrawal. In addition, this study provides compelling evidence that cells within a tumor may be heterogeneous in their response and resistance development. Thus, distinct cells in the same tumor may contain more than one of the mechanisms described above of pre-existing or drug-induced resistance, suggesting the advantage of using drug combinations in the treatment of mutated BRAF melanomas.

5.3 Conclusions and Future Directions

Over the past two decades, a tremendous effort has been made to design effective treatment strategies aiming to reduce ERK activation commonly seen in many cancers. This has given rise to several clinically approved therapeutic drugs, which had a substantially positive impact, mainly on mutated BRAF melanoma patients. However, as effects are short lived and resistance emerged quite rapidly, the search for potent selective small molecules specifically targeting the ERK cascade, to which the cells of these patients are usually addicted for their survival, is still ongoing. The high complexity level of the ERK cascade, along with the different mechanisms by which resistance emerges or preexists, is a challenge for targeted therapies. One strategy to overcome this gave rise to treatment with a combination of therapeutic approaches in order to increase durability of patient's response. This approach may include the combination of chemotherapy with an inhibitor or the combination of inhibitors, which tackle the major oncogene together with the upregulated component of which it activates or the combination of inhibitors simultaneously co-targeting multiple molecular targets of the cascade. Indeed, combining the MEK inhibitor, trametinib, and the RAF inhibitor dabrafenib, showed improved pharmacokinetics, safety and anti-tumor activity when administered to mutated BRAF melanoma patients [13]. Other successful attempts to combine MEK and RAF

inhibitors include combining the MEK inhibitor, cobimetanib, and the RAF inhibitor, vemurafenib, as well as combining MEK162 and LG818 for the treatment of advanced mutated BRAF melanomas [27]. Additionally, the combination of BRAF inhibitors and immunotherapy may be used to provide prolonged responses for metastatic melanoma. Recent clinical studies investigating multi-targeted combinations on melanoma patients are summarized elsewhere [95, 96]. Future developments in this direction and novel drug combinations may delay the onset of resistance and improve drugs efficacy.

Another direction that is currently being pursued is the development of new types of drugs that are not based on the inhibition of the kinase activity. These include interfering with protein interaction of the various signaling proteins as we recently demonstrated for the interaction of ERK with importin7 (Imp7) [97]. Inhibiting this interaction prevented ERK nuclear activity. The stimulated nuclear translocation of signaling proteins is a necessity for many of their functions [98–100], which is particularly important for inducing ERK-dependent proliferation [101]. We have previously demonstrated that the ERK nuclear shuttling machinery is mediated by interaction with the β-like importin 7, through a specific nuclear translocation signal (NTS) in the kinase insert domain of ERK [102, 103]. Based on that, we challenged the concept of whether prevention of ERK interaction with Imp7 should inhibit proliferation without affecting ERK cytosolic functions including the induction of negative feedback loops. For that purpose, we developed an NTS-derived phosphomimetic peptide (EPE peptide) conjugated to myristic acid which was able to block ERK nuclear translocation by inhibiting its interaction with Imp7 [97]. This peptide inhibited the growth of several transformed breast and colon cancers xenografts and completely eradicated the growth of mutated BRAF melanoma tumors in SCID mice without any detectable reoccurrence. Moreover, the EPE peptide was significantly more effective when compared to the clinically used vemurafenib treatment [97]. This proof of concept can lead to the development of drugs that block ERK nuclear translocation, thereby eliminating cancer growth with fewer side effects, while remaining protected against resistance.

In a similar manner, another effort to inhibit ERK, not by the conventional inhibition of its catalytic activity, focused on the prevention of ERK dimer formation [104]. The regulatory protein-protein interaction of ERK as a potential target for antitumor drugs was previously demonstrated as impeding ERK dimerization prevented tumor progression in cell lines harboring oncogenic KRAS [105]. In the search for small molecules capable of preventing ERK dimerization, DEL-22379 was found to inhibit ERK dimerization without affecting its phosphorylation, to block tumor cells proliferation and prevent tumor formation in animal models [104]. Recent efforts to develop better efficacy inhibitors with a wider spectrum of action, which evade paradoxical ERK cascade activation, were recently reported. Next-generation RAF inhibitors, PLX7904 and PLX8394, were shown to suppress mutant BRAF cells without activating the ERK cascade in cells bearing upstream activation [106]. The paradox-breaking pan-RAF inhibitors, CCT196969 and CCT241161, inhibit melanoma cells and patient-derived xenografts that are resistant to BRAF and BRAF/MEK inhibitors. Importantly, these inhibitors where able to inhibit EGFR and SRC-family kinase

(SFK) activities, which are often observed in vemurafenib-resistant melanoma tumors [66]. Although these developments are just in their initial stages and still require further clinical evaluation, they have the potential to result in the development of drugs with improved safety and durable efficacy that cause less side-effects than the ones currently used and which are unaffected by resistance mechanisms.

Acknowledgments This review was supported in part by ISF grant 625/15.

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Chapter 6
Mechanisms of Resistance to PI3K and AKT Inhibitors

Pau Castel and Maurizio Scaltriti

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 PI3Kα 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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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_6

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
PDK1	3-Phosphoinositide Dependent Protein Kinase-1
PGC-1	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1
PIF	PDK1-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	p110α p110β p110δ	p85α/β, p55α, p50α and p55γ	PIP2	Angiogenesis, cell growth, metabolism, motility, transformation, immune cell biology, etc.
Ib	p110γ	p101 and p87	PIP2	
II	PI3K-C2α PI3K-C2β PI3K-C2γ		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 PI3Kα in complex with the regulatory subunit p85α 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 p110β and p110δ 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 PI3Kδ 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

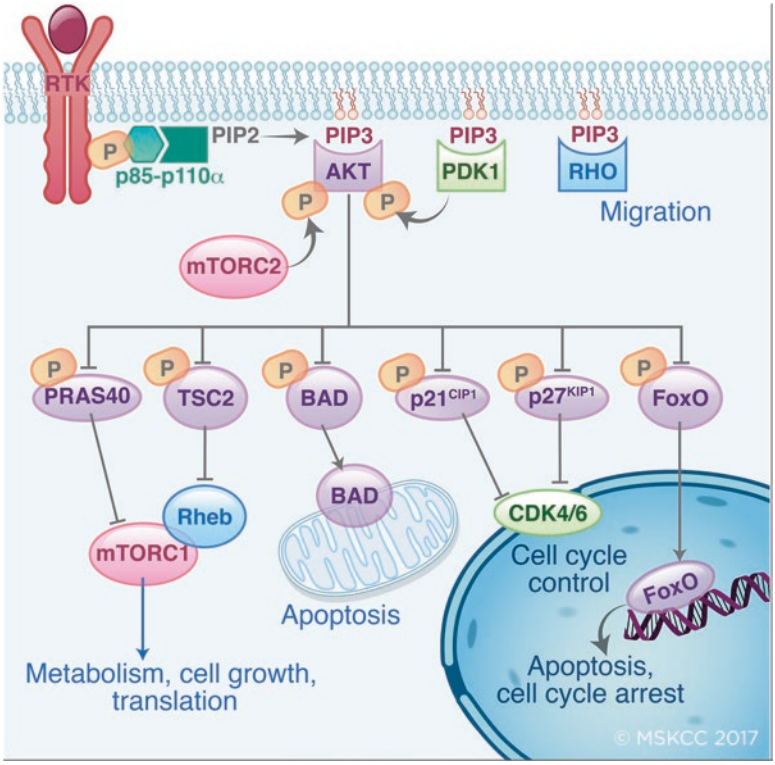

Fig. 6.1 The PI3K/AKT pathway. Activation of the PI3K/AKT pathway is the result of several stimuli. However, it is commonly associated to RTKs activation through the binding of the regulatory subunit p85 to RTK phosphorylated residues. The lipid kinase activity of the catalytic subunit of PI3K (p110) 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 *Pdpk1* (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 RXRXX(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 phopshorylation 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 p110α 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 p110α 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 of 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 antitumor 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 p110α are BYL719 (Novartis Pharmaceuticals [63]) and GDC-0032 (Genentech Inc. [64]). The latter also targets p110γδ and is more potent against the H1047R and E545K p110α 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 p110β or p110δ 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

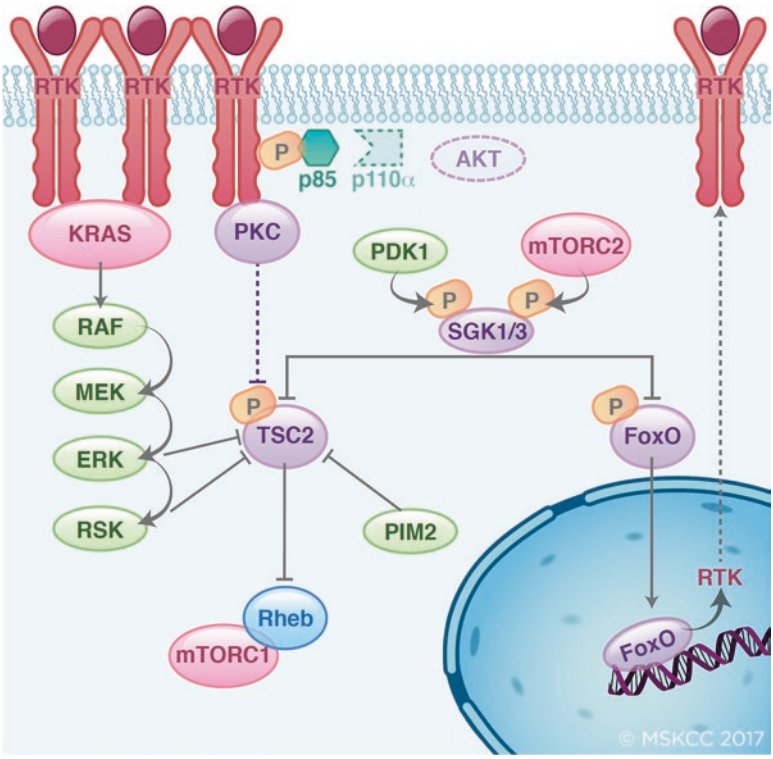

Fig. 6.2 Mechanisms of resistance mediated by kinases. Upon inhibition of PI3K or AKT the pro-survival mechanisms of the cancer cell are challenged, leading in multiple cases to cell death or cell cycle arrest. However, cancer cells can overcome these pharmacological stresses by relying on parallel signaling 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 PI3Kα have been shown to be more effective in malignancies harboring mutations in *PIK3CA* [65–67], while inhibitors targeting the PI3Kδ 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 PI3Kα 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 p110β isoform, since both inhibition and knockdown of such isoform reduce the levels of PIP3 [93]. Moreover, the combination of p110α and p110β 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, p110β 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 p110α and p110β isoforms [95].

There is high prevalence of PTEN loss in prostate cancer, hence treatment with PI3K p110β inhibitors has been considered as a possible therapeutic strategy. However, there are compensatory mechanisms as a result of increased p110α signaling. In LNCAP prostate cancer cells, the increased p110α signaling is mediated by IGFR1 [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 p110α inhibitors identified in the metastatic lesions from a patient that relapsed to the treatment with the p110α 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 p110β 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 p110α 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 RXRXX(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 PI3Kα 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 (PKCα, βI,βII, and γ), novel (PKCδ, ε, θ, and η), and atypical (PKCζ, 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 PI3Kα 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 p110α 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/demethyases 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 p110β 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].

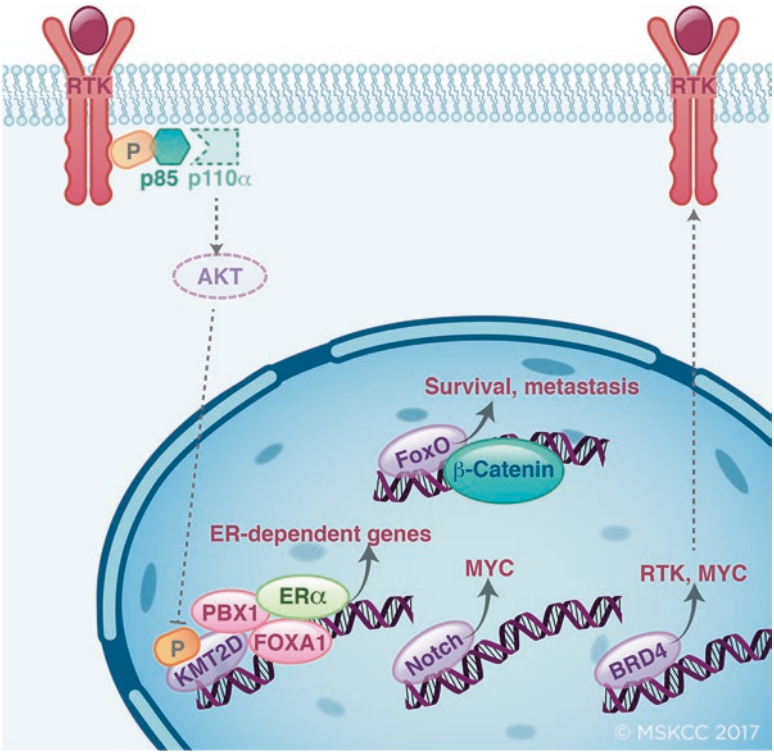

Fig. 6.3 Epigenetic mechanisms of resistance to PI3K/AKT inhibitors. Resistance to PI3K and AKT inhibitors can also be regulated transcriptionally by several transcription factors. Hormone receptors, such as ER and AR, have been shown to drive resistance to PI3K/AKT inhibitors in breast and prostate 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.

Acknowledgments We would like to thank the Breast Cancer Research Foundation and the Geoffrey Beene Cancer Research Center. Pau Castel is a Fellow of the Jane Coffin Childs Memorial Fund. We apologize for the impossibility to cite every author who has contributed to this field of inquiry.

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 7
Sensitivity and Resistance to BH3 Mimetics in Cancer Therapy

Konstantinos V. Floros, Anthony C. Faber, and Hisashi Harada

Abstract Targeted molecular agents have revolutionized cancer care in the adult population. Many of these drugs have been inhibitors of kinases. BCL-2 family members have long been understood to play key roles in mitochondrial integrity, serving as the key signaling nexus between kinase cascade-driven growth and survival signals, and they can also be found genetically altered in human cancers (e.g. IgG-*BCL-2* translocations in follicular lymphoma). Indeed, the FDA-approval of the BCL-2 homology (BH)3 domain mimetic, venetoclax (AbbVie), is the first clinically approved BCL-2 family member targeted therapy of any kind, bringing BCL-2 family member inhibitors into the spotlight. This chapter will highlight the current state of affairs of this exciting time for BCL-2 family member targeted therapies, by focusing on three most advanced types of BCL-2 family inhibitors: the BCL-2 BH3 mimetic, venetoclax; the dual BCL-2/BCL-xL BH3 mimetic, navitoclax; and the recently developed MCL-1 BH3 mimetics. We will also discuss resistant mechanisms that have emerged from the intensification of preclinical and clinical studies of these compounds. The challenges understanding which cancers may most benefit from BH3 mimetics will also be discussed, as will the emergence of BH3 profling to address these challenges. Finally, we will discuss how these drugs may be combined with other currently available drugs to overcome resistance and induce robust clinical responses.

Keywords BH3 mimetics • Resistance • Targeted therapies • Apoptosis • Venetoclax • Navitoclax

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_7

Abbreviations

ALK	Anaplastic lymphoma kinase
BAX	BCL-2 associated X protein
BCL-2	B cell-lymphoma 2
BH3	BCL-2 homology 3
BIM	BCL-2 interacting mediator of cell death
BRAF	V-Raf murine sarcoma viral oncogene homolog B
EGFR	Epidermal growth factor receptor
HER2	Proto-oncogene Neu
MCL-1	Myeloid cell leukemia-1
MEK	Mitogen-activated protein kinase kinase
MOMP	Mitochondrial Outer Membrane Permeabilization
mTOR	Mechanistic target of repamycin
MYCN	V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
PI3K	Phosphoinositide 3-kinase
PUMA	p53 upregulated modulator of apoptosis

7.1 Introduction to BCL-2 Family Members

The mitochondria are ancient organelles, with the endosymbiotic theory postulating roughly 1.5 billion years ago mitochondria were derived from proteobacteria. As such, it should be no surprise that the mitochondria are the prime location for energy transduction, where the major currency is ATP. It therefore should also not be surprising that life and death decisions are centralized at the mitochondria, where the cell can in essence be "short-circuited" if the mitochondria are compromised [1].

To appropriately protect and compromise the mitochondria, stand the BCL-2 family of proteins: a highly homologous and conserved group charged with maintaining the integrity of the mitochondria. In healthy cells, these proteins are instructed by signaling cascades that often originate on the plasma membrane, therefore balancing growth and survival signaling with anti-proliferative and anti-survival signaling. To do so, in humans, the BCL-2 family of proteins has evolved to include three main groups: (1) anti-apoptotic members (e.g., MCL-1, BCL-2, BCL-xL) (2) BH3-only pro-apoptotic members (e.g. BIM, PUMA, NOXA) and (3) terminal pro-apoptotic members (e.g. BAK and BAX). BH3-only members cause cytochrome c release from the mitochondria by activating and oligomerizing BAX and/or BAK, while the anti-apoptotic BCL-2 family of proteins prevents this process (Fig. 7.1). Underlying the important interplay of apoptosis and proliferation, most of the anti-apoptotic and pro-apoptotic BCL-2 family members by themselves are

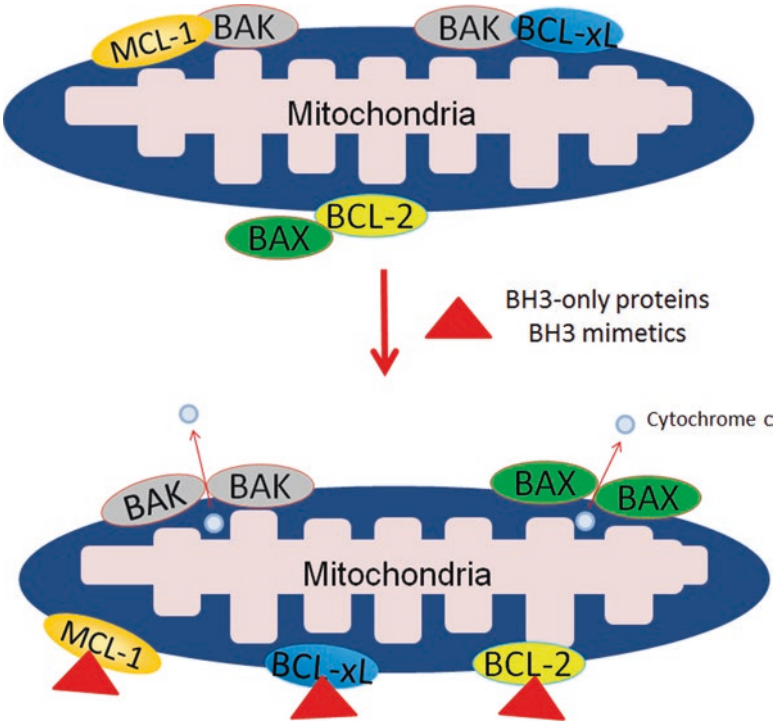

Fig. 7.1 Schematic representation of how BH3-only proteins or BH3 mimetics induce apoptosis. The effector members BAX and BAK are inactivated by the anti-apoptotic members. When the BH3-only proteins are induced or the cells are treated with BH3 mimetics, they bind to the anti-apoptotic members to activate BAX and BAK. BAX and BAK are then oligomerized, resulting in the release of cytochrome c from the mitochondria

weakly oncogenic and tumor suppressive, respectively. However, when combined with a genetic event that confers a proliferative gain in the cell—such has been demonstrated in a mouse model with combined BCL-2 and MYC overexpression—the result is a highly malignant cancer [2].

In diseased cells, the BCL-2 family proteins are altered in their expression, function, subcellular localization and/or protein-protein interaction. In cancer, these alterations often lead to a dominant anti-apoptotic signal. In fact, this is one of the hallmarks of cancer [3].

How the imbalance of BCL-2 family proteins comes about is diverse, nuanced and complex. Sometimes, it is underlined by an overt genomic alteration (e.g. *BCL-2* translocations leading to a gain-of-function anti-apoptotic signal, or *BIM* polymorphisms leading to a loss-of-function apoptotic signal); often, however, it is underlined by more subtle changes, many of which remain poorly understood. Altogether, these processes that hinder cancer cells from dying can be thought as "apoptotic blocks" [4].

7.2 Models Describing the Induction of Apoptosis

Before we consider specific apoptotic blocks, it is important to understand how the BCL-2 family proteins interact as a group. There are three primary models that describe how the interactions between the BCL-2 family members control the apoptotic cascade. In the direct activation model, the pro-apoptotic BH3-only proteins are divided into activators (BIM, tBID and perhaps PUMA that have high affinities for all anti-apoptotic BCL-2 members) and sensitizers (BAD and BMF that bind to BCL-2, BCL-xL and BCL-w, NOXA that binds to MCL-1 and A1, and BIK and HRK that bind to BCL-xL, BCL-w and A1) [5]. The effector molecules BAX and BAK require the input of a BH3-only activator to translocate into the mitochondria (in the case of BAX), oligomerize, and induce permeabilization of the outer mitochondrial membrane, called MOMP, which ultimately leads to apoptosis. In healthy cells, the BH3-only proteins stay inert or are sequestered by the anti-apoptotic proteins. Following the induction of death stimuli, the activators are induced (transcriptionally or post-translationally) or displaced from the anti-apoptotic proteins by the BH3-only sensitizers and finally engage BAX and BAK triggering apoptosis [6, 7]. In the direct activation model, the displacement of BAX and BAK from the pro-survival BCL-2 family proteins by itself is not sufficient to lead to their activation.

The indirect activation model focuses on the interaction between BAX/BAK and the pro-survival BCL-2 proteins: this model stresses that BAX/BAK become capable of permeabilizing the outer mitochondrial membrane only if all the anti-apoptotic BCL-2 family proteins are neutralized and, thus, BAX and BAK liberated [8–10]. Recently, Luo and colleagues reported that BAX and BAK can be spontaneously activated after neutralization of the anti-apoptotic proteins BCL-xL and MCL-1, respectively, supporting the main principles of the indirect activation model [11].

Most likely, however, both models are correct insofar as a unified model has emerged, combining the experimental observations that led to both the direct and indirect models [12]. This model includes the requirements of the anti-apoptotic proteins to sequester the BH3-only proteins that can activate BAX and BAK, as well as the displacement of the effector molecules from the pro-survival proteins to execute apoptosis. These models are depicted in Fig. 7.2.

7.3 Apoptotic Blocks

The inability of cancer cells to undergo apoptosis is a central component of the neoplastic process. Apoptotic blocks emerge, often manifesting as over-activation of a prominent anti-apoptotic BCL-2 family member or under-activation of a prominent pro-apoptotic BCL-2 family member [4]. The phenotypic consequences of these apoptotic blocks have been demonstrated elegantly in mouse models. For instance, the deletion of BIM or PUMA in both *HER2*-amplified breast cancer and *EGFR* mutant lung cancer prevents tumor regressions following HER2 and EGFR

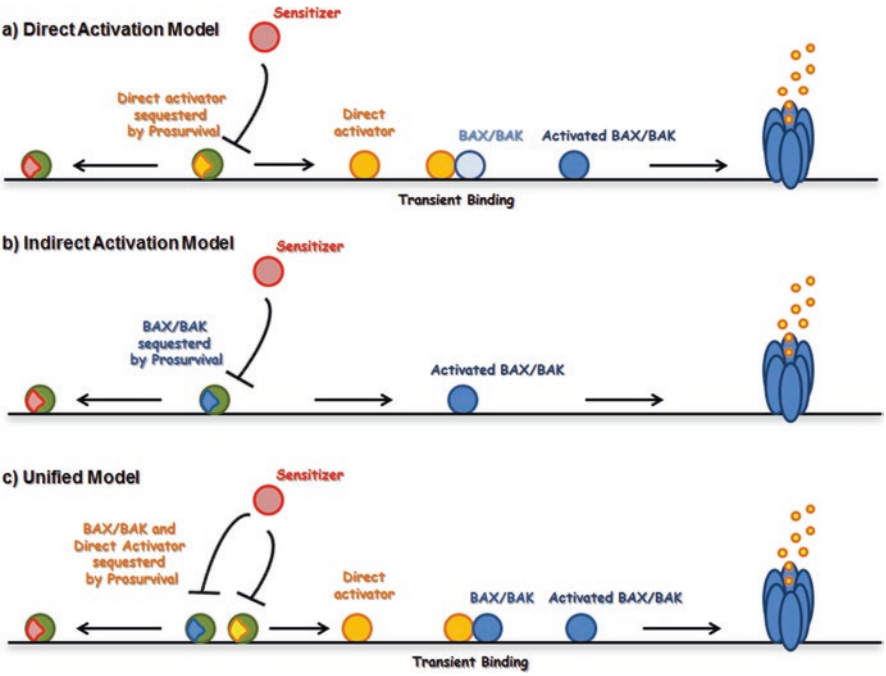

Fig. 7.2 BAX/BAK activation models. (**a**) In the **direct activation model**, the direct activator (BIM/tBID) is initially sequestered by one of the pro-survival proteins (BCL-2, BCL-xL, MCL-1) and needs to get freed by a sensitizer/de-repressor (the remaining BH3-only proteins) in order to activate BAX or BAK and induce mitochondrial outer membrane permeabilization (MOMP). Once a sensitizer binds to the anti-apoptotic BCL-2 family member with a higher affinity, the activator is released and able to promote the appropriate conformational changes in BAX and BAK that are necessary to result in their oligomerization, pore formation in the outer mitochondrial membrane (MOM) and cytochrome c release. (**b**) In the **indirect activation model**, BAX and BAK are in advance activated, but sequestered by the anti-apoptotic proteins through interaction between their already exposed BH3 domains and the hydrophobic grooves of BCL-2, BCL-xL and MCL-1. Their displacement is supposed to be sufficient to trigger apoptosis. (**c**) Since the previous two models result in paradoxical observations regarding the outer mitochondrial membrane permeabilization, a third **unified model** has become more widely accepted. In this model, BH3-only proteins like BAD, BMF and NOXA can de-repress both the activators and the effector proteins. The function of the sensitizers is critical since in some cases the already activated effectors are capable, once liberated, to induce the appropriate changes that result in the execution of apoptosis. The displacement of the direct activators is also important and additionally contributes to the activation of BAX and BAK

inactivation, respectively [13]. In the *Eμ-Myc* B-cell lymphoma mouse model, the heterozygous expression of *BIM* results in accelerated Myc-induced tumorigenesis [14]. In mouse models of acute myeloid leukemia (AML), deletion of MCL-1 results in cure [15].

Clinically, apoptotic blocks have also been demonstrated. For instance, low levels of functional BIM in *EGFR* mutant lung cancer patients [16–20] and *BCR-ABL* translocated chronic myeloid leukemia (CML) [17], as well as *ALK* translocated

lung cancers [21], lead to mitigated efficacy of targeted therapies to induce progression free survival in these patients. Apoptotic blocks have also been demonstrated outside of kinase inhibitors. Immunotoxins—monoclonal antibodies targeting cell surface markers, conjugated to a protein toxin—some of which are used to treat refractory hairy cell leukemia [22], are less effective in patients with low levels of BIM [21]. In advanced gastric cancers, lower levels of BIM confer resistance to multiple chemotherapeutic regimens, leading to poorer overall survival [23].

Given the imperative role of growth factors and BCL-2 family members in cancer development, progression, and therapeutic resistance, it is not surprising that growth factor pathways can in themselves create these apoptotic blocks. Perhaps the best well-studied effect is hyperactivation of the MAPK pathway, which can be conferred by mutant *EGFR* [24, 25], *BRAF* mutant colorectal cancer [26], *HER2* amplified breast cancer [25], *KRAS* mutant lung cancer [27], *BRAF* mutant melanoma [28], and *ALK* translocated lung cancer [29], which in all these models leads to suppression of BIM [25, 27, 29, 30]. Additionally, mutant *PIK3CA* in breast cancer leads to TORC1-mediated translation of the anti-apoptotic and oncogenic MCL-1 [31].

7.4 BH3 Profiling

Among the BCL-2 family proteins, the interactions show differential binding patterns. For example, a BH3-only member, BAD, strongly binds to BCL-2 and BCL-xL, but not MCL-1. In contrast, NOXA has a high affinity only to MCL-1. While BIM, BID, and PUMA interact with all the BCL-2 anti-apoptotic members (reviewed in [32]). Although cancer cells often overexpress anti-apoptotic proteins, these cells are often initially chemosensitive. Examples are chronic lymphocytic leukemia (CLL) and small cell lung cancer (SCLC). Large subsets of these cancer cells overexpress BCL-2, which sequesters pro-apoptotic BIM to prevent BAX/BAK activation and apoptosis. Therefore, these cells are dependent on BCL-2 for survival, i.e. addicted to BCL-2.

In order to better identify and quantify the dependence of different cancers on BCL-2 family members, BH3 profiling has been developed by Letai and colleagues [4, 33–35]. Its utility has grown over the past 10 years and has demonstrated a lot of promise as a companion test for patients diagnosed with cancer. It works, in simple terms, when known concentrations of BH3 peptides are contacted to the mitochondria, allowing for the permeabilization of mitochondrial membrane to be monitored by cytochrome c release; these readouts can then be correlated with response to certain treatments in cancer. BH3 profiling is useful to identify the cellular dependence on individual anti-apoptotic proteins, particularly BCL-2, MCL-1 and BCL-xL [4, 34], and the earliest studies demonstrated its ability to predict responses to BH3 mimetics [4, 34]. Subsequent studies have shown that BH3 profiling can predict cellular responses and clinical patient responses to other toxic stimuli such as chemotherapy [36].

More recent endeavors have demonstrated a further utility of BH3 profiling. For example, BH3 profiling helped demonstrate (1) why *KRAS* mutant lung cancers are refractory to targeted therapy-induced apoptosis [37], (2) a general refractoriness to anti-apoptotic members in several normal tissues [38] (and indirectly expanding the expected therapeutic window of BH3 mimetics for cancer treatment), and (3) a possible use to identify subsets of cancer cells that are more or less vulnerable to apoptosis, an important tool in the backdraft of tumor heterogeneity [39].

An even more intriguing tool has recently been debuted, known as "dynamic BH3 profiling" [40]. This technique, which has the ability to detect alterations in the apoptosis-primed state prior to and following cancer therapy by performing *ex vivo* assessments in the two states, appears both feasible and successful at predicting anti-cancer therapies across a number of paradigms [41]. Given the importance of modification of BCL-2 family proteins by kinase inhibitors for drug response (please see below), the dynamic profiling in parallel with early patient treatments appears to be an exciting and immensely promising technique to understand early how patients will be expected to respond to different anti-cancer therapies, thus providing the opportunity to make early changes in their treatment regimens. For a more thorough review on the current state of BH3 profiling, please see review [42].

7.5 Evidence that Apoptosis is Critical in Targeted Therapy Responses

The importance of a robust apoptotic response following kinase inhibitor therapy has been the subject of a number of recent reports of both preclinical and clinical nature. Some of the most compelling studies have been conducted in non-small cell lung cancer (NSCLC), where approximately 15% of Caucasians and up to 50% of Asians harbor an *EGFR* mutation, a driving oncogenic event in their cancers. Intriguingly, there exists a germline polymorphism (that has come to be known as the "*BIM* deletion polymorphism") that results in splicing out the BH3 domain, and occurs in 15.5% of the Asian population; the presence of which is a harbinger for poor response of *EGFR* mutant lung cancers to EGFR inhibitors, as well as other cancers to multiple targeted therapies [43–45]. The phenotype may be further exacerbated in some cases by a hit to the second allele of *BIM*.

Although relatively straightforward detection of a genetic deletion is via a blood diagnostic test, this exemplifies the exception and not the rule: there have yet to be other ubiquitous genetic causes of alterations of BCL-2 family members in solid tumors driven by either receptor-tyrosine kinases, PI3K, KRAS or BRAF. Perhaps the closest scenario is in *BRAF* mutant melanoma, where loss of phosphatase and tensin homolog (PTEN), as detected by a tissue array, confers resistance to BRAF inhibitors, but interestingly not to anti-proliferation, but strictly to apoptosis [46]. This is the result of PTEN-mediated suppression of BIM through the PI3K pathway, and can be reversed by the addition of PI3K inhibitors that transcriptionally induce BIM through downregulation of phospho-AKT3 and the downstream BIM tran-

scription factor, FOXO3A [46]. Theoretically, PTEN-negative IHC staining could triage *BRAF* mutant melanoma patients to the combination of PI3K and BRAF inhibitors, although the best opportunity for a therapeutic window may be if/when isoform specific AKT3 inhibitors are available, or if PI3K isoform specific inhibitors can successfully lead to BIM upregulation.

7.6 First Generation BH3 Mimetics: Specificity and Non-specificity

While many of the first targeted therapies developed and consisted of antibodies and small molecules targeting kinases [47–50], an important addition to targeted molecular therapeutics have been BH3 mimetics. A BH3 mimetic small molecule is one that interferes with protein-protein interaction (PPI) by directly binding the BH3 binding pocket of its respected anti-apoptotic target(s), resulting in the activation of BAX and BAK (Fig. 7.1). While a number of BCL-2 family inhibitors have been around for over 10 years, many of these have proven to be non-specific, killing in BAX and BAK independent manners [51–56]. This includes the once promising pan BCL-2 inhibitor [57, 58], obatoclax, which had unexpected clinical neurotoxicity [59, 60], probably due to a result of its off-target effects [53, 61–63]. The problem has been so ramped that Lessene et al. [64] and more recently, Soderquist and Eastman [54], have offered new and more stringent criteria for drugs to be categorized as true BH3 mimetics: For instance, this includes that putative BCL-2 inhibitors and BCL-xL inhibitors should demonstrate quick killing of CLL cells *ex vivo* and platelets *ex vivo*, respectively [54], and the putative BH3 mimetic should have nanomolar activity in cell-free binding assays against its putative target(s) [64]. For a thorough review of off-target activity of putative BCL-2 family inhibitors and ways to avoid mischaracterization, see Soderquist and Eastman [54].

7.7 BH3 Mimetics as Single Agents: Efficacy and Resistance

7.7.1 BCL-2/BCL-xL Dual Inhibitors

Navitoclax [65] (ABT-263, AbbVie) was the first BCL-2 family inhibitor in clinical trials that has (along with the structurally analogous ABT-737) been demonstrated to be very specific as a BCL-2, BCL-xL and BCL-w antagonist/inhibitor [53, 66]. ABT-263 interferes with the PPI of BCL-2, BCL-xL and BCL-w with pro-apoptotic BCL-2 family members, by mimicking the BH3 domain of these latter proteins. This leads to sequestration of these anti-apoptotic proteins. Pre-clinically, navitoclax showed impressive activity against several blood cancers, including chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL) [34, 67], multiple myeloma [68] and acute lymphoblastic leukemia (ALL) [69]. In solid tumors, it has

demonstrated impressive pre-clinical activity against SCLC [70–73] and neuroblastoma [74], and is preferentially active in *MYCN*-amplified neuroblastoma [75]. Navitoclax appears to kill in a BIM-dependent manner, as this has been demonstrated mostly through the use of siRNA or shRNA designed against BIM. These include studies in CLL [69], ALL [76], and SCLC [70].

7.7.2 Intrinsic Resistance to Navitoclax

7.7.2.1 *In Vitro* Correlatives to Sensitivity and Resistance to Navitoclax

Many of the preclinical studies with navitoclax have led to important insights on not only how cancers can be expected to become resistant to navitoclax, but also the requirements for different anti-apoptotic BCL-2 family members for survival in different cancers. Indeed, it has become abundantly evident that high MCL-1 expression, or low expression of the endogenous MCL-1 inhibitor, NOXA, confers resistance to navitoclax, since navitoclax itself has a low affinity to MCL-1 and cannot inhibit its function [65]. An initial siRNA screen from AbbVie demonstrated the MCL-1 was critical to sensitize cancers to navitoclax [77]. Our recent work demonstrated through a high-throughput navitoclax screen in ~800 solid tumor cell lines (i.e. the Genomics of Drug Sensitivity in Cancer (GDSC)) [78–80], that high MCL-1 expression conferred resistance, while high expression of BIM (pro-apoptotic) conferred sensitivity [70]. We have also demonstrated that high level of NOXA was important to reduce MCL-1 guided resistance in SCLC [73], one of the only solid tumors (the other being neuroblastoma) showing broad sensitivity against navitoclax.

Other studies have also shown that BCL-2 family expression and modification are correlated with *in vitro* sensitivity of CLL to the navitoclax analogue ABT-737; Al-harbi et al. [67] correlated *in vitro* sensitivity to MCL-1 plus BFL-1/BCL-2 levels. The most well-studied post-translational modification of BCL-2 is the phosphorylation of Ser70 and other residues in the loop region, with likely candidate kinases ERK and PKC [81]. In a separate study looking at biomarkers for response and resistance in CLL, Song et al. concluded that the ratio of (phospho-BCL-2 + MCL-1)/BCL-2 best predicted response to navitoclax. Importantly, they demonstrated a 100–300-fold decrease in binding of BCL-2 to navitoclax when BCL-2 was phosphorylated [82].

As BH3 mimetics that target BCL-xL are dependent on their ability to disrupt BCL-xL complexes (please see above), Pecot et al. [83] recently used a bioluminescence resonance energy transfer (BRET) to study BCL-xL interactions in whole cell lysates (through evaluation of energy transfer between BCL-xL and partnering proteins, as opposed to the traditional membrane restricted studies), and provided the insight that BH3 mimetics may be more inferior than it was previously thought to disrupt certain BCL-xL complexes, such as BCL-xL:PUMA complexes. Indeed, the effects of BH3 mimetics seem to depend at least partly on the subcellular localization of BCL-xL complexes.

7.7.3 Acquired Resistance to Navitoclax

By deriving resistant cell lines from initially sensitive lymphoma cells, Yecies et al. [84] demonstrated that both MCL-1 and BFL-1 were upregulated in the ABT-737-resistant cells, replacing BCL-2 as the preferred binding partner to BIM. *In vitro* experiments in ALL demonstrated that ABT-737-induced resistance also led to increased MCL-1 expression, which was underlined by post-translational changes to MCL-1. Again, this led to an increase of BIM:MCL-1 complexes, preventing BIM-mediated apoptosis upon BCL-2 inhibition, and engendering the cell for apoptosis following MCL-1 inhibition [85].

7.7.4 Clinical Trial Correlatives to Navitoclax Sensitivity and Resistance

Biomarker analyses for navitoclax have been conducted as well. The analysis by Roberts et al. in CLL patients treated with navitoclax [86] indicated that the levels of BIM:MCL-1, as determined by gene expression, but not other single BCL-2 family members or ratios investigated, were statistically correlated with the response to navitoclax.

7.7.5 BCL-2 Specific Inhibitors

Clinically, however, the excitement for navitoclax was slowed when early clinical trial data demonstrated that navitoclax induced dose-limiting thrombocytopenia—a result of BCL-xL inhibition followed by cell death in platelets [87]—likely limiting the ability of navitoclax to potently inhibit BCL-2 [86, 88, 89]. Addressing these concerns was the next AbbVie BH3 mimetic, which potently inhibited BCL-2 while sparing BCL-xL [90].

Indeed, there have been fewer targeted therapies that have made a bigger impact in the past few years than the pure BCL-2 inhibitor, venetoclax (ABT-199). Venetoclax is a BCL-2 specific antagonist, designed by reengineering navitoclax, that has demonstrated robust clinical activity and a large therapeutic window in BCL-2-dependent blood cancers, including CLL, AML (including blastic plasmacytoid dendritic cell neoplasm), and TCF3-HLF-positive ALL [90–93]. In solid tumors, substantial activity of venetoclax across tumor types is more limited, with studies demonstrating activity against a substantial subset of *MYCN*-amplified neuroblastoma [75, 94]. Similarly to navitoclax, venetoclax also appears to kill in a predominantly BIM-dependent manner [75, 90, 94], although other factors are clearly involved.

Venetoclax received breakthrough therapy designation by the FDA for 17p-deleted CLL, which lacks *TP53* as a result of the deletion event. The approval

was supported from two single arm, dose-escalating studies, where venetoclax demonstrated close to an 80% response rate in both trials [95, 96]. These clinical trial data are also consistent with the mechanism of venetoclax killing in CLL, and presumably other cancers, in a p53-independent manner [97] and with no or limited off-target BCL-xL inhibition [90, 98].

In non-hodgkin's lymphoma (NHL), a phase I study demonstrated activity across multiple subtypes, with a 75% percent overall response rate (ORR) in MCL. As in the CLL studies, venetoclax was well tolerated, with neutropenia in 11% and thrombocytopenia in less than 10% of patients [99].

Still other hematological cancers have shown responses to venetoclax. Recently, in a rare but aggressive malignancy, blastic phasmacytoid dendritic cell neoplasm, Montero et al. demonstrated significant pre-clinical activity in a patient-derived xenograft (PDX) model, and clinical activity with venetoclax in two patients [93].

As a true BCL-2 inhibitor, it would be expected that the efficacy of venetoclax is significantly restricted, particularly within solid tumors, where many of these cancers are dependent on BCL-xL and/or MCL-1 [31, 100–103]. Indeed, we have found many solid tumors that are sensitive to navitoclax [70] are completely resistant to venetoclax as a single agent (unpublished data). These data suggest the use of venetoclax will be most promising in solid tumors in rational combinations, which are discussed below.

7.7.6 Intrinsic Resistance to Venetoclax

7.7.6.1 *In Vitro* Correlatives to Sensitivity and Resistance to Venetoclax

In a preclinical biomarker study in multiple myeloma, Punnoose et al. [104] found that the co-expression of BCL-xL or MCL-1 conferred resistance to venetoclax. In diffuse large B-cell lymphoma (DLBCL) cells, low BCL-xL and low MCL-1 conferred sensitivity to venetoclax [105]. We recently reported that low NOXA conferred resistance to venetoclax, while high NOXA, as a result of amplification of *MYCN*, conferred sensitivity [75]. This finding was in neuroblastomas, which had predominantly low BCL-xL levels. The regulation of NOXA was direct, as MYCN increased the transcription of *NOXA* by binding to its promoter. As would be expected, inhibition of MCL-1 further sensitized the high NOXA, *MYCN*-amplified neuroblastomas to venetoclax, while overexpression of MCL-1 or transduction of virus-containing shNOXA blunted the venetoclax response [75]. In contrast, forced expression of exogenous NOXA in *MYCN*-low neuroblastomas sensitized the low NOXA, *MYCN*-low neuroblastomas to venetoclax. This raises the possibility that the amplification of *MYCN*, and possibly *MYC* which has also been demonstrated to directly upregulate NOXA [106], may be a biomarker for response to venetoclax in BCL-xL low cancers through the inhibition of functional MCL-1 (via NOXA), and venetoclax-based therapy may be an interesting option in *MYC* family-amplified solid tumors that possess low levels of BCL-xL.

7.7.7 *In Vivo Correlatives to Sensitivity and Resistance to Venetoclax*

Biomarker analysis was recently performed in conjunction with an investigation of venetoclax in a heavily pretreated AML population, which yielded a 20% objective response rate. Sensitivity correlated with the presence of *IDH1/2* mutations; *IDH1/2* mutations result in production of the oncometabolite (R)-2-hydroxygluterate (2-HG), which confers BCL-2 dependence by decreasing the activity of cytochrome c oxidase (COX), which in effect lowers the apoptotic threshold to venetoclax [107].

7.7.8 *Acquired Resistance to Venetoclax*

As venetoclax is now FDA approved, understanding how cancers acquire resistance to continuous venetoclax exposure is imperative to eventually find pharmaceutical solutions to overcome this resistance, including perhaps re-designed BH3 mimetics. Fresquet et al. [108], studying acquired resistance to venetoclax in mouse lymphoma models, identified two mutations in *BCL-2*—both missense mutations at Phe101 of the BH3 domain—which hindered the ability of venetoclax to bind BCL-2. These cells did not have cross-resistance to a number of other anti-cancer drugs, including taxol and cisplatin, further suggesting these mutations were BH3 mimetic-specific. In parallel, the investigators established an *in vitro* model of resistance to venetoclax in a sensitive human mantle cell lymphoma cell line. After 7 months of venetoclax exposure, the resistant line was established both *in vitro* and when injected into mice. While there was no mutation in *BCL-2*, strikingly, there was a point mutation in the downstream effector BAX (Fig. 7.1), which resulted in resistance to BCL-2 family-mediated apoptosis. This mutation conferred cross-resistance to several other anti-cancer drugs, as would be expected from a mutation in the terminal BCL-2 member, BAX.

In DLBCL cells that were sensitive to venetoclax and acquired resistance through chronic exposure, BCL-xL and MCL-1 levels increased [105]. MCL-1 changes were both at the RNA and protein stability levels, indicating in several ways cells increase MCL-1 levels to acquire resistance to venetoclax. A summary of the mechanisms of resistance to navitoclax and venetoclax can be found in Table 7.1.

7.7.9 *MCL-1 Specific Inhibitors*

One of the interesting observations from a large number of preclinical studies of navitoclax and venetoclax has been the demonstration that MCL-1 is a major resistant mechanism against these compounds [70, 75, 109–115]. Other studies

Table 7.1 Intrinsic and acquired resistance to Navitoclax and Venetoclax

Intrinsic resistance to	Condition	Mechanism of resistance	References
Navitoclax	High expression of MCL-1 or low expression of NOXA	Navitoclax has low affinity to MCL-1, thus cannot disrupt BIM:MCL-1 and BAK:MCL-1 complexes	[64, 69]
Navitoclax and Venetoclax	Phosphorylation of BCL-2 at Ser70	Phosphorylation prevents direct binding of BH3 mimetics to BCL-2 molecule	[82]
Venetoclax	High expression of MCL-1 and BCL-xL	Venetoclax has low affinity to MCL-1 and BCL-xL, thus cannot disrupt BIM:MCL-1, BIM:BCL-xL BAK:MCL-1, and BAX:BCL-xL complexes	[103]
Venetoclax	Low expression of NOXA	The pro-apoptotic partner of MCL-1 is missing, and venetoclax cannot disrupt BIM:MCL-1 and BAK:MCL-1 complexes	[75]
MCL-1 inhibition	High expression of BCL-xL	MCL-1 inhibition cannot disrupt BIM:BCL-xL and BAX:BCL-xL complexes	[100]
Acquired resistance to	**Condition**	**Mechanism of resistance**	**References**
Navitoclax	In ALL, upregulation of MCL-1 and BFL-1	The formation of complexes BIM:MCL-1 and BIM:BFL-1 that cannot be disrupted by navitoclax	
Venetoclax	After treatment with venetoclax in DLBCL, upregulation of MCL-1 and BCL-xL	The formation of complexes BIM:MCL-1 and BIM:BCL-xL that cannot be disrupted by venetoclax	[105]
Venetoclax	Acquired mutations in BCL-2	Venetoclax cannot bind to BCL-2	[108]
Venetoclax	Acquired point mutation in BAX	Inactivation of the terminal BCL-2 protein prevents MOMP and apoptosis	[108]

utilizing siRNA have also implemented MCL-1 as an important survival signal in different cancers; for instance, a subset of high MCL-1/low BCL-xL NSCLCs [116], melanoma cells [117], myeloma cells [118], and CML cells treated with imatinib [119]. While many groups have designed MCL-1 BH3 mimetics, the first widely testable MCL-1 BH3 mimetic was recently debuted, the AbbVie compound, A-1210477 [120]. As noted by Soderquist and Eastman [121], in retrospect, the increase in cellular MCL-1 observed after A-1210477 treatment should have been expected with other putative MCL-1 inhibitors, since MCL-1 is highly regulated through ubiquitin-mediated degradation by several ligases. A-1210477 is effective as a single-agent against MCL-1 dependent NSCLCs [120] and breast cancers [122].

A second BH3 mimetic, MCL-1 inhibitor S63845 [123], was more recently developed. Similarly, it demonstrated single-agent activity against some cancers, namely AML, a few low BCL-xL expressing NSCLCs, breast and melanoma cell lines [123]. In freshly derived AML samples, there was activity with S63845 that sometimes approached 100–1000 fold greater than in normal human CD34+ progenitor cells. In addition, S63845 was demonstrated to kill MCL-1-dependent multiple myeloma, leukemia and lymphoma cells; S63845 also showed activity in combination with kinase inhibitors in solid tumors, further demonstrating that enhancement of clinical targeted therapies may be achieved with the inhibition of anti-apoptotic members.

The most clinically advanced MCL-1 inhibitor, however, is from Amgen, AMG176. The first in-human trial is currently enrolling for multiple myeloma (clinical trial: NCT02675452). The results from this clinical trial will be exciting to see, with the hopes that MCL-1 inhibitors will be tolerated in patients, especially given the reliance of MCL-1 on many normal hematopoietic cells [124]), including B and T lymphocytes [125], macrophages and neutrophils [126]. Of additional concern, MCL-1 is essential for neuronal survival, at least during development [127]. In fact, mouse genetic studies have demonstrated that MCL-1 is unique among the three major anti-apoptotic members as it is essential for embryonic development, with lethality noted at 3.5 days [124]. It should be noted here as well that obatoclax, a once promising therapeutic candidate that inhibited MCL-1 [57], demonstrated neuropsychiatric dose-limiting toxicities and ataxia in clinical trials [59, 128]. As obatoclax is not a specific inhibitor of MCL-1 [121], it will be interesting to see if there are any neurotoxicities in the upcoming AMG176 trial.

7.7.10 Non-BH3 Mimetic MCL-1 Inhibitors

In addition to the development of specific MCL-1 inhibitors, there are a number of clinically-relevant compounds that have the ability to act as MCL-1 inhibitors. In order to appreciate these strategies, it is helpful to understand how MCL-1 is regulated. Due to its very short half-life of mRNA and protein, MCL-1 is one of a few oncogenes—and the only BCL-2 family member—that is exquisitely dependent on short bursts of transcription and translation, which makes it an attractive primary target of CDK9 inhibitors (transcriptional) and TORC1 inhibitors (translational) [129, 130]. For instance, multiple CDK inhibitors result in acute RNA polymerase II-mediated transcriptional inhibition [131–134], and acute translational inhibition through inhibition of mTORC1 [70, 129, 135]. In addition, mitotic arrest can lead to a decrease in MCL-1 expression [75, 136].

In fact, many studies on inhibitors of CDK9 have demonstrated that MCL-1 is a key, early target of these compounds [131, 132, 137]. Given the exposure time of CDK9 inhibitors like dinaciclib (Merck) to tumors, it is likely that the primary effects of these drugs are on genes with short-lived mRNA—like

MCL-1 [138]. In mixed lineage leukemia (MLL)-AF9 driven cancers, dinaciclib was found to induce potent apoptosis and anti-cancer efficacy in mouse models, as a result of decreased expression of MCL-1 [139]. Gregory et al. [131] demonstrated similar results in a MYC-driven B cell lymphoma mouse model. We and others have also demonstrated that inhibition of MCL-1 is a primary target of pure TORC1/2 inhibitors [70, 101], which will soon be investigated in planned clinical trials in combination with navitoclax. While long-term treatments in culture with either CDK9 inhibitors or TORC1/2 inhibitors are almost universally anti-proliferative or toxic, given the short time from drug exposure to effect on MCL-1, these drugs may be beneficial given favorable pharmacodynamics (PD) and pharmacokinetics (PK). Altogether, there are a number of approaches to inhibit MCL-1 in humans, and careful examination of PD/PK measures in patients may further clue us to which of these compounds may really be a surrogate MCL-1 inhibitor.

7.7.11 Intrinsic Resistance to MCL-1 Inhibitors

Prior to the availability of specific MCL-1 inhibitors, Golub and colleagues demonstrated through the combination of shRNAs and transcriptional repressors, that MCL-1 inhibition was ineffective in high BCL-xL expressing cancers [100]. These data are in line with the lack of single-agent MCL-1 inhibitors in solid tumors [123] and the apparent need for MCL-1 inhibitor-based combination therapies.

7.8 Sensitizing Cancers to Intrinsic BH3 Mimetic Resistance

7.8.1 Growth Factor Pathway Regulation by BCL-2 Family Members

As the information connectors from intracellular kinase cascades to the mitochondria, BCL-2 family members are highly and ubiquitously regulated by different growth factor pathways (for a recent review, please see ref. [32]). As such, depending on the activity levels of these particular pathways, BCL-2 family member expression and interactions are vastly altered. These alterations can also connect aberrant growth and survival. For instance, the expression of BIM_{EL}, the most prevalent isoform of the tumor suppressor BIM [140], is post-translationally modified on Ser69 phosphorylation by ERK, leading to its degradation and subsequent apoptotic resistance [140]. The RAF/MEK/ERK pathway is a major growth factor signal transduction pathway in cancer, and as such, co-regulates growth and survival in many cancers. On the other hand, the oncogenic anti-apoptotic protein, MCL-1 is a major effector molecule of the PI3K/mTORC1 axis, due to the heavy reliance of

MCL-1 on cap-dependent translation [129]. As a major metabolic hub and translational impetus in the cell, TORC1 is the nexus for the major growth factor pathways in the cell, namely the PI3K [31] and the RAF/MEK/ERK pathways. Therefore, aberrant proliferative and survival signals through the BCL-2 family are intimately linked in cancer, encouraging the transformation of normal cells through mutations and/or aberrant activation of these pathways.

7.8.2 Helping Each Other Out

From the perspective of the kinase inhibitor, identifying apoptotic blocks like the ones highlighted above, even if the causes are not fully understood, are first steps in assigning one of the BH3 mimetics to add to the kinase inhibitor to sensitize it. Mechanistically, these different targeted therapies commonly block key growth factor/survival signaling pathways that are intracellular and downstream of the offending oncogenic lesion. As such, they converge on the BCL-2 family proteins. The changes that occur following this convergence are vital to a robust apoptotic response. Among them are upregulation of pro-apoptotic proteins, PUMA, NOXA and BIM and downregulation of anti-apoptotic proteins BCL-2, BCL-xL and MCL-1.

Some examples of these strategies are BCL-2/BCL-xL inhibition in combination with mutant *BRAF* inhibition in melanoma [141, 142] and colorectal cancer [30], the combination of BCL-2/BCL-xL inhibition with EGFR inhibitors to treat *EGFR* mutant lung cancer [17, 143, 144], the combination of PI3K inhibitors with BCL-xL inhibition in *PIK3CA* mutant breast cancer [31], and BCL-2/BCL-xL inhibition in combination with MEK inhibitors in *KRAS* mutant pancreatic cancer [145]. While the common thread in these therapies may be targeting the important oncogenic driver in these cancers in combination with specifically targeting the BCL-2 family, there are other instances where this doesn't necessarily have to be the case for this strategy to be effective. For instance, Wali et al. [146] has recently demonstrated that the ALK inhibitor, Crizotinib in combination with navitoclax is effective in (non-*ALK* mutant) triple negative breast cancers. It has also been demonstrated that inhibition of PI3K with GDC-0941 leads to MCL-1 loss and sensitization to ABT-737 in breast cancers with different oncogene addictions (e.g. *KRAS*) [147]. Therefore, strategies involving co-targeting kinases with BH3 mimetics need not be limited to kinase inhibitors targeting the oncogene that the cell is addicted to. Lastly, strategies that involve co-targeting a specific epitope of a cancer cell, in combination with a BH3 mimetic, may be effective. Along these lines, clinical trials with the FDA-approved Rituximab and Obinutuzumab, targeting CD20 found on B cells (and B-cell malignancies), in combination with venetoclax has demonstrated promise [148]. In total, many of these co-targeting strategies are highlighted in Fig. 7.3. From the perspective of the BH3 mimetic, identifying growth factor pathways in which downregulation can overcome resistance contributed by other BCL-2 family members.

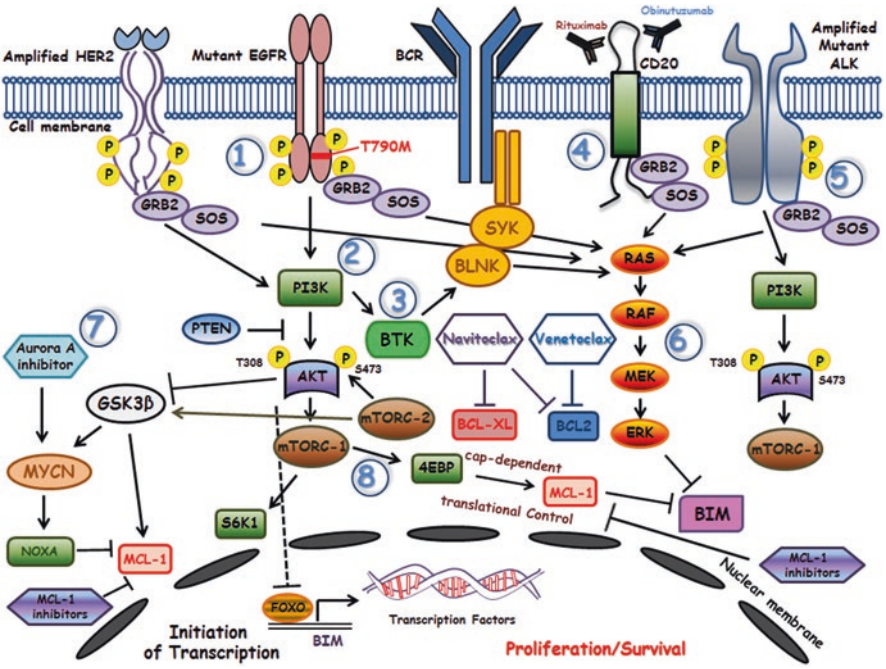

Fig. 7.3 BCL-2/BCL-xL as therapeutic targets in combination therapies. (*1*) Osimertinib (AZD9291), a third generation EGFR inhibitor, is being explored in clinical trials in combination with navitoclax for the treatment of EGFR-mutant in a basket trial of solid tumors (Phase I (NCT01009073)). (*2*) The addition of PI3K inhibitors reverses BIM suppression through upregulation of its transcriptional factor, FOXO3A. The navitoclax twin compound ABT-737 in combination with the PI3K inhibitor GDC-0941 is effective in breast cancer models, through GDC-0941-mediated downregulation of MCL-1 (pre-clinical). (*3*) Bruton's tyrosine kinase (BTK) is a vital enzyme in the BCR pathway. Ibrutinib, a BTK inhibitor, is being used in the treatment of patients with chronic lymphocytic leukemia (CLL) and follicular lymphoma (FL), reducing the levels of BCL-xL and MCL-1. The combination of ibrutinib with venetoclax demonstrated increased efficacy in CLL (Phase II (NCT02756897) (NCT02910583) for CLL and Phase I/II (NCT02956382) for FL). (*4*) Monoclonal antibody drugs (Rituximab, obinutuzumab) are used to treat CLL targeting the CD20 antigen, a protein found on the surface of B-lymphocytes. Recently, Rituximab in combination with venetoclax showed significant efficacy in clinical trials (Phase Ib (NCT01682616)). (*5*) The ALK inhibitor, Crizotinib, has synergistic effects with navitoclax in triple-negative breast cancers (pre-clinical). (*6*) BIM is phosphorylated and degraded by the MEK/ERK pathway: The BRAF inhibitor dabrafenib and MEK inhibitor trametinib have been used in clinical trials (Phase I/II (NCT01989585)) in combination with navitoclax for the treatment of BRAF mutant melanomas. (*7*) *MYCN*-amplified neuroblastomas are sensitive to venetoclax due to low levels of NOXA, the pro-apoptotic partner of MCL-1. The combination of MLN8237, an Aurora A inhibitor, with venetoclax displayed synergism in *MYCN*-amplified neuroblastomas due to concomitant suppression of BCL-2 and MCL-1 (pre-clinical). (*8*) The TORC1/2 inhibitor AZD8055 reduces MCL-1 levels and sensitizes *KRAS* and *BRAF* mutant colorectal cancers and *p53/Rb* deleted SCLCs to dual BCL-2/BCL-xL inhibition (navitoclax) (pre-clinical)

Indeed, the addition of histone deacetylase inhibitors, both pan [149] and isoform-specific [150], have been demonstrated to be effective to re-sensitize *EGFR* mutant NSCLCs with the *BIM* deletion polymorphism to EGFR inhibitors, through increased expression of BIM from the wild-type allele. Data from an ongoing clinical trial will shed light on whether pan HDAC inhibitors in combination with EGFR inhibitors will be tolerated and effective (clinical trial NCT02151721), and future trials will likely tackle whether isoform-specific HDAC inhibitors may be effective. The notion that the *BIM* deletion polymorphism is helping triage patients to optimal treatment plans speaks to the importance of functional BIM for EGFR inhibitor efficacy [16–19]. Even though a clinical trial of EGFR inhibitor and BH3 mimetics has not yet initiated for *BIM* deletion polymorphism positive patients, there are ongoing trials of this combination in the general *EGFR* mutant NSCLC population (clinical trial NCT02520778).

7.9 Combinations to Overcome Resistance

7.9.1 Navitoclax

7.9.1.1 Navitoclax and MCL-1 Inhibition

While MCL-1 inhibitors are rapidly developed for clinical translation (please see "MCL-1 inhibitors" section above), most instances of sensitizing navitoclax and venetoclax through MCL-1 blockage has been through indirect MCL-1 inhibitors. As mentioned above, due to its short mRNA and protein half-life, MCL-1 is very sensitive to acute transcriptional inhibitors and cap-dependent translation inhibitors. Along these lines, several years ago, it was demonstrated that in SCLCs inhibiting MCL-1 with the general transcription factor inhibitor, actinomycin D (Dactinomycin) sensitized to ABT-737 [135]. In addition, Klanova et al. [114] described a strategy involving the clinically-approved Homoharringtonine (Omacetaxine mepesuccinat) in combination with venetoclax to treat DLBCL. Homoharrangtonine markedly reduced MCL-1 expression, and sensitized DLBCL mouse models to venetoclax. Importantly, some of the efficacy is based on DLBCLs in general not to be very reliant on BCL-xL expression [114].

Another strategy to reduce MCL-1 levels is through TORC1/2 inhibitors, which block eIF4E-mediated cap-dependent translation [129, 151]. SCLC is the only solid tumor we are aware where navitoclax was specifically tested clinically as a monotherapy [89] based on strong pre-clinical activity [65, 72, 110]. However, clinical responses were disappointing with only one patient demonstrating a partial response [89]. After verifying the activity of navitoclax in SCLC by comparing it to hundreds of other cancer cell lines through the genomics of drug sensitivity in cancer (GDSC) platform [78–80], we demonstrated that SCLCs

can be sensitized by newer pure TORC1/2 inhibitors, and the mechanism is through inhibition of TORC1-dependent MCL-1 expression [70]. These data demonstrate not only an important role in SCLC resistance to navitoclax, but that certain cancers can be sensitized to navitoclax through inhibiting TORC1-mediated MCL-1 translation. Grant and colleagues demonstrated a similar strategy was efficacious in hematological cancers as well [112]. Underlying the importance of MCL-1 downregulation to the efficacy of TORC1/2 inhibitors in cancer care, cancers acquire resistance to these drugs through amplification of eIF4E, and subsequent restoration of MCL-1 translation [152].

While many cancers may be reliant on all three major anti-apoptotic members, it is possible that one may be able to achieve selective downregulation of MCL-1 in cancers that regulate MCL-1 either more heavily or dominantly by TORC1. To this point, we recently demonstrated that while *KRAS* and *BRAF* mutant colorectal cancers are insensitive to navitoclax, similar to in SCLC [70], MCL-1 downregulation can be achieved with mTORC1 inhibitors, and as such, these cancers are very responsive to navitoclax and TORC1 co-inhibition [101]. Interestingly, neither *KRAS* mutant lung cancers nor *KRAS/BRAF* wild-type colorectal cancers markedly downregulated MCL-1 following TORC1 inhibitor treatment, demonstrating selectivity and a potential therapeutic window [101]. In contrast to *KRAS*/*BRAF* mutant colorectal cancers, as well as most SCLCs, we found that neuroblastomas were also significantly more reliant on BCL-2 and MCL-1, and less reliant on BCL-xL [75]. As such, co-treatment of venetoclax-sensitive *MYCN*-amplified neuroblastomas with the Aurora A inhibitor, MLN8237 led to co-loss of BCL-2 and MCL-1 functions, marked apoptosis, and tumor regressions in multiple mouse models of *MYCN*-amplified neuroblastoma [75].

These combination studies demonstrate that alternative strategies, either by treating with drugs that can lead to downregulaton of MCL-1 in the cancer of interest but not other cells, or identifying cancers that are uniquely reliant on only one or two of the major three anti-apoptotic members, may create therapeutic windows that can translate to effective cancer therapy. Some of these strategies are currently headed to clinical trials, and we should know soon whether they will be tolerated and/or effective.

As mentioned above, MCL-1 or reduced expression of NOXA are major resistant mechanisms in many cancer types to BCL-2/BCL-xL dual inhibition. There are some cancers that are very sensitive to inhibiting BCL-2/BCL-xL/MCL-1 simultaneously, such as in luminal breast cancer, where navitoclax has limited activity, but sensitization with MCL-1 inhibition is effective. Similarly, *KRAS* mutant colorectal cancer, but not *KRAS* mutant lung or *KRAS* wild-type colorectal cancer, is hypersensitive to the triple inhibition.

7.9.2 Venetoclax

7.9.2.1 Venetoclax and MCL-1 Inhibitors

Phillips et al. [153] demonstrated high BCL-2 expressing NHL was sensitized to venetoclax via pharmaceutical MCL-1 inhibition (via the AbbVie tool compound, A-1210477). Low BCL-2 cancers, however, were not sensitized and were sensitive to navitoclax, reiterating the importance that venetoclax sensitive cancers are dependent on BCL-2, and not BCL-xL nor both.

7.9.2.2 Venetoclax and Other Rational Drug Combinations

As venetoclax efficacy is similarly mitigated by MCL-1, but also by BCL-xL, strategies aimed at sensitizing cancers to venetoclax have accordingly involved co-inhibiting these proteins. Cervantes-Gomez et al. demonstrated Ibrutinib, a Bruton Tyrosine Kinase (BTK) inhibitor which is approved for CLL [154], effectively combined with venetoclax in these cancers by partially downregulating BCL-xL and MCL-1 [155]. The combination of Ibrutinib (obinutuzumab) and venetoclax has already reached clinical testing (Table 7.2 and Fig. 7.3). In 13 previous untreated CLL patients [156], after a 22 day period of Ibrutinib treatment, venetoclax was administered at 20 mg the first week, and gradually ramped up to 400 mg daily. At 12 months of treatment, 11 patients were accessible, with one patient having an infusion related reaction to Ibrutinib, and one who declined to participate in the trial after cycle 8. Among these 11 patients, all had responses, and at least five of them had complete responses, with three others not being fully accessible. Neutropenia was seen in about 60% of patients, and a tumor lysis syndrome (TLS) that were both manageable, was seen in two patients. These data indicate efficacy and tolerability of the combination, with caveats that slowly ramping up venetoclax following Ibrutinib treatments may reduce the risk of TLS, but expanded data with more patients will be needed to make those determinations.

Pre-clinical studies have also demonstrated that the combination of Ibrutinib and venetoclax has activity in both DLBCL and Follicular Lymphoma, and venetoclax re-sensitized Ibrutinib-resistant cells, demonstrating again an important component of effective kinase inhibitors is apoptosis induction [157].

Others have demonstrated co-targeting of MCL-1 along with venetoclax can be effective in other cancers. For instance, in DLBCL, co-targeting MCL-1 with the CDK9 inhibitor, dinaciclib, demonstrates efficacy *in vitro* and in a mouse model of MYC/BCL-2 activated lymphoma [137].

Table 7.2 Current clinical trials with venetoclax or navitoclax in combinations

Venetoclax combination	Clinical trial	Conditions	References
Rituximab	Phase Ib (NCT01682616)	Relapsed or refractory CLL	[148]
Obinutuzumab	Phase Ib (NCT02242942)	Relapsed or refractory CLL	[155]
Obinutuzumab	Phase II (NCT02987400)	Relapsed or refractory DLBCL	
Obinutuzumab	Phase I (NCT02877550)	Treatment-naïve FL	
Obinutuzumab + lenalidomide	Phase I (NCT02992522)	Relapsed or refractory B-cell NHL	
Obinutuzumab + ibrutinib	Phase Ib/II (NCT02427451)	CLL	
Obinutuzumab + ibrutinib	Phase I/II (NCT02558816)	Relapsed or refractory MCL	
Obinutuzumab or rituximab	Phase I/II (NCT02055820)	NHL	[158]
Bendamustine + rituximab or Bendamustine + obinutuzumab	Phase Ib (NCT01671904)	Relapsed or refractory CLL	
Bendamustine + rituximab	Phase I (NCT01594229)	Relapsed or refractory NHL	[159]
Bendamustine + rituximab	Phase II (NCT02187861)	Relapsed or refractory FL	[160]
Polatuzumab vedotin + rituximab or polatuzumab vedotin + obinutuzumab	Phase Ib/II (NCT02611323)	Relapsed or refractory FL or DLBCL	
Decitabine or azacitidine	Phase Ib (NCT02203773)	Treatment-naïve AML	[161]
Cytarabine	Phase I/II (NCT02287233)	Treatment-naïve AML	[162]
Azacitidine	Phase Ib (NCT02966782) Phase II (NCT02942290)	MDS	
Azacitidine	Phase III (NCT02993523)	Treatment-naïve AML	
Bortezomib + dexamethasone	Phase III (NCT02755597)	Relapsed or refractory MM	
Carfilzomib + dexamethasone	Phase II (NCT02899052)	Relapsed or refractory MM	
Ibrutinib	Phase II (NCT02756897) (NCT02910583)	CLL	
Ibrutinib	Phase I/II (NCT02956382)	Relapsed or refractory FL	
Ibrutinib	Phase II (NCT02471391)	MCL	[163]
ABBV-838 + dexamethasone	Phase I (NCT02951117)	Relapsed or refractory MM	
Dexamethasone	Phase I (NCT01794520)	Relapsed or refractory MM	[164]

(continued)

Table 7.2 (continued)

Navitoclax combination	Clinical trial	Conditions	References
Sorafenib tosylate	Phase I (NCT02143401)	Relapsed or refractory solid tumors	
Osimertinib	Phase I (NCT02520778)	EGFR-positive NSCLC	
Trametinib	Phase Ib/II (NCT02079740)	Advanced or metastatic solid tumors	
Rituximab	Phase II (NCT01087151)	CLL	[165]
Rituximab	Phase I (NCT00788684)	CD20-positive lymphoid malignancies	
Gemcitabine	Phase I (NCT00887757)	Solid tumors	[166]
Trametinib + dabrafenib	Phase I/II (NCT01989585)	BRAF mutant melanoma	
Paclitaxel	Phase I (NCT00891605)	Solid tumors	
Docetaxel	Phase I (NCT00888108)	Solid tumors	
Erlotinib	Phase I (NCT01009073)	Solid tumors	[167]

7.10 Concluding Remarks

It has been an exciting last few years for BH3 mimetics, highlighted by the FDA-approval for Venetoclax, the first BH3 mimetic to reach such status. With the accelerated development of MCL-1 inhibitors, and with some of these reaching clinical trial testing, it seems BH3 mimetics are beginning to achieve their lofty potentials. Like other successful targeted therapies, the biggest challenges will be to gain a better understanding of how cancers will acquire resistance to these drugs, and to better understand how to use BH3 mimetics in rational combinations with other drugs not only to thwart off acquired resistance but also to expand its use in the upfront setting. Tools like BH3 profiling, as they inch closer to clinical use, should play a large role in helping to solve these problems.

Acknowledgments This review was partly funded by NIH/NCI grant 5K22CA175276 (ACF), an American Cancer Society Research Scholar Grant (ACF), and a Massey Cancer Center Pilot grant (HH and ACF).

Conflict of Interest No potential conflicts of interest were disclosed.

Note added in proof We recently published that venetoclax is effective in small cell lung cancer with high BCL-2 expression [168], which, for the first time, demonstrates the eff ect of venetoclax in solid tumors.

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Chapter 8
Resistance Mechanisms to Cyclin-Dependent Kinase Inhibitors

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Abstract Pharmacological cyclin-dependent kinase inhibitors (CDKi) target a class of kinases (CDKs) that are critical mediators of cell cycle progression. Cell cycle deregulation is a hallmark of cancer, and CDKs have long been considered attractive targets for anti-cancer agents. After initial failures of pan-CDKi in clinical trials, two highly specific CDK4/6 inhibitors, palbociclib and ribociclib, are now FDA-approved in hormone receptor-positive breast cancer and an integral part of the treatment regimen. A third CDK4/6 inhibitor, abemaciclib, has also demonstrated promising clinical activity and has been granted break through status by the FDA. CDK4/6 inhibitors in combination with endocrine therapy significantly improve progression-free survival in patients with estrogen receptor-positive breast cancer. CDK1/2 inhibitors, such as dinaciclib, have also advanced to late-stage clinical trials but have yet to be FDA-approved in human cancers. Preclinical studies are beginning to shed light on inherent and acquired mechanisms of resistance to CDKi. Several cancer-relevant signaling pathways, such as the MAPK and PI3K pathways, drive cell cycle progression and contribute to CDK activity, suggesting that concomitant inhibition of these pathways may delay the outgrowth of CDKi-resistant cells. Mutation or deletion of RB1 is associated with inherent resistance to CDK4/6 inhibitors and may also play a role in acquired CDKi resistance. Similarly, amplification or overexpression of cyclin E1 may render cancer cells less dependent on CDK4/6. Ultimately, putative mechanisms of CDKi resistance will need to be validated by the analysis of clinical samples from CDKi-treated cancers.

Keywords Cyclin-dependent kinase 4 and 6 • Drug resistance • Receptor tyrosine kinase

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_8

Abbreviations

BLBC	Basal-like breast cancer
CAK	CDK activating kinase
CCND1	Cyclin D1
CCNE1	Cyclin E1
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
CIP	CDK interacting protein
ER	Estrogen receptor
ERBB2	Human epidermal growth factor receptor 2 (HER2)
FDA	Federal Drug Administration
HGSC	High-grade serous ovarian cancer
IGFR1	Insulin-like growth factor 1 receptor
INK4	Inhibitor of Cyclin-Dependent Kinase 4
KIP	Kinase interacting protein
NTRK1	Neurotrophic tyrosine kinase receptor type 1 (TrkA)
PDX	Patient-derived xenograft
PI3K	Phosphatidylinositol-3-Kinase
RB1	Retinoblastoma 1
RTK	Receptor tyrosine kinase
SERD	Selective estrogen receptor degrader
TCGA	The Cancer Genome Atlas

8.1 Introduction

Cyclin-dependent kinases (CDKs) are evolutionarily conserved enzymes and essential components of the complex machinery that governs the proliferation of cells, i.e. the cell cycle. The cell cycle is controlled by a tightly regulated system of positive and negative regulators whose interplay ultimately decides the proliferative fate of a cell. These regulators, in turn, respond to extracellular signals, such as growth factors bound to membrane receptors, cell–cell or cell–matrix interactions, and intracellular signals, such as the availability of nutrients, as well as cell size. Cell cycle deregulation and increased net proliferation are classic hallmarks of cancer cells, and many prominent oncogenes, such as mutant *RAS* or amplified *ERBB2*, drive proliferation by mediating the expression or protein stability of critical cell cycle regulators. These include cyclins—the required CDK binding partners found in catalytically active CDK protein complexes, and endogenous CDK inhibitors (CDKis), including the INK4 family and the CIP/KIP family. Tumor suppressor pathways frequently culminate in the expression and protein stabilization of cell-endogenous CDKi. For example, the CDK1/2 inhibitor $p21^{CIP}$ is a target of p53 and expressed in response to DNA damage, while the CDK4/6 inhibitor $p16^{INK4A}$ is an important marker of aging and senescence.

In yeast, a single constitutively expressed CDK is sufficient to drive the cell cycle via sequential binding of a number of different cyclins, whose expression levels are highly regulated and oscillate throughout the cycle. In proliferating yeast cells, cyclins undergo a cycle of synthesis and degradation, whereby they are sequentially transcribed, then bind and activate the CDK and are subsequently degraded to allow for binding of the next cyclin. In human cells with several interphase CDKs and an even greater number of cyclins, the cell cycle is an intricate network of proteins that integrate upstream signals in order to either induce the molecular events required for proliferation, DNA replication and cell division, or to transiently or permanently exit the cell cycle (quiescence). While most cells in the human body are in a quiescent state and divide infrequently or never, many of these cells retain their capacity to proliferate (proliferative potential) in response to certain stimuli. For example, fibroblasts can reenter the cell cycle during wound healing. Indeed, a number of functional cell cycle studies have been performed in quiescent fibroblasts and have helped shape our view of the cell cycle today. To what extent the general concepts can be applied to different cell types, including genetically heterogeneous cancers, remains to be elucidated. It is becoming increasingly clear that some cancer cells employ non-canonical mechanisms of proliferation that may render them intrinsically resistant to CDK inhibition. On the other hand, many of the molecular effects seen in CDKi-treated cells, as well as some of the emerging mechanisms of resistance, are in good accordance with our current model of the cell cycle. As more specific CDKis enter clinical settings, the analysis of response and resistance mechanisms will help us define tissue-specific and cancer-specific variations of the cell cycle regulation.

Targeting the cell cycle in order to cause cell cycle arrest, senescence or cell death has long been a major goal in molecular cancer therapeutics. However only now, after several decades and the arrival of more specific and potent small molecule CDKis, are we beginning to see therapeutic successes. Pharmacological CDK4/6 inhibitors, led by Pfizer's Palbociclib (IBRANCE®, formerly known as PD0332991) and Novartis' Ribociclib (KISQALI®) now have firmly established roles in the treatment of estrogen receptor (ER)-positive breast cancer, where they work in combination with ER antagonists, such as the aromatase inhibitor, letrozole, or the selective estrogen receptor degrader (SERD), fulvestrant. Here, I will discuss emerging mechanisms of response and resistance to CDK4/6 inhibitors, a more complete understanding of which may pave the way for their use in other cancer types, and in combination with other anti-cancer agents. We may also be able to infer effective combinations and target populations for CDK1/2 inhibitors, which have yet to make their breakthroughs in the realm of molecular cancer therapeutics.

8.2 Cyclin-Dependent Kinases

The human genome contains at least 20 CDK genes, only some of which are directly involved in cell cycle regulation (*CDK1/2/3/4/6*), whereas the majority performs other functions, such as phosphorylation of transcriptional regulators, that may

indirectly affect the cell cycle (reviewed in [1]). Cell cycle CDKs, unlike some of the other members of the family such as CDK12 and CDK13, are relatively small proteins that largely consist of a kinase domain containing the active ATP-binding site. The structure of the active site, and thus the enzymatic activity of the CDK, is modified by cyclins, necessary cofactors that confer kinase activity to CDKs and are also involved in target recognition and specificity. CDKs are serine-threonine kinases that phosphorylate substrates with a consensus amino acid sequence of [S/T*]PX[K/R] [2, 3], where S/T* is the phosphorylated serine or threonine, P is proline, X is any amino acid, K is lysine, and R is arginine, although exceptions to this rule have been published [4]. The first CDK was discovered as *cdc28* in the budding yeast Saccharomyces cerevisiae by Leland H. Hartwell and colleagues among a set of cell cycle genes with temperature-sensitive mutants [5]. Soon after, *cdc28* homologs were identified as *cdc2* in Schizosaccharomyces pombe [6], Drosophila melanogaster, and in human cells (*CDC2*, now *CDK1*) [7, 8]. CDKs are highly conserved across different species and phyla, so that both human CDK1 and CDK2 can rescue mutant cdc28 in S. cerevisiae [9]. For their discovery of the role of CDKs and cyclins in cell cycle regulation, Leland H. Hartwell, Sir Paul Nurse and Sir Richard Timothy Hunt, who first discovered cyclins in sea urchins, received the Nobel prize in medicine in 2001 [10]. In addition to the human CDK1/2/4/6, whose roles in cell cycle progression have been studied extensively, CDK3/5 may also regulate the cell cycle but their contributions are less well understood [1]. Nonetheless, CDK5 has been identified in a screen as a modulator of sensitivity to the proteasome inhibitor, bortezomib, and is a potential therapeutic target in multiple myeloma [11]. CDK5 is potently inhibited by several current CDKis (Table 8.1). Kinase-independent functions of CDKs and cyclins further contribute to cancer phenotypes and have been reviewed elsewhere [4].

In order for the cell cycle CDKs to become catalytically active, at least two activation steps are necessary: binding of a specific cyclin and phosphorylation by a CDK-activating kinase (CAK). In humans, this function is performed by CDK7, which phosphorylates the activation segment (T loop) of cell cycle CDKs (CDK1/2/4/6), specifically threonine 160 in human CDK2 [12–15]. CDK7-cyclin H is also a part of the general transcription factor TFIIH, resulting in a dual role for CDK7 in cell cycle activation and transcription [12]. CDK7 is targeted by several pharmacological CDKis (SNS032, flavopiridol, roscovitine, Table 8.1) but the significance of CDK7 inhibition in the context of anti-cancer activity of CDKis is unknown. Further, activation of CDK1 and CDK2 is negatively regulated by the WEE1 kinase, first identified by Sir Paul Nurse in fission yeast [16], where as a result of premature mitotic entry *wee1* loss of function produces smaller yeast cells that gave the kinase its name. WEE1 adds an inhibitory phosphate group to the tyrosine 15 residue of CDK1/2, close to its ATP-binding pocket. The inhibitory phosphate group is removed by phosphatases of the CDC25 family during later stages of the cell cycle. Interestingly, pharmacological WEE1 inhibitors, such as MK-1775, are also in development as anti-cancer agents. WEE1 inhibition following chemotherapy can potentiate DNA damage and enhance cytotoxicity via induction of mitotic catastrophe [17]. It is a strong testament to the complexity and

Table 8.1 CDK inhibitors

	CDK4/6 inhibitors					
Class	Palbociclib		Abemaciclib		Ribociclib	
CDK1		(>1.4 μM)	>1 μM	(330 nM)		(>1.4 μM)
CDK2		(>2.5 μM)	>500 nM	(150 nM)		(>2.5 μM)
CDK4	9–11 nM	(0.26 nM)	2 nM	(0.07 nM)	10 nM	(0.53 nM)
CDK5		(>2 μM)		(86 nM)		(>2 μM)
CDK6	15 nM	(0.26 nM)	5 nM	(0.52 nM)	39 nM	(2.3 nM)
CDK7		(>2 μM)	300 nM	(220 nM)		(>2 μM)
CDK9		(150 nM)	57 nM	(4.1 nM)		(190 nM)
Other known targets			(DYRK, PIM, HIPK, CaMK)			
References	[34]	[74]	[77]	[74]	[129]	[74]

	CDK2 inhibitors					
Class	SNS032		Dinaciclib		CYC065	
CDK1	280 nM		3 nM	(18 nM)	578 nM	
CDK2	20 nM[a]		1 nM	(1 nM)	5 nM	
CDK4	940 nM			(4.6 nM)	232 nM	
CDK5	340 nM		1 nM	(0.85 nM)	21 nM	
CDK6	>1 μM			(1.7 nM)		
CDK7	60 nM			(21 nM)	123 nM	
CDK9	5 nM		4 nM	(0.13 nM)	26 nM	
Other known targets	GSK3α,β		CDK3		CDK3	
References	[130]	[131]	[75]	[74]	Cyclacel	

[a]CDK2-cyclin E (70 nM for cyclin A)

heterogeneity of advanced human cancers that two seemingly diametrically opposed approaches – inhibition of CDK1/2 and inhibition of WEE1- are being investigated as alternative therapeutic strategies, with evidence of success for both in different contexts.

In the canonical model of cell cycle progression, sequential activation of CDK4/6, CDK2 and CDK1 propels the cell through different phases of the cell cycle (Fig. 8.1). CDK4 and CDK6 are active in the early G1 phase of the cell cycle and require D-type cyclins for activation. CDK2 activity peaks in late G2 (when bound to cyclin E) and early S phase (when bound to cyclin A), followed by CDK1 activity in the S, G2 and M phases (Fig. 8.1). Importantly, there appears to be significant redundancy among cell cycle CDKs. While S. cerevisiae contains two CDK2 genes (*cdc28* and *pho85*), cdc28 is sufficient to drive the cell cycle by sequential binding of different cyclins. In line with this, animal models have shown that only Cdk1 is essential for proliferation in all cell types of the mouse [18]. *Cdk1−/−* embryos do not develop past the two cell stage. In contrast, mice deficient for either Cdk4,

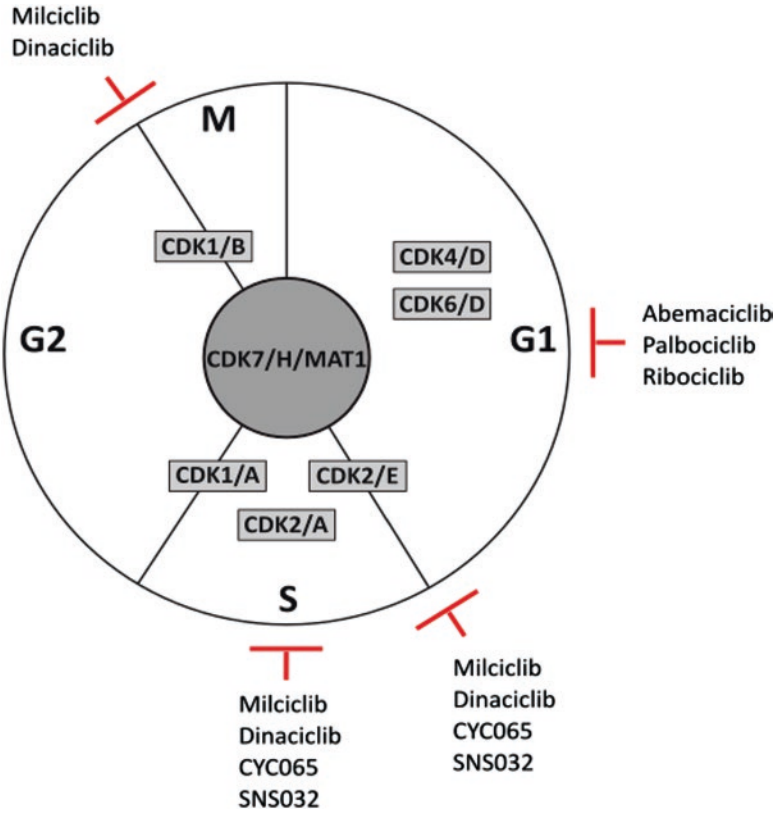

Fig. 8.1 The cell cycle in human cells, important CDK/cyclin pairs driving cell cycle progression and available CDK inhibitors

Cdk6, or Cdk2 develop normally with relatively minor phenotypes [19]. In *Cdk4−/−* mice, the impaired proliferation of pancreatic β-cells and pituitary hormone-producing cells leads to diabetes and reduced size [20]. *Cdk6−/−* mice have mild anemia due to defects in the hematopoietic system that are more severe in CDK4/6 double knockout mice [21], resulting in embryonic lethality by E16.5-E17.5. This phenotype accurately predicted the observed toxicity in early dose escalation trials with CDK4/6 inhibitors, where grade 3–4 neutropenia is the most common adverse event [22, 23].

Cdk2/4/6 triple knockout mice die by mid-gestation (E12.5–E13.5) due to decreased numbers of hematopoietic precursors and cardiomyocytes. Overall though, this study showed that most cell types can divide despite the lack of all interphase CDKs [18]. As in yeast, CDK1 can bind to cyclins D, E and A (albeit with lesser affinity) when the preferred binding partners (CDK4/6 and CDK2) are missing. Similarly, CDK2 can bind D-type cyclins, particularly in the absence of CDK4/6 [21, 24], or in the context of CDK4/6 inhibition [25]. Collectively, a picture emerges where CDK1 is absolutely essential for cell cycle progression, whereas all

other CDKs perform cell type- or context-specific functions. Therefore, pharmacological inhibition of CDK1 is expected to be toxic in a number of dividing cell types, resulting in a narrow therapeutic window. Similarly, the combined inhibition of all other interphase CDKs (CDK2/4/6) is likely associated with considerable toxicity, providing strong arguments in favor of highly selective CDKi that may then be combined with other targeted agents, chemotherapeutic drugs, or immunotherapy. We are now beginning to identify synergistic combinations and genetic patterns associated with response and resistance to specific inhibitors of CDK4/6. At the same time, there is a growing appreciation of the contributions of additional CDK family members to cell cycle progression. For example, CDK3 may contribute to RB inactivation [1] but has not been studied as a therapeutic target, although it is inhibited by some CDKis, such as dinaciclib. Similarly, the subfamily of CDK5-related CDKs, including CDK14/15/16/17/18, may contribute to cancer phenotypes, for example by activation of the WNT pathway [26].

8.3 Cell-Endogenous CDK Inhibitors

The third mechanism of CDK regulation is via cell-endogenous CDKis, such as members of the INK4 and CIP/KIP families. The INK4 family comprises four members, $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$ [27]. All INK4 proteins are specific inhibitors of CDK4 and CDK6, and when overexpressed either via cell-endogenous mechanisms or ectopically arrest responsive cells in the G1 phase of the cell cycle. Two of the four members, $p16^{INK4A}$, $p15^{INK4B}$, are encoded by two neighboring genes on human chromosome 9p21, *CDKN2A* and *CDKN2B*. *CDKN2A* further produces an alternate reading frame gene product, $p14^{ARF}$, which is a specific inhibitor of the MDM2 ubiquitin ligase that degrades p53. Expression of $p14^{ARF}$ thus results in stabilization of p53 and activation of its tumor suppressor function. While all three gene products from the *CDKN2A*-*CDKN2B* locus are regulated independently of one another and therefore able to relay different growth inhibitory signals, the entire region is frequently deleted in human cancers, resulting in at least partial inactivation of both the RB and the p53 pathways [28]. In line with this, $p16^{Ink4a}$ knockout mice [29, 30], in contrast to mice null for both $p16^{Ink4a}$ and $p19^{Arf}$, have a relatively mild phenotype and develop tumors late in life [31], suggesting that additional genetic hits are necessary for cancer initiation. Moreover, advanced human cancers frequently have acquired multiple genetic alterations within the signaling network surrounding the retinoblastoma (RB) protein ("the RB pathway") [32, 33]. This is important in the context of the RB pathway targeting and suggests that single agent CDKi may not be sufficient to arrest cells with multiple genetic lesions within the RB pathway, or in other signaling pathways that contribute to RB inactivation. The *CDKN2A* locus is of particular importance for treatment with CDK4/6 inhibitors, such as palbociclib, as its deletion is an important predictor of sensitivity to CDK4/6 inhibition, at least *in vitro*. *CDKN2A*-deleted cancer cells of various cancer types are almost uniformly responsive to palbociclib and, when

grown in two-dimensional conditions in cell culture, undergo proliferation arrest in the short term [33–37]. Ironically though, in the clinical setting that led to palbociclib's breakthrough status, ER-positive breast cancer, *CDKN2A* deletion does not appear to predict a better response [22, 23]. Thus, positive $p16^{INK4A}$ staining in cancer cells, rather than its absence, may be useful as a predictive marker of resistance to CDK4/6 inhibitors for two reasons: first, high levels of $p16^{INK4A}$ are found in cells with the RB pathway inactivation and second, cancer cells that are able to proliferate in the presence of $p16^{INK4A}$ likely do not require the CDK4/6 kinase function.

The CIP/KIP family of CDKis are pan-CDK inhibitors that form ternary complexes with cyclin-bound CDKs. Both $p21^{CIP}$ and $p27^{KIP1}$ are potent inhibitors of CDK1 and CDK2 while their role in controlling CDK4/6 activity is more complex. Where studied, the majority of p27 was found to be associated with CDK4 rather than CDK2, and *Cdk2−/−*;*Cdkn1b−/−* double knockout mice revealed that deletion of Cdk2 did not rescue the tumor-prone phenotype of p27-null mice [38, 39], suggesting that the tumor suppressor function of p27 and p21 is not entirely dependent on its inhibition of Cdk2 [38]. In the cytoplasm, p27 can facilitate the formation of CDK4-cyclin D complexes and their import into the nucleus. In the nucleus, p27 inhibits all cell cycle CDKs, including CDK7. The subcellular localization of p27, as well as its ability to inhibit CDK-cyclin complexes, is regulated by phosphorylation. Oncogenic tyrosine kinases, such as Abl and Src, can phosphorylate p27 and prevent it from blocking the ATP-binding pocket of the associated CDK molecule [40, 41]. In addition, AKT can phosphorylate p27 and prevent it from entering the nucleus and causing G1 arrest [42]. Similarly, AKT can phosphorylate p21 and alleviate its inhibitory effect on CDK2, highlighting the importance of the PI3K-AKT pathway in cell cycle progression. Not surprisingly, activation of this pathway is also emerging as a mechanism of resistance to CDKis. The basic helix loop helix transcription factor and proto-oncogene MYC is also required for CDK2-cyclin E activity in late G1 in some systems.

8.4 Cell Cycle Activation by the MAPK Pathway

Growth-stimulatory signals such as interactions of growth factors and their receptors at the cell surface set in motion a number of signaling cascades inside the cell that are relayed to the nucleus and ultimately result in gene expression changes underlying proliferation. Receptor tyrosine kinases such as ERBB2 and IGFR1 activate, among other pathways, the mitogen-activated kinase (MAPK) pathway and the phosphatidyl-inositole-3-kinase (PI3K) pathway. Both signaling pathways contribute to cell survival and proliferation (Fig. 8.2).

The MAPK pathway features sequential activation of the kinases RAS, RAF, MEK and ERK, resulting in transcriptional activation of cyclin D. Activated ERK can enter the nucleus and directly phosphorylate transcription factors of the MYC and ETS families, among other targets [43]. MYC is a transcriptional activator of

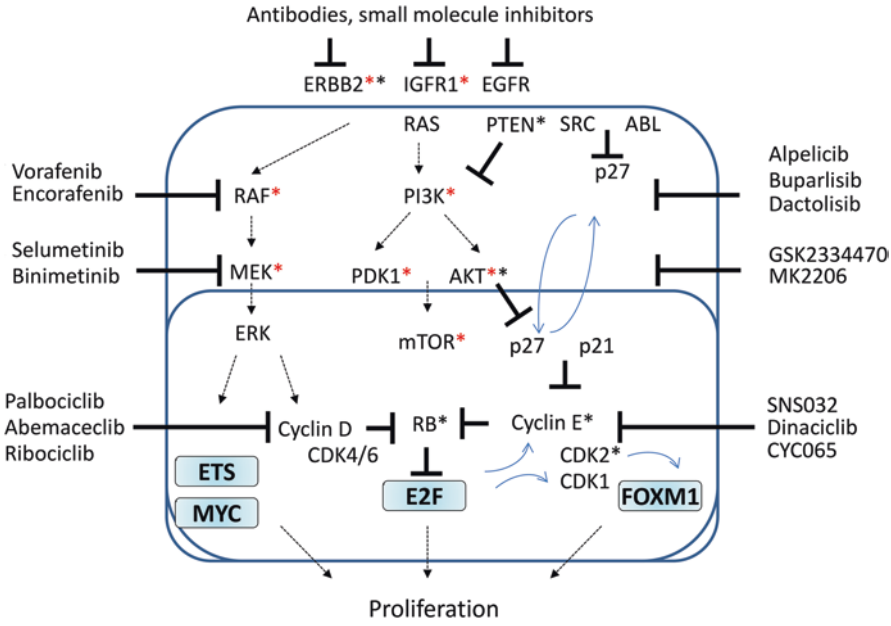

Fig. 8.2 Signaling pathways involved in proliferation. Red asterisks mark therapeutic targets for CDKi combination therapy, black asterisks mark genes involved in CDKi resistance

cyclin D2 and repressor of cyclin D1, while ETS factors act in concert with AP-1 (JUN/FOS) to stimulate cyclin D expression in the early G1 phase. Activation of the PI3 kinase pathway contributes to stabilization of cyclin D protein by inactivating the kinase GSK3β, which targets the cyclin D protein for proteasomal destruction. Cyclin D proteins then bind and activate CDK4/6. In contrast to CDK1/2, which have hundreds of known targets, the target spectrum of CDK4/6 is narrow, with the retinoblastoma (RB protein, *RB1* gene) protein and the closely related pocket proteins, p107 (*RBL1*) and p130 (*RBL2*) as its main targets. Pocket proteins, and the RB protein in particular, are powerful transcriptional repressors and mediators of cell cycle arrest [44]. RB interacts with more than 100 proteins and most of these interactions are poorly understood, but its well-known inhibitory effect on E2F-mediated transcription is central to its role as a tumor suppressor and negative regulator of the cell cycle. Phosphorylation of RB by CDK4/6-cyclin D complexes at multiple residues partially inactivates RB and allows for the transcription of some E2F target genes, such as *cyclin E*, *CDK1/2*, and *CDC25*, resulting in activation of CDK2-cyclin E complexes in late G1. As discussed above, additional signals are required to remove p21 and p27 from CDK2-cyclin E complexes and trigger CDK2 activity. Once active, CDK2 phosphorylates RB at additional serine residues as part of a positive feedback loop, thus amplifying the signal and further stimulating E2F-mediated transcription. The E2F transcription factor family comprises at least eight members belonging to different subgroups. Activator E2Fs (E2F1/2/3) drive transcription of genes

involved in DNA replication, cell cycle control, and DNA repair [45], such as *thymidine kinase* (*TK1*), *dihydrofolate reductase* (*DHFR*), *cyclin E2* (*CCNE2)*, *CDK1*, *CDC6*, *CDC25A*, and *BRCA1* [46, 47]. The importance of E2F target genes in cell cycle progression is demonstrated by the fact that overexpression of an activator E2F1 in quiescent fibroblasts is sufficient to induce S phase [48, 49]. Therefore, the complete inactivation (hyperphosphorylation) of RB at the end of the G1 phase has been termed the restriction point, after which the cell cycle progression occurs independently of growth factor stimulation.

E2F transcription factors have been considered the main mediators of G1–S phase progression, however mouse models have demonstrated that E2Fs are dispensable for proliferation in some systems [50, 51]. Thus, the redundancy observed among cell cycle CDKs may similarly apply to downstream mediators. For example, oncogenic ETS factors that become activated by translocation events in prostate cancer mimic mutant RAS and may directly regulate E2F target genes [52, 53]. Similarly, MYC family members have been found to compensate for loss of E2F function [50], and gain of the *MYC* locus on chromosome 8q24 was observed in ovarian cancer cell lines with acquired resistance to both palbociclib and SNS032, even though the functional relevance of this genomic gain was not investigated [47]. Downregulation of E2F target genes by CDKis is an important measure of on-target efficacy and is commonly observed in responsive cell lines and xenograft tumors treated with CDKis. Affected genes include *CCNE2*, *CDC6*, *CDC25* and others [47]. Moreover, cancer cells with acquired resistance to CDKis frequently restore E2F target gene expression [25, 47], and a RB loss signature, containing a number of E2F targets, is predictive of resistance to palbociclib in cell lines and patients [54]. When investigating the molecular mechanisms underlying restored E2F target gene expression in CDKi-resistant cells, possible compensation by non-E2F transcription factors should be considered. These may be the result of adaptations outside the CDK/cyclin-RB-E2F axis. The complex nature of cell cycle regulation, including the existence of numerous signaling pathways converging on CDK-cyclin activation (MAPK, PI3K, WNT, integrin signaling, etc.), coupled with functional redundancy among the transcription factors that drive cell cycle progression (E2F, ETS, MYC, FOXM1etc.) may explain why CDKis as single agents have limited potency in advanced human cancers. The combination of CDKis with agents targeting the MAPK pathway is currently being explored in various settings, such as in BRAF-mutant melanomas and in several RAS-mutant cancers [55] (Fig. 8.2).

Another transcription factor, FOXM1, is involved in cell cycle progression (reviewed in [4]. Recent studies have shown that FOXM1 is an important target of CDK1/2 phosphorylation in late G1 and S phases [56]. FOXM1, in turn, transcribes a number of genes required for successful assembly of the spindle apparatus and completion of mitosis. FOXM1 has also been identified as a direct substrate of CDK4 [57], and may play a role in suppressing senescence, thus warranting further investigation of its potential involvement in CDK inhibitor resistance.

8.5 Genomic Alterations Targeting the CDK-Cyclin-RB-E2F Axis in Cancer

Genomic alterations in the signaling network upstream and downstream of the RB pathway are frequent, if not universal, in advanced human cancers. Studies by The Cancer Genome Atlas (TCGA) have determined that the RB pathway, along with the p53 signaling network and the PTEN/PI3K network, is among the most commonly altered signaling pathway in glioblastoma (GBM), high-grade serous ovarian cancers (HGSC), breast and other cancers [58–60]. The type and frequency of a specific RB pathway lesion varies considerably among different cancers. For example, *CDKN2A/B* deletions resulting in loss of $p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$, are very common in GBM tumors. Moreover, deletion of *CDKN2A* is mutually exclusive with amplification of CDK4 and deletion or mutation of *RB1* in GBM [61, 62]. However, in addition to these signature lesions, further genetic alterations within the RB pathway and outside the RB pathway contribute to RB inactivation and E2F-mediated transcription. For example, in GBM the closely related INK4 member *CDKN2C* ($p18^{INK4C}$) is co-deleted with *CDKN2A* in some primary tumors and a larger proportion of cell lines [32]. Alternatively, genomic gain of *CDK6* or loss of one allele of *RB1* may contribute to RB inactivation, and the specific pattern of the RB pathway lesions in a given tumor may determine its response to CDKis [33].

In HGSC, on the other hand, deletions of *CDKN2A* are rare while amplification of *CCNE1* (cyclin E1) is observed in 20% of all primary HGSC [58, 63], often in conjunction with loss (rather than deletion) of *RB1*. In contrast, *RB1* mutations appear to be mutually exclusive with *CCNE1* amplification. In invasive breast cancer carcinomas, *CCNE1* amplifications and loss of *RB1* are enriched in the triple-negative or basal-like subtype while they are rare in the ER-positive subtype [60], where amplification of *CCND1* is the most common RB pathway lesion, while *CDK4* amplification, *CDKN2A* deletion and *RB1* mutation are relatively rare events. These differential patterns of the RB pathway inactivation may explain why most HGSC and triple-negative breast cancers are intrinsically resistant to CDK4/6 inhibition—they have genetic lesions downstream of CDK4/6 (*CCNE1* gain or amplification, *RB1* loss) that render them less dependent on CDK4/6. Cyclin E1 overexpression independently of genomic amplification is also frequent in HGSC and basal-like breast cancer (BLBC) but the underlying mechanisms are less clear. They may involve deletion of *PTEN* or other genomic events that result in the activation of the PI3K-AKT pathway, which may then activate CDK2-cyclin E via p27-dependent and independent mechanisms. Finally, both HGSC and BLBC are associated with p53 mutation, which is ubiquitous in HGSC and frequent in BLBC, and may indirectly contribute to CDK4/6 resistance. There is direct crosstalk between the p53 pathway and the RB pathway, e.g. via $p21^{CIP}$. In addition, p53 mutation contributes to genomic instability, thereby increasing intratumoral heterogeneity, which in turn may result in cells with different RB pathway alterations within a given tumor. It will be interesting to apply single cell approaches in the context of acquired CDK4/6 inhibitor resistance in order to further test this hypothesis.

8.6 CDK4/6-Dependent Cancers and CDK2-Dependent Cancers

An important question is whether we can predict CDK4/6-dependent cancers and CDK2-dependent cancers. Preclinical studies in mice and human cell lines strongly suggest that *CDKN2A* deletion indicates sensitivity to palbociclib and other CDK4/6 inhibitors. The underlying assumption is that cells that select for *CDKN2A* deletion must be dependent on the CDK4/6 function. However, in advanced solid tumors the existence of numerous genetic alterations within a single cell and multiple genetically different cell clones within a tumor increases the odds that some cancer cells are inherently resistant to a given therapeutic agent. Another complication stems from the fact that within the complex tumor architecture, protective niches may prevent the drug from accessing all cancer cells at effective concentrations, thus facilitating the rise of resistant cells. Finally, CDK4/6 inhibitors such as palbociclib are mainly cytostatic and enable cancer cells that are not being eradicated to rewire their proliferative machinery over time. Given these caveats, it may be easier to exclude tumors with certain genetic alterations, such as *RB1* deletion in the case of CDK4/6 inhibitors, than to actively select for CDK4/6-dependent cancers. Gene signatures associated with resistance may also become important diagnostic tools in this context.

ER-positive breast cancer appears to be a perfect scenario for CDK4/6 inhibition, as (1) there is a proven dependence of ER signaling on CDK4/6-cyclin D (2) CDK4/6 inhibitors mechanistically synergize with ER antagonists and (3) ER-positive breast cancers generally lack genomic alterations associated with CDK4/6 inhibitor resistance. This was realized by Richard Finn and colleagues in their groundbreaking paper that first described the differential sensitivity of different breast cancer subtypes to palbociclib [35].The identification of susceptible subtypes among other cancers is the subject of intense research and should result in the FDA approval of CDK4/6 inhibitors for additional indications.

Parallel efforts seek to identify CDK2-dependent cancers. Currently, *CCNE1*-amplified HGSC and BLBC may be among the best candidates. Several publications by the David Bowtell's group have shown that *CCNE1*-amplified ovarian cancer cell lines are dependent on cyclin E1, CDK2, and AKT [64–66]. Moreover, *CCNE1*-amplified cancers form a distinct subgroup that does not overlap with *BRCA*-mutant cancers and is intrinsically more resistant to platinum-based chemotherapy, the standard of care in HGSC, which is increasingly used for the treatment of BLBC. *CCNE1*-amplified HGSC overexpress a number of DNA repair genes, including *BRCA1* and *RAD51* [46, 67] and are dependent on the intact homologous recombination (HR)-mediated DNA repair. A genome-wide shRNA screen found that BRCA1 depletion in *CCNE1*-amplified cancer cell lines results in synthetic lethality [68]. Thus, therapeutic approaches aimed at inhibiting the HR machinery may be specifically effective in *CCNE1*-amplified cancers. Etemadmoghadam et al. used the proteasome inhibitor, bortezomib, which blocks the HR machinery, to specifically target *CCNE1*-amplified cancers. In an alternative

approach, it was shown that CDK1/2 inhibitors can sensitize ovarian cancer cells to platinum-based chemotherapy [47, 69], mediated at least in part by inhibition of BRCA1 and HR-mediated DNA repair. BRCA1 is a transcriptional target of E2F transcription factors [70], and both the BRCA1 and the BRCA2 proteins are directly phosphorylated by CDK2 and CDK1, suggesting that CDK1/2 inhibitors, such as dinaciclib, may have a dual effect on BRCA1/HR. Consistent with this notion, dinaciclib was found to impair HR in multiple myeloma cells, where it sensitized cells to PARP inhibition [71]. However, clinical studies with more selective CDK2 inhibitors are needed to conclusively ascertain if certain cancers are indeed dependent on CDK2.

8.7 Small Molecule Cyclin-Dependent Kinase Inhibitors

CDKis targeting cell cycle CDKs can be distinguished into CDK4/6 inhibitors and CDK2 inhibitors based on their intended target proteins, mechanism of action and responsive cancer types (Fig. 8.1). However, while palbociclib (PD0332991, IBRANCE®) and the two other CDK4/6 inhibitors, abemaciclib (LY2835219) and ribociclib (LEE011, KISQALI®) are highly target-specific (Table 8.1), there are currently no available compounds that exclusively inhibit CDK2. Dinaciclib (SHS727965) is a very potent inhibitor of CDK1 and CDK2 but in addition, targets CDK5 and CDK9, and possibly also CDK4/6 [72–74]. Thus, dinaciclib should be considered a potent pan-CDK inhibitor [74], which may limit its systemic use in combination with other agents, including chemotherapy. Another CDK2 inhibitor for which mechanisms of resistance have been investigated, SNS032 (BMS387032), appears to be more selective in its target spectrum but still inhibits CDK5 and CDK7 at nanomolar concentrations [75]. SNS032 had potent activity against ovarian cancer cell lines and xenografts with high cyclin E1 expression in a preclinical study [76]. However, after early clinical trials showed modest activity as a single agent, the clinical development of SNS032 was discontinued. A number of other CDKis are in development, including CYC065, an orally available inhibitor of CDK2/7/9 (Table 8.2), as well as novel specific inhibitors of CDK7 and CDK9 [77]. The specific inhibition of CDK7 or CDK9 is emerging as a novel therapeutic concept [78, 79], and agents originally developed as CDK2 inhibitors may find a new purpose as CDK9 inhibitors.

8.7.1 CDK4/6 Inhibitors

Several excellent recent reviews have covered the current state of CDK4/6 inhibitors in cancer treatment in great detail [80–83]. In their Cancer Discovery review, Charles Sherr, David Beach and Geoffrey Shapiro provide a fascinating historical perspective on the discovery of CDK4/6 and D-type cyclins, their identification as

Table 8.2 Mechanism of resistance of CDK inhibitors

CDKi	Mechanism of resistance	References
Palbociclib	*RB1* deletion/loss/mutation	[34] [35] [93] [33] [36] [47] [25]
	CCNE1 amplification/overexpression	[121] [47] [25]
	Activation of CDK2	[112] [25]
	Loss of p27 protein expression	[112] [93]
	MDM2 amplification	[94]
Abemaciclib	*CDK6* amplification	[132]
Dinaciclib	*ERBB2* amplification	[47]
	Activation of AKT signaling	[47] [66]
SNS032	*PTEN* deletion	[47]
	ERBB2 amplification	[47]
PHA-533533	Activation of CDK2	[65]
	Polyploidy	[65]

therapeutic targets and the setbacks that almost halted the development of palbociclib (then PD0332991) as a cancer drug [84]. Palbociclib was FDA-approved for ER-positive breast cancer in 2015, in combination with the aromatase inhibitor, letrozole. Early clinical trials established a dosing schedule of 125 mg/kg orally per day for 3 weeks followed by 1 week without drug. Palbociclib is currently in a number of clinical trials as a single agent or in combination with letrozole or fulvestrant. In RAS-driven lung cancers and melanoma, palbociclib is tested in combination with RAF and MEK inhibitors, and in mantle cell lymphoma it is tested in combination with ibrutinib (Imbruvica®), a Bruton's tyrosine kinase inhibitor. Palbociclib is generally well-tolerated; the most common side effects are neutropenia and thrombocytopenia, which are in accordance with phenotypes observed in CDK4/6−/− mice and the known requirement of CDK6 during myeloid development [81]. Results from a double-blind phase III study (PALOMA-2) testing palbociclib plus letrozole versus placebo plus letrozole in 666 postmenopausal women with ER-positive, HER2-negative breast cancer and withour prior systemic treatment for advanced disease revealed a significant increase in the mean progression-free survival for the palbociclib-letrozole combination compared to letrozole alone (mean PFS: 24.8 months for palbociclib/letrozole versus 14.5 months for placebo/letrozole) [22]. In the earlier PALOMA-1 trial with 165 women, the mean PFS was 10.2 months with letrozole alone and 20.2 months for the combination [23]. This trial initially maintained a second, independent cohort of patients with *CCND1*

amplification and *CDKN2A* loss but accrual was stopped after an interim analysis and both cohorts were analyzed together. Given the caveat of small numbers and early termination, *CCND1* amplification or *CDKN2A* loss did not predict benefit from palbociclib in this trial.

The PALOMA-3 trial, a double-blind, randomized phase 3 study, investigated the combination of palbociclib with fulvestrant in 521 women with ER-positive, HER2-negative metastatic breast cancer that had progressed on previous endocrine therapy [85]. As observed in the letrozole trials, palbociclib in combination with fulvestrant significantly extended the median progression-free survival (9.5 months for fulvestrant plus palbociclib versus 4.6 months for fulvestrant plus placebo), while resulting in more grade 3 or 4 adverse events (73% in the fulvestrant plus palbociclib group and 22% in the fulvestrant plus placebo group, the most common adverse event was neutropenia).

Similarly, ribociclib and abemaciclib have entered early and late stage clinical trials in breast and other cancers [80]. The MONALEESA-2 trial resulted in FDA approval for ribociclib in combination with an aromatase inhibitor in March 2017. In postmenopausal women with hormone receptor –positive, HER2-negative advanced or metastatic breast cancer, ribociclib (600 mg daily for 21 days, followed by 7 days off) in combination with letrozole (2.5 mg daily continuously) resulted in significantly improved progression-free survival compared to letrozole plus placebo. Notably, ribociclib is also being tested in a phase I/II study in combination with the BRAF inhibitor encorafenib (LGX818) in *BRAF*-mutant melanoma and in combination with the MEK inhibitor binimetinib in *NRAS*-mutant melanoma. While the efficacy and toxicity profiles among the three CDK4/6 inhibitors are very similar, there are some notable differences: palbociclib and ribociclib are most selective for CDK4/6, while abemaciclib co-targets CDK9 and may have the best single agent efficacy. Whether its efficacy is related to co-inhibition of CDK9 remains to be elucidated. Abemaciclib may also be readily absorbed across the blood-brain barrier, which would make it the preferred CDK4/6 inhibitor for the treatment of glioblastoma and other brain tumors, although palbociclib has been reported to cross the blood-brain barrier as well. Of note, two glioblastoma patients have achieved long term benefit on abemaciclib [86]. Abemaciclib is also being studied for patients with breast cancer in combination with both endocrine therapies and agents targeting the ERBB2 or PI3K–AKT–mTOR pathways.

8.7.2 CDK2 Inhibitors

Dinaciclib (MK-7965) versus the chemotherapeutic drug capecitabine was tested in a phase II clinical trial in women with previously treated advanced breast cancer [87]. In contrast to CDK4/6 inhibitors, dinaciclib is not available orally but administered as an infusion every 21 days at 50 mg/m^2. The dosing schedule is reflective of the considerably higher toxicity of dinaciclib compared to CDK4/6 inhibitors, which are administered daily for 21 consecutive days. In this setting, dinaciclib as a single agent

was less effective than capecitabine, even though antitumor activity was seen in two of seven patients with ER–positive and HER2–negative metastatic breast cancer, and even in a patient with triple-negative breast cancer. As with CDK4/6 inhibitors, grade 3 or 4 adverse events, such as neutropenia and leukopenia, were common. A phase III study in chronic lymphocytic leukemia (CLL) comparing dinaciclib and the anti-CD20 antibody ofatumumab reported partial responses in 40.0% of dinaciclib-treated patients and 8.3% of ofatumumab-treated patients, with stable disease in 35.0% and 45.8%, respectively. Dinaciclib also resulted in longer median survival (21.2 months with dinaciclib versus 16.7 months with ofatumumab). In another phase III trial in relapsed multiple myeloma, single agent dinaciclib resulted in partial responses in 3 out of 27 patients [88]. Its efficacy in this context may be attributable to CDK5 inhibition. CDK5 has a critical role in multiple myeloma and was identified as a potential resistance factor to bortezomib [11]. Therefore, the combination with bortezomib may be an effective strategy for dinaciclib in multiple myeloma. Dinaciclib is also tested in combination with the PARP inhibitor, veliparib.

Other CDK2 inhibitors have not progressed far in clinical trials. Earlier CDKis, such as flavopiridol and roscovitine, failed in the clinic due to lack of potency in relation to associated toxicity. SNS032, an inhibitor of CDK2, CDK5, CDK7 and CDK9, was tested in several phase I trials in refractory metastatic solid tumors, multiple myeloma and CLL [89]. Sporadic responses resulting in stable disease were observed but the compound is no longer in clinical development. Milciclib is a potent, ATP-competitive CDK inhibitor for CDK2-cyclin A with an IC_{50} of 45 nM. However, it is less potent at inhibiting CDK2-cyclin E (IC_{50} = 363 nM), and it co-targets a number of other CDKs, as well as TrkA, a receptor for nerve growth factor. In a phase II trial milciclib has shown some activity in patients with thymic carcinoma. Another novel CDK2 inhibitor, CYC065, is currently being tested in early clinical trials.

8.7.3 CDK Inhibitors: Mechanism of Action

In order to define mechanisms of resistance to CDKis it is important to identify favorable outcomes. Specific inhibition of CDK4/6 or CDK2 induces a temporary cell cycle arrest that is entirely reversible unless it can be manipulated to drive the cell into a permanent state of arrest (e.g. senescence) or cell death. Quiescence (also referred to as G0 state) is characterized by an indefinite exit from the cell cycle. Quiescent cells lower their metabolism but remain responsive to mitogenic stimuli, i.e. they can reenter the cell cycle after receiving appropriate stimuli. In contrast, senescence is usually a permanent state of arrest, characterized by high metabolic activity and secretion of cytokines that may result in clearance by the immune system [90–92]. Although the role of senescence in tumor initiation and progression is somewhat controversial, chronic CDK4/6 inhibition can induce senescence in sensitive cells, and is associated with a favorable response in this context [25, 47, 93–96]. It has been difficult to establish consistent biomarkers of senescence. However,

senescence-associated β-galactosidase activity, senescence-associated heterochromatin foci, and a senescence-associated hyper-secretory phenotype, as well as high levels of endogenous CDKi, such as $p16^{INK4A}$ and $p21^{CIP}$, are frequently found in senescent cells [97–99]. Senescence may be an important outcome in cancer therapy but has been difficult to detect *in vivo*.

Most current CDKis are ATP-competitive inhibitors that reversibly inhibit their CDK targets. In most sensitive cells, CDK4/6 inhibition by palbociclib, ribociclib or abemaciclib results in a clean RB-dependent G1 arrest without significant induction of apoptosis. The G1 arrest can be easily visualized by FACS profiling, detection of hypophosphorylated RB-pSer780/807/811, and transcriptional downregulation of E2F target genes, such as *CCNE2*, *CDC25*, *CDK1* and *BRCA1/2*. This form of cell cycle arrest in response to short term exposure to CDK4/6 inhibitors is detectable *in vitro* and in treated xenograft tumors. The lack of cytotoxicity associated with CDK4/6 inhibitors translates to cytostasis in treated tumors; as single agents CDK4/6-specific inhibitors frequently result in stable disease while tumor regressions are rare. Similarly, specific inhibition of CDK2 is associated with G1 arrest in susceptible cells. However, since current CDK2 inhibitors such as dinaciclib co-target other CDKs, they elicit a mixed cell cycle profile with cells in G1, G2/M and significant induction of cell death depending on the drug. For that reason, the combined inhibition of CDK4/6 with current CDK1/2 may be counterproductive. While CDK4/6 inhibitors and CDK1/2 inhibitors are expected to be effective against different cell populations within a tumor, and while this may have complementary effects in theory, in practice it has been hard to achieve high enough doses that are tolerated by the patient while still being effective against the tumor. If the desired outcome is cell death (e.g. following mitotic catastrophe) then cell cycle arrest may have an inhibitory effect as it gives the cell time to recover and endure in a quiescent state until it has adapted (e.g. by activation of DNA repair pathways and concomitant upregulation of anti-apoptotic proteins such as Mcl-1). In CDK4/6-independent cancers, the protective effect of CDK4/6 inhibition may actively protect normal cells from cytotoxicity during radiation treatment [100]. Furthermore, in a pharmacological screen for agents synergistic with CDK4/6 inhibition, several cytotoxic agents displayed antagonistic effects [25]. In contrast, the combination of CDK1/2 inhibitors with chemotherapy or PARP inhibitors has been successful in preclinical studies. The administration of CDKis in between chemotherapy cycles may be another option.

The combination of CDK4/6 inhibitors with other agents can induce a more permanent cell cycle arrest or shift the mechanism of response from cell cycle arrest to cell death or senescence. Selina Chen-Kiang's work not only helped resurrect CDKis as anti-cancer drugs by pioneering studies on palbociclib (then PD0332991) long before its potential became widely recognized but also resulted in several clinical trials in mantle cell lymphoma and multiple myeloma. Her group has demonstrated the prolonged early G1 arrest induced by palbociclib can sensitize lymphoma cells to ibrutinib killing [101–104]. In a recent publication by Kovatcheva et al., depletion of MDM2 triggered senescence in well-differentiated and dedifferentiated liposarcoma cell lines that were treated with palbociclib [94]. MDM2 depletion

shifted the response to palbociclib from quiescence to senescence in a p53-independent manner. Palbociclib has already shown activity in a liposarcoma trial [105]. Interestingly, the *MDM2* gene is frequently co-amplified with *CDK4*, as both genes reside on human chromosome 12q14 and may thus modulate CDK4/6 inhibitor sensitivity in *CDK4*-amplified cancers. Combinations of CDK4/6 inhibitors with MAPK pathway inhibitors may similarly result in senescence or cell death, which induce tumor shrinkage (partial responses) rather than cytostasis (stable disease).

8.8 Mechanisms of CDKi Resistance

Since CDKis have only entered clinical treatment regimens relatively recently, there is little information available from the analysis of tumor specimens that were treated with CDKis, had a temporary response, and then became drug-resistant. In contrast to other targeted agents, such as inhibitors of ER, RTK and MAPK, our current knowledge of resistance mechanisms to CDKis is limited and mostly derived from preclinical studies. Another complicating factor is that CDK4/6 inhibitors are usually administered in combination with letrozole or fulvestrant, so that resistant tumors have adapted to the combination of both drugs. However, resistance to ER antagonists has been studied for decades, so it will be interesting to see what additional mechanisms arise in response to treatment with CDK4/6 inhibitors. As with other targeted agents, several strategies can be pursued to delay or revert acquired resistance to CDKis, although acquired drug resistance appears inevitable when treating advanced solid tumors, given their inherent genetic and cellular heterogeneity and adaptability. Several approaches have been taken to identify rational drug combinations that enhance the efficacy of CDKis and delay the onset of resistance. Since cyclin D can associate with CDK2 in CDK4/6 inhibitor-treated cells, the acquired resistance to CDK4/6 may be delayed by inhibition of pathways that activate cyclin D, such as the MAPK pathway, the PI3K pathway, or estrogen receptor signaling. However, these combinations usually cannot resensitize cells that have acquired resistance.

8.8.1 Inhibition of the PI3K-AKT-mTOR Pathway to Delay CDK4/6 Inhibitor Resistance

Herrera-Abreu et al. investigated resistance mechanisms for palbociclib and ribociclib in models of breast cancer [25]. They performed a pharmacological screen to identify agents that synergize with palbociclib and found that inhibitors of the RTK-PI3K-AKT-mTOR pathway were among the best combination drugs. Specifically, the AKT inhibitor MK2206, the mTOR inhibitor everolimus, the PDK1 inhibitor GSK2334470 and the IGFR1 inhibitor OSI906, as well as other PI3K/mTOR

inhibitors, all acted synergistically with palbociclib. Biochemically, co-treatment with a PI3K inhibitor or IGFR1 inhibitor resulted in a more complete reduction of phospho-pRB Ser807/811, and suppression of downstream targets, such as cyclin E2 protein. Interestingly, co-inhibition of PI3K shifted the primary response from cell cycle arrest to cell death, which resulted in tumor regressions in patient-derived xenograft (PDX) models. Synergy was observed in ER-positive cell lines with concomitant deletion of *PTEN* or mutation of *PIK3CA*, and in the cell line, BT474, a HER2-positive/*PIK3CA*-mutant cell line. In contrast, no synergy was observed in ER-positive/*PIK3CA*-wildtype cell lines (sensitive to palbociclib but no added effect by PI3K inhibition), three triple-negative breast cancer cell lines, and in HCC1569, a basal-like cell line that is positive for HER2 and *CCNE1*-amplified (resistant to palbociclib). On the other hand, several drugs showed antagonist effects when combined with palbociclib. These included cytotoxic drugs, such as topoisomerase inhibitors, the microtubule inhibiting agent paclitaxel, and the chemotherapeutic agent gemcitabine. Interestingly, a WEE1 inhibitor also displayed antagonism with palbociclib, which is in line with the role of WEE1 kinase as an inhibitor of CDK activity. In a PDX model, combining ribociclib, fulvestrant and the PI3K inhibitor alpelisib (BYL719) caused greater tumor regression compared with fulvestrant paired with either ribociclib or alpelisib [25]. Based on these data, a phase II clinical trial assessing the triplet combination was recently initiated. Another publication by the Arteaga lab describes a kinome screen that identified PDK1 activation as a mechanism of resistance to ribociclib, confirming the importance of the PI3K pathway in CDKi resistance [106].

8.8.2 *Inhibition of the MAPK Pathway to Delay CDK4/6 Inhibitor Resistance*

The combination of agents targeting the MAPK pathway and CDK4/6 inhibitors has been suggested for cancers that depend on the MAPK signaling axis, namely mutant RAS-driven cancers and mutant BRAF-driven melanomas. The RAS-RAF-MEK-ERK pathway is a powerful inducer of cyclin D, and preclinical models of RTK/RAS-driven cancers have suggested a requirement for cyclin D-CDK4/6. For example, CDK4 ablation is synthetic lethal in *KRAS*-mutant lung cancer cells [107]. Effective combination of palbociclib with a MEK inhibitor, selumetinib or trametinib (GSK1120212), was first demonstrated in an NRAS-driven mouse model of melanoma, where it displayed potent synergy *in vitro* and *in vivo*, leading to regression in established tumors [108], suggesting that combined inhibition of CDK4/6 and the MAPK pathways has a dual effect on proliferation and survival. A phase I/II clinical trial with ribociclib and binimetinib in NRAS-mutant melanomas has delivered promising preliminary results that included partial responses or stable disease in the majority of patients. Similar results were subsequently obtained in *KRAS*-mutant colorectal cancer cell lines and xenografts using the combination of palbociclib and trametinib [55].

8.8.3 Combined Inhibition of CDK2 and CDK4/6

At present, combined targeting of CDK2 and CDK4/6 is essentially equal to pan-CDK inhibition, since all currently available CDK2 inhibitors co-target CDK1, CDK5, CDK7, or CDK9. Flavopiridol, the first pharmacological CDKi to be tested in clinical trials, had a similar target profile, and ultimately failed in the clinic due to its unfavorable therapeutic window. The CDK2/4 double knockout mouse is embryonic lethal, suggesting that if a highly specific CDK2 inhibitor were available, one would expect considerable potency but also moderate to severe toxicity. *In vitro* studies suggest that in the short term, inhibition of CDK2 by RNAi or pharmacological inhibitors can significantly prevent or delay the outgrowth of CDK4/6 inhibitor-resistant colonies. This was demonstrated in ovarian cancer cell lines [47] and in breast cancer cell lines [25].

8.8.4 Acquired Resistance to CDK Inhibitors

Our current knowledge of mechanisms of acquired resistance to CDKis is largely based on preclinical models. With the conclusion of several phase III clinical trials for CDK4/6 and CDK1/2 inhibitors it will be interesting to see which of the proposed mechanisms can be recapitulated in recurrent tumor samples. While hitherto unreported, we may expect to see novel mutations in CDK genes that render the kinases resistant to CDKis. Such mutations have been reported for a number of kinase targets, including EGFR, BTK and the estrogen receptor. ER mutations have also been detected in the blood of patients treated with the palbociclib/letrozole combination but it is unknown if these contribute to palbociclib resistance [109]. However, restoration of estrogen signaling likely results in increased CDK2/4 activity [110]. CDK mutations are rare in primary tumors, one exception being the R24C mutation found in familial melanoma. This mutation renders mutant CDK4 refractory to inhibition by $p16^{INK4A}$ and other members of the INK family. In mouse models, deficiency for $p27^{KIP}$ but not $p18^{INK4C}$ strongly cooperates with the R24C mutant in the formation of pituitary tumors [111]. Indeed, loss of p27 expression has been proposed as on mechanism of resistance to CDK4/6 inhibitors that contributes to activation of CDK2 [93, 112].

Analysis of palbociclib-resistant cells has revealed that there are several distinct mechanisms of acquired CDK4/6 resistance, each one possibly requiring a different therapeutic strategy to counter or reverse resistance. Several themes are emerging: 1. Loss of at least one copy of *RB1*, possibly decreasing the threshold for CDK activity required to inactivate RB. 2. Amplification of cyclin E1 and subsequent activation of CDK2. Overexpressed cyclin E1 may also bind to and activate CDK1 directly, thus bypassing the need for the earlier cell cycle kinases. And, 3. Activation of CDK1/2 by other mechanisms, such as PI3K signaling upstream of the CDK-RB-E2F axis. This signaling pathway may result in decreased p27 tumor suppressor

activity and activation of CDK2 but, in addition, affects a number of other cellular targets, including mTOR signaling. For CDK2 inhibitors, it has been more difficult to establish clean mechanistic resistance mechanisms due to the aforementioned promiscuity of current CDK2 inhibitors. However, receptor tyrosine kinase signaling and activation of the PI3K-AKT axis are emerging mechanisms of acquired resistance to CDK2 inhibitors [47, 66].

8.8.5 Loss of RB1

RB deficiency is the only consistent biomarker of CDK4/6 inhibitor resistance. With the narrow target spectrum of CDK4/6, RB and the related pocket proteins are their main targets, and RB deficiency accurately predicts resistance to palbociclib in all tested cell systems. Moreover, an 87 gene "RB loss" signature, containing many E2F target genes, predicts sensitivity to palbociclib in cancer cell lines and treated patients [54]. This signature may be of help in identifying emerging resistance in patients treated with CDK4/6 inhibitors. Loss of *RB1* has been observed in breast and ovarian cancer cell lines treated with palbociclib [47, 93]. There is currently no therapeutic strategy to reverse the effects of *RB1* loss. Since the human gene is in close vicinity to the *BRCA2* gene on chromosome 13, deletions affecting both genes could sensitize palbociclib-resistant cells to platinum-based chemotherapy or PARP inhibition (reviewed in [46]).

8.8.6 Amplification of CCNE1

The *CCNE1* gene on chromosome 19q is frequently amplified in human cancers, including breast, ovarian, lung and other cancers [58, 113–116], with frequency of amplification ranging from 2 to 40% [114, 117, 118]. Some publications correlate *CCNE1* amplification with poor overall survival but this correlation remains controversial and its mechanistic basis poorly understood [119, 120]. Amplification of the *CCNE1* and subsequent overexpression of cyclin E1 is associated with resistance to CDK4/6 inhibition in high-grade serous ovarian and basal-like breast cancers. Ectopic expression of cyclin E1 conferred partial resistance to palbociclib in HEY ovarian cancer cells (*CDKN2A*-null, *p53* wildtype). While proliferation remained impaired in the presence of palbociclib, ectopic cyclin E1 prevented palbociclib-induced senescence [47]. Ectopic expression of cyclin E1 or cyclin E2 also rendered T-47D breast cancer cells less sensitive to anti-estrogen treatment and palbociclib [121]. These studies established *CCNE1* as a *bona fide* CDK4/6 inhibitor resistance gene and were validated by the detection of *CCNE1* amplification events in breast cancer cells that had acquired resistance to palbociclib [25]. In these cells, siRNA-mediated depletion of CDK2 rescued the palbociclib-resistant phenotype and resensitized the cells to palbociclib, suggesting the combined inhibition of CDK2/4/6

as a therapeutic strategy in this context. However, as discussed above, this strategy may require more specific and less toxic CDK2 inhibitors. In addition, Scaltriti et al. demonstrated that the *ERBB2*-amplified breast cancer cell line BT474 selectively amplifies the *CCNE1* gene as a mechanism of resistance to trastuzumab [122]. Trastuzumab-resistant clones become highly dependent on cyclin E1, and siRNA-mediated knockdown or treatment with a CDK2 inhibitor drastically reduced the growth of xenografts [122]. Interestingly, other published data show that ectopically overexpressing ERBB2 renders *CCNE1*-amplified cancer cells resistant to CDK2 inhibitors [47], suggesting that cyclin E1 and ERBB2 cooperate in acquired CDKi resistance. Therapeutic strategies against *CCNE1*-amplified cancers are discussed in detail in a recent review [63] and may be applied as follow-up treatment in cancers that amplify *CCNE1* in the process of acquired CDK4/6 inhibitor resistance.

8.9 Activation of CDK1/2 by Other Mechanisms

Activation of CDK1 and CDK2 independently of *CCNE1* amplification play a role in acquired resistance to CDK4/6 and CDK2 inhibitors but the underlying mechanisms are less clear and may involve several cooperating events, including activation of the PI3K-AKT pathway by loss of the PI3K antagonist, *PTEN*, genomic gains of one of the three *AKT* genes, and gain of PI3K genes [47]. AKT inhibition resensitized CDKi-resistant cell lines to dinaciclib [66], while PI3K inhibition did not. Polyploidy may also contribute to CDK2 inhibitor resistance [65], as well as alterations in signaling pathways converging on the endogenous CDKi, $p27^{KIP}$ and $p21^{CIP}$ (Fig. 8.2).

8.9.1 Epilogue

We are constantly adjusting and refining our knowledge of the mechanisms of cellular proliferation and their deregulation in cancer. Due to the high prevalence of RB pathway alterations in human cancers it seems certain that CDK inhibitors will play an important role as anti-cancer agents. They may serve as sensitizers in combination with chemotherapy, maintenance therapy in between chemotherapy intervals or, due to the anti-apoptotic function of CDK4/6 inhibition, protect non-cancer cells from chemotherapy-related or radiation-induced cell death. Novel therapeutic combinations will be identified for the treatment of advanced human cancers but it is also conceivable that CDKi may assume a role in cancer prevention. Recent reports suggest that metastasis may occur earlier than previously thought [123, 124], and dinaciclib was shown to prevent metastasis in preclinical model of breast cancer [125]. As CDKi become more prevalent in clinical settings, novel mechanisms of resistance will emerge that restore CDK function and E2F activity. Almost

certainly, additional signaling pathways with roles in CDKi resistance will be uncovered. For example, the YAP1 oncogene can bypass oncogenic KRAS addiction in pancreatic cancer cells and may also contribute to cell cycle deregulation [126]. Non-canonical cell cycle CDKs, such as CDK8, may indirectly drive progression via the WNT signaling pathway [127, 128]. Finally, CDKi may also be useful as immunomodulatory agents due to their ability to induce cell cycle arrest and senescence, which alter the secretory profile of affected cells.

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 9
Resistance to Inhibitors of Angiogenesis

Nili Dahan, Ksenia Magidey, and Yuval Shaked

Abstract Angiogenesis, a process that is predominantly driven by the vascular endothelial growth factor (VEGF) signaling pathway, plays an essential role in tumor progression and metastasis. Accordingly, a range of anti-angiogenic agents, most of which block VEGF or its receptor, have been approved for the treatment of various malignant diseases. However, the clinical benefits of anti-angiogenic therapy are relatively modest for several reasons, some of which are related to the development of therapy resistance. Since anti-angiogenic agents target the tumor-supporting vascular system rather than the tumor cells themselves, resistance is dependent on the interplay between the host- and tumor-mediated pathways. In general, the activation of various evasive mechanisms allows for sustained tumor vascularization and growth despite the therapeutic blockade of the drug target. These mechanisms include the upregulation of bypass angiogenic pathways, pro-angiogenic activity of infiltrating stromal cells and alternative vascularization processes. In addition, off-target effects of anti-angiogenic drugs have implications for tumor aggressiveness. In this chapter, we discuss the molecular and cellular mechanisms contributing to therapy resistance as well as possible strategies to improve the clinical outcome.

Keywords Chemokines • Chemokine receptors • Tumor microenvironment • Angiogenesis • Bone marrow-derived cells

Abbreviations

BMDC	Bone marrow-derived cell
CAF	Cancer-associated fibroblast
CRC	Colorectal cancer

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Y. Yarden, M. Elkabets (eds.), *Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways*, Resistance to Targeted Anti-Cancer Therapeutics 15, https://doi.org/10.1007/978-3-319-67932-7_9

ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GBM	Glioblastoma multiforme
G-CSF	Granulocyte colony stimulating factor
GIST	Gastrointestinal stromal tumor
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor-1
MDSC	Myeloid-derived suppressor cell
NSCLC	Non-small-cell lung cancer
OS	Overall survival
PDGF	Platelet-derived growth factor
PFS	Progression-free survival
PlGF	Placental growth factor
PNET	Pancreatic neuroendocrine tumor
RCC	Renal cell carcinoma
SCF	Stem cell factor
SDF-1α	Stromal derived factor-1α
TAM	Tumor-associated macrophage
TEM	Tie2-expressing monocyte
TH17	T helper type 17
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

9.1 Introduction

Angiogenesis, the formation of new blood vessels from existing vasculature, plays an essential role in tumor progression and metastasis. The angiogenic process involves the activation, proliferation and migration of endothelial cells toward angiogenic stimuli produced by the tumor and supporting stromal cells within the tumor microenvironment. This ultimately results in the formation of new blood vessels that supply the growing tumor with nutrients and oxygen. This “angiogenic switch” is recognized as a rate-limiting event in tumor progression [1]. The concept of anti-angiogenic therapy was first proposed over four decades ago by Judah Folkman. He postulated that since the growth of all solid tumors is dependent on angiogenesis, inhibiting this process should suppress tumor growth [2]. It is now well-established that one of the most potent factors driving angiogenesis, and especially tumor angiogenesis, is the vascular endothelial growth factor-A (VEGF-A).

Based on the key role of this factor in tumor angiogenesis, numerous therapies that block various components of the VEGF signaling pathway have been developed [3]. Several such therapies have been approved for the treatment of a variety of human cancers and there are more in preclinical and clinical trials. However, despite the potent activity of these agents and the high expectations for this therapeutic strategy, the clinical benefits are proving to be relatively mild. In the majority of patients, anti-angiogenic therapy achieves transient tumor control, with only a modest gain in long-term survival [4]. This can be explained by several mechanisms of resistance that allow the tumor to evade the therapeutic inhibition of angiogenesis. Here we discuss the molecular and cellular events underlying resistance in different tumor contexts, distinguishing between tumor- and host-mediated mechanisms.

9.2 Inhibitors of Angiogenesis: Mode of Action and Clinical Use

Although angiogenesis is a highly complex process, it is driven by one predominant key player, VEGF-A (hereafter, referred to as VEGF) in both physiological and pathological conditions. VEGF signals through its main receptor expressed on endothelial cells, VEGFR2, thereby coordinating the biological processes necessary for new vessel formation. These processes include: endothelial cell proliferation, migration, invasion and survival; chemotaxis and homing of bone-marrow-derived endothelial precursor cells; vascular permeability; and vasodilation [3, 5]. Whereas autocrine VEGF, released by endothelial cells, maintains vascular homeostasis [6], paracrine VEGF, released by both tumor cells and stromal myeloid cell types, increases vessel branching resulting in abnormal, tortuous vasculature [7]. VEGF is upregulated in most solid tumors. Furthermore, slight increases in tumor VEGF levels are sufficient to promote angiogenesis and tumor growth. Accordingly, it was proposed that neutralizing circulating VEGF would suppress tumor growth, as demonstrated by a number of cancer models in mice [3, 8].

Since 2004, several drugs that target VEGF or its receptor have been approved by the Food and Drug Administration (FDA) for the treatment of various malignant diseases (Table 9.1), and there are more in clinical trials. These drugs include neutralizing antibodies against VEGF and VEGFRs, soluble VEGF receptor hybrids (VEGF traps) and tyrosine kinase inhibitors (TKIs) with selectivity for VEGFRs. It should be noted that, due to their mode of action at the ATP-binding pocket, TKIs designed to target VEGFRs may also significantly inhibit other kinases. Nevertheless, their potent anti-angiogenic activity has been demonstrated in preclinical studies [3]. Bevacizumab, a monoclonal antibody against VEGF, was the first anti-angiogenic drug to be approved by the FDA. It is currently used as first-line therapy in metastatic colorectal cancer (CRC), non-small-cell lung cancer (NSCLC) and renal cell carcinoma (RCC), as second-line therapy in CRC and glioblastoma multiforme (GBM), and as maintenance therapy in advanced ovarian cancer (Table 9.1).

Table 9.1 Clinical benefits of approved anti-angiogenic drugs

Drug	Drug class	Approved use	Treatment combination	Improvement in RR (%)	Improvement in PFS (months)	Improvement in OS (months)	Ref.
Bevacizumab	VEGF-A antibody	Metastatic CRC	Chemotherapy	10	4.4	4.7	[139]
				14.1	2.6	2.1	[140]
				0	1.4	1.4	[141]
		Metastatic NSCLC	Chemotherapy	20	1.7	2	[142]
				10.3–14	0.4–0.6	NS	[143, 144]
		Metastatic RCC	IFNα	12.4	3.3	NS	[145, 146]
				18	4.8	2	[147, 148]
		Advanced ovarian cancer	Chemotherapy	19	NS	NS/4.8[a]	[149, 150]
				NA	3.8	NS	[151]
		GBM	Monotherapy	Only phase II data reported			[152]
Ramucirumab	VEGFR2 antibody	Metastatic GEJ	Chemotherapy	12	1.5	2.2	[153]
			Monotherapy	0.8	0.8	1.4	[154]
Aflibercept	VEGF trap	Metastatic CRC	Chemotherapy	8.7	2.2	1.4	[155]
Sorafenib	TKI	Metastatic RCC	Monotherapy	8	2.7	NS	[156, 157]
		Metastatic HCC	Monotherapy	1	NS	2.8	[158]
Sunitinib	TKI	Metastatic RCC	Monotherapy	25	6	4.6	[159, 160]
		Metastatic GIST	Monotherapy	NA	20.9	NA	[161]
		PNET	Monotherapy	9.3	5.9	NA	[162]
Pazopanib	TKI	Metastatic RCC	Monotherapy	27	5	NS	[163, 164]

Drug	Drug class	Approved use	Treatment combination	Improvement in RR (%)	Improvement in PFS (months)	Improvement in OS (months)	Ref.
Axitinib	TKI	Advanced RCC	Monotherapy	NA	2.6	NS	[165][b]
Regorafenib		Metastatic CRC	Monotherapy	0.6	0.2	1.4	[166]
Nintedanib	TKI	Advanced NSCLC	Chemotherapy	NA	0.7	1	[167][c]
Vandetanib	TKI	Advanced medullary thyroid cancer	Monotherapy	43	6.2	NA	[168]
Cabozantinib	TKI	Advanced medullary thyroid cancer	Monotherapy	28	7.2	NS	[169]

CRC colorectal cancer, *GBM* glioblastoma multiforme, *GEJ* gastric and gastroesophageal junction cancer, *GIST* gastrointestinal stromal cancer, *HCC* hepatocellular carcinoma, *NA* not available, *NS* not significant, *NSCLC* non-small-cell lung cancer, *OS* overall survival, *PFS* progression-free survival, *PNET* pancreatic neuroendocrine tumors, *RCC* renal cell carcinoma, *RR* response rate, *TKI* tyrosine kinase inhibitor

[a]In poor-prognosis patients

[b]Axitinib vs. sorafenib

[c]Approved only in Europe

Of note, bevacizumab generally failed to provide significant benefits when used as monotherapy. However, with the exception of GBM, it has been approved for use as combination therapy for the treatment of the above-mentioned advanced-stage cancers [9]. The TKIs, sorafenib, sunitinib, pazopanib and axitinib are approved as monotherapies for the treatment of metastatic RCC, a highly vascularized tumor type. In addition, sunitinib is approved for gastrointestinal stromal tumors (GIST) and pancreatic neuroendocrine tumors (PNET), and sorafenib for hepatocellular carcinoma (HCC), for advanced-stage disease in all cases (Table 9.1). Other anti-angiogenic therapies approved for late-stage, metastatic disease are described in Table 9.1. These include: ramucirumab, a VEGFR2 monoclonal antibody; aflibercept, a VEGF-trap that binds 3 VEGF family ligands; and other VEGFR TKIs. Anti-angiogenic agents have also been evaluated for early-stage disease, specifically in the adjuvant setting, when treatment is administered after surgical removal of the primary tumor. It has been postulated that inhibiting angiogenesis after tumor resection would prevent local relapse or growth of micrometastases [10]. However, two large phase III post-operative adjuvant trials of bevacizumab in combination with chemotherapy in patients with early-stage CRC failed to provide significant benefits when compared to treatment with chemotherapy alone [11–13]. The use of anti-angiogenic therapy in the neo-adjuvant setting in order to downsize or downstage a tumor before resection has also been evaluated. However, two large trials testing the efficacy of neoadjuvant bevacizumab in combination with chemotherapy in comparison to neoadjuvant chemotherapy alone in patients with primary breast cancer revealed conflicting findings in terms of long-term benefits [14, 15]. The diverse outcomes of anti-angiogenic therapy in different clinical scenarios highlight the effects of specific parameters, such as disease stage and cancer type, on therapy efficacy. However, our understanding of the underlying mechanisms is still incomplete.

In general, protein-based anti-angiogenic drugs, such as bevacizumab and aflibercept, have only shown significant activity when combined with cytotoxic chemotherapy, whereas TKIs are effective when used as monotherapy, without an additive effect when combined with chemotherapy [10]. Conceivably, in cases where single-agent activity is observed, such as in RCC, therapy-induced vessel regression is the major mechanism of action contributing to the efficacy of therapy. In cases where anti-angiogenic therapies only show activity when combined with cytotoxic chemotherapy, such as in CRC, mechanisms other than vessel regression may play a role [3]. A widely-held view is that anti-angiogenic therapy improves the delivery of co-administered chemotherapy through a process known as “vascular normalization”. This is based on the principle that the abnormal tumor vasculature, which is known to be dysfunctional, leaky and tortuous, can be “normalized” by suppressing VEGF signaling. The resulting improvement in vessel function and blood flow is presumed to increase delivery of cytotoxic agents [16]. An alternative possibility explaining the benefit of combined therapy is that anti-angiogenic agents block the activity of bone marrow-derived endothelial progenitor cells that have been shown

to infiltrate tumors in response to chemotherapy drugs [8, 17–19]. However, given that the clinical relevance of such phenomena is dependent on cancer type and drug class, additional unknown mechanisms likely play a role [4]. A recent study suggests that the vessel phenotype of tumors contributes to the response to different treatment strategies. Using preclinical models and clinical samples, it was shown that cancers that are more responsive to bevacizumab in combination with chemotherapy have a stromal-vessel phenotype, where the vessels are surrounded by a well-developed stroma. In contrast, cancers that are more responsive to TKI monotherapy have a tumor-vessel phenotype, where the vessels are in close proximity to the tumor cells [20]. In addition, tumor-specific differences likely account for why certain anti-angiogenic therapies show efficacy in some cancers, but not in others, although the precise molecular mechanisms are not known [10].

Although anti-angiogenic therapy has been incorporated into the standard protocol for certain cancer types, there are a number of concerns, the foremost being its modest clinical benefits. The gain in progression-free survival (PFS) and overall survival (OS) is generally in the order of months. In addition, initial response rates and gains in PFS do not always translate into significant improvements in OS (Table 9.1). These limited clinical benefits strongly suggest that tumors treated with anti-angiogenic agents develop resistance to therapy. Such resistance can be classified as intrinsic, where tumors are unresponsive from the beginning of treatment, and acquired, where tumors initially respond but then progress during the course of treatment [21]. Thus, there is an urgent need to overcome these limitations and to develop improved strategies for the treatment of cancer at all stages of the disease.

9.3 Mechanisms of Resistance to Inhibitors of Angiogenesis

There is a growing interest in understanding the mechanisms underlying both acquired and intrinsic resistance to anti-angiogenic therapy. Classical drug resistance mechanisms involve the clonal selection of tumor cells harboring genomic mutations that either alter the drug target or affect drug uptake or efflux [22]. However, since anti-angiogenic therapy targets the vascular supply of the tumor mass rather than the tumor cells themselves, resistance in this case is mainly indirect and involves an interplay between tumoral cues and host-mediated pathways. In addition, given that endothelial cells are more genetically stable than tumor cells, they are less likely to acquire mutations after exposure to such drugs [23]. In general, resistance to anti-angiogenic therapy is manifested by the activation of alternative mechanisms that sustain tumor vascularization and growth while the specific target of the drug remains inhibited [21]. These evasive mechanisms are described in detail below. A graphical summary is shown in Fig. 9.1.

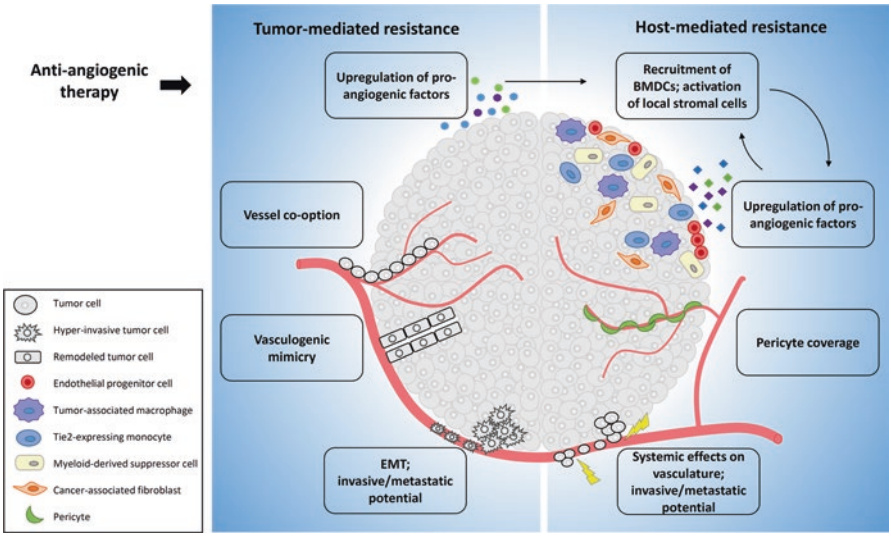

Fig. 9.1 Mechanisms of resistance to anti-angiogenic therapy. Tumors develop resistance to anti-angiogenic therapy via a range of tumor- and host-mediated processes. These evasive mechanisms sustain tumor vascularization and/or progression despite the blockade of VEGF signaling imposed by anti-angiogenic agents. Increased tumor hypoxia, which occurs as a direct result of anti-angiogenic therapy, drives many of these processes. The processes are not mutually exclusive, and some are interdependent (indicated by thin arrows). BMDC, bone marrow-derived cell; EMT, epithelial-mesenchymal transition

9.3.1 Upregulation of Alternative Angiogenic Pathways

Tumor angiogenesis is mainly driven by the VEGF signaling pathway. However, there are also numerous complementary non-VEGF pathways that contribute to blood vessel formation. Tumor hypoxia, which occurs as a direct result of anti-angiogenic therapy, modulates the interplay between these various angiogenic pathways via the master regulator, hypoxia inducible factor-1 (HIF-1), a transcription factor that regulates the expression of multiple pro-angiogenic genes [24]. The activation of alternative or compensatory angiogenic pathways allows for persistent neovascularization despite VEGF inhibition and represents the most common means by which tumors evade the blockade of angiogenesis. Preclinical trials in a murine pancreatic cancer model demonstrated an initial response to anti-VEGFR2 therapy (DC101) followed by restoration of tumor growth and vascularization shortly after initiation of therapy. Interestingly, at the time of progression, these tumors expressed higher levels of various pro-angiogenic factors such as fibroblast growth factor (FGF) 1 and 2, ephrin A1 and A2 and angiopoietin 1. Similarly, tumor cells subjected to hypoxic conditions upregulated most of these genes. Blocking both VEGF and FGF signaling attenuated revascularization and slowed tumor growth, suggesting that upregulation of FGF signaling contributes to anti-angiogenic therapy resistance [25]. Several additional pro-angiogenic factors have been implicated in

resistance to anti-angiogenic therapy in various murine tumor models. These include: placental growth factor, PlGF [26]; platelet-derived growth factor, PDGF [27]; hepatocyte growth factor, HGF, and its receptor, c-Met [28, 29]; epidermal growth factor, EGF [30]; interleukin-8, IL-8 [31]; granulocyte colony stimulating factor, G-CSF, and Bv8 [32], among others (recently reviewed in [33]). Similar to the seminal study of Casanovas et al. [25], the above-mentioned studies report elevated levels of the specific factor in resistant tumors and that dual inhibition of the VEGF pathway and the specific factor or its pathway enhances therapeutic outcome. It should be noted that these upregulated pro-angiogenic factors may be derived from tumor cells or host stromal cells residing within the tumor microenvironment. The former case involves a direct response of tumor cells to hypoxia. In the latter case, stromal cells may be responding to cues from the tumor, environmental signals or systemic effects of the drug [10, 34].

There is a wealth of clinical evidence showing that circulating levels of pro-angiogenic factors are elevated just prior to disease progression or during the relapse phase in cancer patients treated with angiogenesis inhibitors suggesting that these factors contribute to the development of acquired resistance [35–40]. There are also cases in which patients do not respond at all to anti-angiogenic therapy suggestive of intrinsic resistance. In late stage malignancies, pre-existing upregulation of alternative pro-angiogenic pathways may compensate for the inhibition of VEGF signaling [21].

9.3.2 Pro-angiogenic Effects of Local and Bone Marrow-Derived Stromal Cells

The release of pro-angiogenic factors in response to anti-angiogenic therapy activates local stromal cells and stimulates the recruitment of bone marrow-derived cells (BMDCs) to the tumor environment. BMDCs include vascular progenitors, which differentiate into cells that make up physical components of the blood vessel walls, and pro-inflammatory monocytes, which produce a diverse assortment of soluble factors that regulate vascular cell survival, proliferation and motility as well as extracellular matrix (ECM) remodeling [21, 41].

The effect of hypoxia on BMDC recruitment was described by Du et al. in an orthotopic model of GBM. They demonstrated that HIF-1α, the direct effector of hypoxia, promotes angiogenesis and tumor growth by inducing an influx of various pro-angiogenic bone-marrow derived $CD45^+$ myeloid cells as well as endothelial and pericyte progenitor cells to the tumor [42]. In addition, treating tumor-bearing mice with vascular-disrupting agents, which cause massive tumor hypoxia, triggers an acute mobilization of circulating endothelial progenitor cells that home to the tumor margins in sufficient numbers to facilitate revascularization [17]. Thus, therapy-induced hypoxia represents a major contributing factor to resistance via the action of recruited BMDCs.

The involvement of specific BMDC types and local stromal cells in resistance to anti-angiogenic therapy is described below:

Immature myeloid cells, also known as CD11b$^+$Gr1$^+$myeloid-derived suppressor cells (MDSCs), produce a variety of factors that influence endothelial cell behavior resulting in new vessel formation [41]. Shojaei et al. demonstrated that tumors resistant to anti-VEGF therapy exhibit higher levels of infiltrating MDSCs in comparison to therapy-sensitive tumors [43]. This is due to an upregulation of G-CSF and Bv8 [32], factors that promote the mobilization of MDSCs from the bone marrow and their infiltration to tumor tissue [44]. In pancreatic tumor models that are resistant to anti-VEGF therapy, increased levels of proinflammatory factors including several CXCR2 ligands, IL-1α, IL-1β and Angptl-2 stimulate the recruitment of CD11b$^+$ myeloid cells to the tumor environment [45]. In agreement with the above-mentioned studies, blocking chemotherapy-induced infiltration of MDSCs to tumors using Bv8 neutralizing antibodies enhances therapy outcome in mouse models of pancreatic cancer [46]. In another study, it was suggested that tumor-infiltrating T helper type 17 (TH17) cells and IL-17 induce the recruitment of immature myeloid cells to the tumor microenvironment. Blocking TH17 cell function renders resistant tumors sensitive to anti-VEGF therapy [47].

Tumor-associated macrophages (TAMs) are recruited to tumors as monocytes from the circulation and, as they extravasate across the tumor vasculature, they differentiate into macrophages. In the tumor environment, TAMs are predominantly polarized towards an M2-like phenotype underlying their ability to promote tumor growth and angiogenesis [48]. TAMs promote angiogenesis mostly through their production of VEGF [41]. However, TAM-derived PlGF can also stimulate angiogenesis in some tumors, representing a possible mechanism for acquired resistance to VEGF/VEGFR-targeted therapies [49]. In HCC xenografts, sorafenib increases CXCL12 levels and TAM infiltration. Furthermore, depletion of TAMs enhances the inhibitory effect of therapy on tumor angiogenesis, growth and metastasis demonstrating the contribution of TAMs to therapy resistance [50].

Tie2-expressing monocytes (TEMs) represent a distinct subpopulation of TAMs expressing the angiopoietin receptor, Tie2. They physically associate with vessels and secrete growth factors and matrix-remodeling proteins that stimulate the angiogenic process in a paracrine manner [51]. TEMs are recruited and activated via endothelial cell- and tumor-secreted chemoattractants, Ang2 and CXCL12, respectively [52, 53]. Their recruitment to spontaneously growing tumors promotes angiogenesis [54]. Furthermore, TEMs infiltrate hypoxic tumors treated with a vascular-disrupting agent, and inhibiting such infiltration enhances treatment efficacy [53]. Lastly, dual targeting of VEGF and Ang2 has been shown to delay tumor growth and improve the outcome of anti-angiogenic therapy in preclinical studies [55–57]. These collective findings highlight the possible contribution of TEMs to resistance to anti-angiogenic therapy.

Pericytes, the periendothelial support cells of the microvasculature, are derived from local or bone marrow-derived mesenchymal stem cells. They provide important support for blood vessel formation, structure and function. Furthermore, tight cross-talk between pericytes and endothelial cells maintains blood vessel integrity

[58]. While anti-angiogenic therapy reduces tumor vascularity, the vessels that remain are functional, distinctively thin and tightly covered with pericytes [21, 59, 60]. Owing to their important role in maintaining vessel integrity, the remaining pericytes, along with basement membrane-associated cells, facilitate a rapid regrowth of blood vessels after cessation of treatment with angiogenesis inhibitors [61]. Importantly, pericytes mediate endothelial cell quiescence and survival and therefore their presence presumably reduces responsiveness to anti-angiogenic therapy [21]. The underlying molecular mechanism involves pericyte-induced survival signals that induce an autocrine activation loop of VEGF signaling and anti-apoptotic Bcl-w expression in tumor endothelial cells [62]. Additional pericyte-derived endothelial survival signals, specifically via the Ang1/Tie2 and EGF pathways, may also contribute to anti-angiogenic therapy resistance [30, 63]. Accordingly, it has been suggested that targeting both endothelial cells and pericytes may improve the efficacy of anti-angiogenic therapy. Indeed, such dual targeting improves therapy outcome in a variety of murine tumor models [63–65]. However, severe reduction in pericyte coverage damages the integrity of the vasculature, enabling local intravasation of tumor cells thereby facilitating metastasis [66]. In support of this concept, a recent study demonstrated that TKI-induced pericyte depletion enhances metastasis due to increased vessel leakiness and hypoxia-associated epithelial-mesenchymal transition (EMT) [67]. Collectively, enhanced as well as reduced pericyte coverage contribute to anti-angiogenic therapy resistance via different mechanisms.

Cancer-associated fibroblasts (CAFs) are tumor-localized, activated fibroblasts originating from connective tissue fibroblasts proximal to neoplasms or from local and bone marrow-derived mesenchymal stem/progenitor cells. They promote angiogenesis by producing a variety of pro-angiogenic signaling factors, chemoattractants and ECM-degrading enzymes [41]. Crawford et al. showed that the upregulation of PDGF-C in CAFs from anti-VEGF resistant tumors compensates for the inhibition of VEGF-dependent angiogenesis. Furthermore, CAFs isolated from resistant tumors can stimulate the growth of therapy-sensitive tumors even when VEGF is inhibited. This suggests that, once activated by the tumor environment, CAFs retain their ability to induce angiogenesis independent of tumor cells [27].

9.3.3 Alternative Vascularization Mechanisms

Primary tumors and metastases may gain access to a blood supply via mechanisms that are independent of classical sprouting angiogenesis. These alternative vascularization mechanisms are not affected by antiangiogenic drugs and therefore represent another mode of resistance to such therapy [68].

Vessel co-option refers to the migration of tumor cells along existing and established blood vessels in the host organ to invade healthy tissue. This process is mostly observed in highly vascularized tissues such as brain, lungs and liver, where tumor cells can co-opt the abundant pre-existing blood vessels [69]. Preclinical and clinical

data show that glioblastomas become more infiltrative with the use of anti-angiogenic therapy, facilitating vessel co-option [42, 70–74]. In addition, vessel co-option has been implicated in resistance to anti-angiogenic therapy in HCC [75] and metastases in lymph nodes [76], brain [77], liver [78] and lung [79].

Vasculogenic mimicry is a mechanism by which highly aggressive tumor cells form vessel-like structures in an angiogenesis-independent manner. These vessel-like structures may connect to the endothelial-lined vasculature to provide a perfusion pathway for the transport of fluid, nutrients and oxygen to the core of the malignant mass [68, 80]. Since its first description in uveal melanoma [81], vasculogenic mimicry has been observed in several tumor types and is associated with poor prognosis [82]. By virtue of their plasticity, tumor cells can dedifferentiate and acquire expression of vascular markers thereby "mimicking" endothelial cells during this process [81, 83, 84]. However, despite expression of various vascular markers, such tumor cells are resilient to treatment with angiogenesis inhibitors [84–87]. Furthermore, antiangiogenic treatment has been shown to induce vasculogenic mimicry in preclinical models of various cancers [86, 87]. This may be due to treatment-induced hypoxia that upregulates vasculogenic mimicry pathways in tumor cells [88, 89]. Collectively, anti-angiogenic therapy not only triggers alternative vascularization mechanisms, but may also select for more aggressive tumor cells with an intrinsic ability to evade the blockade of angiogenesis.

9.3.4 The Host Response to Inhibitors of Angiogenesis: Implications for Tumor Aggressiveness

Targeting the host-mediated angiogenic process that supports tumor growth has its limitations. As detailed in the previous sections, anti-angiogenic therapies may trigger an array of evasive mechanisms that involve the activity of host cells such as pro-inflammatory myeloid cells and endothelial progenitor cells in the tumor microenvironment. Furthermore, anti-angiogenic therapy has been shown to augment the invasive and metastatic potential of tumors despite overall inhibition of tumor growth [90, 91]. This seemingly paradoxical phenomenon is proposed to arise, at least in part, from a direct response of the host to anti-angiogenic therapy, independent of the tumor. Ebos et al. showed that short-term sunitinib treatment of mice prior to intravenous injection of tumor cells accelerates metastasis and reduces survival. Similarly, adjuvant short-term sunitinib treatment after resection of the primary tumor enhances spontaneous metastatic tumor burden [90]. The mechanisms underlying this effect may involve a drug-induced change in the levels of circulating factors implicated in tumor progression. For example, healthy, tumor-free mice treated with VEGF receptor TKIs exhibit a dose-dependent increase in the levels of circulating G-CSF, SDF-1α, SCF and osteopontin demonstrating a systemic tumor-independent response to therapy [92]. Similarly, cancer patients treated with sunitinib exhibit increased circulating levels of

pro-angiogenic factors [93, 94]. In theory, such systemic host-mediated responses could promote the formation of "pre-metastatic niches" in distant organs, thereby facilitating metastasis [95]. The deleterious effect of anti-angiogenic therapy on the host vasculature represents another factor that may explain increased metastasis in response to such therapy. The systemic action of VEGF receptor TKIs may damage the integrity of the vasculature by targeting endothelial cells as well as pericytes. This facilitates local intravasation of invasive tumor cells and creates permissive niches for extravasation of tumor cells in target organs [67, 96, 97].

It should be emphasized that several steps are required for disease progression from a local primary tumor to established metastatic disease. These include loss of cellular adhesion, increased motility, intravasation, survival in the bloodstream, homing, extravasation, seeding of micrometastases, and finally colonization and growth at a distant site [98]. Therefore, it is conceivable that the above-mentioned host-mediated responses act in concert with tumor-derived effects to promote overall tumor aggressiveness in response to anti-angiogenic therapy. Paez-Ribes et al. demonstrated that the anti-VEGFR2 antibody, DC101, and VEGF receptor TKI, sunitinib, promote local primary tumor invasion and metastasis in mouse models of pancreatic neuroendocrine carcinoma and glioblastoma. The researchers suggest that therapy-induced hypoxia in the primary tumor triggers a switch to a hyperinvasive condition in tumor cells [91]. In agreement with this, several preclinical studies demonstrate that VEGF-targeted therapies cause tumor cells to undergo hypoxia-associated EMT, thereby promoting invasion and metastasis [67, 99, 100]. Collectively, both host- and tumor-dependent responses to anti-angiogenic therapy contribute to the invasive and metastatic potential of treated tumors.

Whether anti-angiogenic therapy causes increased tumor aggressiveness in patients is still a debatable issue. A retrospective analysis found no evidence for accelerated tumor growth in metastatic RCC patients treated with sunitinib [101]. Similarly, a meta-analysis of several randomized phase III trials of bevacizumab found no evidence for accelerated disease progression after discontinuation of therapy in patients with metastatic renal, pancreatic, breast and colorectal cancer [102]. On the other hand, rapid tumor regrowth has been reported after treatment discontinuation in RCC patients receiving sunitinib or sorafenib [103, 104], and in CRC patients receiving bevacizumab and chemotherapy [105]. In addition, several clinical studies describe an increased infiltrative growth pattern of glioblastomas in response to anti-angiogenic therapy [70, 71, 73]. The differences in preclinical and clinical findings may be explained by the animal model used, tumor type, disease stage, drug type, dosage, duration of treatment, or combination with chemotherapy [10].

Other anti-cancer treatment modalities, such as chemotherapy, radiation and surgery, can also produce undesirable pro-angiogenic and pro-metastatic effects that arise from the response of the host to therapy. Accordingly, blunting this host response using combinatorial therapies may improve treatment outcomes [34]. For example, the elevation in circulating endothelial progenitor cell levels following treatment with chemotherapeutic or vascular-disrupting agents can be blocked using anti-VEGF or anti-VEGFR2 neutralizing antibodies. This combinatorial treatment

enhances therapy efficacy and delays tumor regrowth in comparison to cytotoxic therapy alone [18]. Recent preclinical studies suggest that the reverse may be true as well; cytotoxic therapy can be used to blunt tumor aggressiveness induced by anti-angiogenic drugs thereby improving treatment efficacy. For example, concurrent paclitaxel chemotherapy was shown to block the increase in primary tumor local invasion and distant metastases induced by anti-VEGFR2 antibody (DC101) therapy in mouse models of breast cancer [106]. In addition, co-administration of chemotherapy counteracted the sunitinib-induced increase in metastasis in mice bearing early stage Lewis lung carcinoma [107]. Thus, add-on therapy that counteracts host- or tumor-dependent responses represents a possible strategy to overcome increased tumor aggressiveness and resistance in response to anti-angiogenic therapy.

9.4 Future Directions

The limited clinical benefits of anti-angiogenic therapy contrast with findings of preclinical studies conducted over the last two decades that demonstrate treatment efficacy. This can be explained by the disparity between preclinical models used to test efficacy and clinical scenarios. Due in part to ethical issues, patients enrolled in clinical trials are generally at an advanced stage of the disease. In contrast, preclinical experimental setups mostly involve localized primary tumors, with suppression of tumor growth after a short-term drug exposure considered a sign of efficacy. Therefore, more relevant preclinical models should be used to study the effects of anti-angiogenic therapy at all stages of disease, including metastatic and adjuvant settings, with clinically-relevant endpoints [108].

In theory, alternative pro-angiogenic pathways upregulated in response to anti-angiogenic therapy may be targeted as a strategy to overcome resistance. Multi-targeted inhibitors such as brivanib, a dual VEGFR and FGFR TKI, and nintedanib, a triple angiokinase inhibitor for VEGFR, FGFR and PDGFR, are being tested in clinical trials [109, 110]. Importantly, host-mediated evasive mechanisms induced in response to anti-angiogenic therapy may also be targeted in order to improve anti-angiogenic therapy outcomes. The major BMDC recruiting factor, SDF1α (CXCL12), represents a potential target for cancer therapy. Recent preclinical and clinical data support the use of anti-CXCL12 agents to reduce BMDC infiltration as a potential strategy to overcome resistance to anti-angiogenic therapy [18, 111]. Macrophages are key regulators in the tumor microenvironment, and have been implicated in resistance to anti-angiogenic therapy. Therefore, specifically blocking macrophage infiltration is also a potential means for overcoming resistance. Antibodies against the monocyte chemotactic protein, CCL2, and the macrophage-expressed CSF-1 receptor are being tested in clinical trials as monotherapies [112–114]. It will be interesting to test whether such agents synergistically increase efficacy when combined with anti-angiogenic agents in the clinical setting [115].

As detailed throughout this review, hypoxia resulting from anti-angiogenic therapy drives tumor aggressiveness and therapy resistance via tumor- and host-mediated mechanisms. Therefore, alleviating hypoxia or targeting HIF-1 represent avenues for future investigation [116]. The former case would involve optimizing the dosage and scheduling of anti-angiogenic agents with the aim of normalizing the abnormal tumor vasculature as opposed to inducing rapid and excessive vessel pruning [16]. Indeed, tumor perfusion and oxygenation correlates with clinical benefit in GBM patients treated with anti-angiogenic therapy [117–120]. Alleviating hypoxia would reduce processes such as EMT, vasculogenic mimicry and the selection of more aggressive tumor cells as well as affect immune and stromal cells within the tumor microenvironment. It is well-established that a hypoxic tumor environment induces BMDC recruitment and reprograms TAMs towards a pro-tumorigenic phenotype. Therefore, alleviating hypoxia through vascular normalization could potentially reprogram the entire tumor microenvironment [16]. Histone deacetylase inhibitors have been shown to strongly repress HIF-1 expression and their use as anti-cancer drugs is currently being explored [121]. A recent phase I clinical trial evaluating the use of a histone deacetylation inhibitor in combination with the anti-angiogenic agent, pazopanib, demonstrated durable tumor regression in 70% of patients with pazopanib-refractory disease [122]. Thus, epigenetic targeting represents a potential strategy to reverse resistance to anti-angiogenic therapy, possibly by targeting HIF-1. The precise molecular mechanisms and clinical benefits should be further characterized.

The combination of anti-angiogenic drugs with immunotherapy represents an emerging strategy for cancer treatment. The rationale for using this combination is based on the systemic influence of VEGF on immune cell function. Specifically, several studies have demonstrated that an elevated level of circulating VEGF in tumor-bearing hosts impedes immune surveillance and destruction of tumor cells [123–125]. Accordingly, anti-angiogenic drugs may be used to neutralize the immunosuppressive activity of VEGF. Moreover, the combination of anti-angiogenic therapy with immunotherapy could potentially offer a synergistic anti-cancer effect. In addition, it has been proposed that alleviating tumor hypoxia via vascular normalization would reprogram the phenotype of the tumor microenvironment from immunosuppressive to immunosupportive, thereby improving the efficacy of anti-cancer immunotherapies [16, 126]. A number of preclinical and clinical studies have demonstrated the benefits of this combination strategy [127–133].

Lastly, a major challenge is to identify robust biomarkers predictive of clinical efficacy of anti-angiogenic therapy. Currently, no validated biomarkers exist to select patients who will benefit from such therapy. Biomarkers under consideration in various cancers include circulating VEGF-A, VEGF-D, Ang2, HGF, osteopontin, IL6 and IL8, among others [4, 134]. With respect to VEGF as a predictive biomarker for bevacizumab-based treatment benefit, phase III trials have reported a correlation between high circulating levels of VEGF and survival benefit in metastatic breast and gastric cancer patients [135, 136], but not in CRC, RCC and lung cancer patients [137]. Other emerging areas for biomarker identification include tumor vessel imaging with dynamic contrast-enhanced MRI, measurement of circulating endothelial

cells, expression arrays, single nucleotide polymorphisms and early pharmacodynamic response to treatment, such as hypertension [138]. The incorporation of predictive biomarkers into routine clinical practice would maximize clinical benefit, reduce unnecessary toxicity and improve costs of cancer care.

9.5 Conclusions

The development of anti-angiogenic agents is an important milestone in the field of cancer research. However, their clinical use is proving to be more complex than originally anticipated with major ongoing challenges. A prominent issue in the clinic is resistance to therapy resulting in only modest gains in long-term survival in the majority of patients. Given that anti-angiogenic agents target the tumor-supporting vascular system comprised of a variety of host cells, and that tumor progression is regulated by tumor-host cell cross-talk, resistance is dependent on both tumor- and host-mediated mechanisms (Fig. 9.1). Understanding these mechanisms is key to developing strategies to overcome therapy resistance and improve clinical outcome.

Acknowledgments This book chapter is primarily supported by the European Research Council (#260633) and Rappaport Institute funds given to YS. KM is supported by a student fellowship from the Lyon Sachs University of Toronto— Technion Collaboration Fund.

Conflict of Interest The authors declare that they have no conflict of interest.

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