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Review

Mutational and network level mechanisms underlying resistance to anti-cancer kinase inhibitors

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ABSTRACT

Tyrosine-specific and other protein kinases are embedded in signaling networks critical for progression of tumors of all types. Hence, kinase inhibitors have nucleated a major arm of personalized cancer therapy. Unfortunately, almost all kinase inhibitors evoke resistance within a year or two, due to secondary mutations, and other alterations within the targeted kinase, or due to emergence of feedback regulatory loops that compensate for extinguished kinases. We review clinically approved kinase inhibitors and the emergence of resistance in leukemia, melanoma, lung and breast tumors, and draw parallel lines in terms of secondary mutations and compensatory mechanisms. Currently emerging are pharmacological strategies able to circumvent resistance and re-sensitize patients to therapeutic treatments. They include second and third generation inhibitors that overcome new mutations, novel drug combinations that simultaneously block the primary oncogenic pathway and compensatory routes, as well as monoclonal antibodies. Deeper understanding of biological signaling networks and their responses to perturbations will aid in the development of effective therapies for patients with cancer.

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Abbreviations: ALK, anaplastic lymphoma kinase; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; CNS, central nervous system; CRC, colorectal cancer; DTC, differentiated thyroid carcinoma; DUSP, dual specificity phosphatase; EGFR, epidermal growth factor receptor; EMT, epithelia-to-mesenchymal transition; ERK, extracellular regulated kinase; FGF, fibroblasts growth factor; GIST, gastrointestinal stromal tumor; HER2, human EGF receptor 2; HGF, hepatocyte growth factor; IGF1, insulin-like growth factor 1; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; NRG, neuregulin; NSCLC, non-small cell lung cancer; PI3K, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; PKI, protein kinase inhibitor; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PV, polycythemia vera; RCC, renal cell carcinoma; RTK, receptor tyrosine kinase; SCLC, small-cell lung cancer; VEGF, vascular endothelial growth factor.

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1. Introduction

The first 15 years of the new millennium heralded a new era in cancer therapy: close to 50 new drugs, each targeting a specific molecule involved in cancer progression, entered routine use in oncology wards around the world. This new wave is the outcome of two concepts. The 1906 'Magic Bullet' concept of Paul Ehrlich predicted that it would be possible to pharmacologically target a pathologic tissue while avoiding nearby healthy tissues. The 2002 concept of Irwin Weinstein [1], called 'Oncogene Addiction', argued that cancers containing multiple genetic and epigenetic abnormalities are dependent on (or 'addicted' to) one or a few genes for maintenance of the malignant phenotype. Hence, reversal of only one or a few of these abnormalities might inhibit cancer cell growth and translate to improved survival rates. Excessive reliance of tumor cells on specific intracellular pathways is best exemplified by dependencies on oncogenic driver mutations such as BCR-ABL and RAS mutations, or aberrant tumor suppressors like p53 and PTEN, which might be identified using genomic analyses [2]. Another class of dependencies is the non-oncogene addictions [3]. This class is accompanied by no somatic mutation(s) and it might be exemplified by reliance of some colorectal tumors on EGF-like growth factors [4] or dependency of many breast tumors on estrogen [5].

Importantly, many oncogenic mutations alter functions of specific protein kinases, and some non-oncogene addictions are regulated by phosphorylation of critical proteins. Accordingly, clinically approved small molecule protein kinase inhibitors (PKIs) currently outnumber other classes of targeted cancer drugs: to date 27 PKIs have been approved for a broad spectrum of tumors (see Table 1), and many more inhibitors are being tested in advanced clinical trials. Moreover, PKIs have been approved also for other diseases, such as cardiovascular and inflammatory diseases [6]. Protein kinases catalyze the transfer of the gamma-phosphate of ATP onto protein substrates, including self-phosphorylation. And although kinases account for only 5% of the total number of protein-coding genes, they offer several advantages for pharmacological interception: many kinases are configured in linear organization permitting stepwise transfer of biochemical signals, for example along the RAF-MEK-ERK cascade of the MAPK pathway, which drives specific types of melanoma and adenocarcinomas [7]. In addition, although all nucleotide-binding pockets of protein kinases bind ATP, kinase-specific three-dimensional attributes confer reasonably high selectivity and sufficiently high affinity toward carefully selected PKIs [8]. Unlike PKIs, clinically approved monoclonal antibodies (mAbs) are endowed with much higher target specificity, which is independent of antibody concentration. This is not the case for PKIs; at high concentrations PKIs often display progressively relaxed specificity [9], which may result in increased toxicity and adverse effects. Yet another pharmacological difference between therapeutic mAbs and PKIs entails mechanisms that confer patient resistance to these two fundamentally different classes of cancer drugs. For example, secondary mutations confined to the targeted kinase domains often confer resistance to PKIs, such as a secondary replacement of threonine 790 of EGFR by a methionine (T790M), which blocks entry of some kinase inhibitors [10–12]. By contrast, resistance to mAbs rarely involves secondary mutations [13]. One notable exception is an acquired EGFR ectodomain mutation (S492R) that prevents binding of an anti-EGFR mAb, called cetuximab, and confers drug resistance [14]. Another type of resistance, which is shared by PKIs and mAbs, might be due to compensatory network alterations that preempt the pharmacological blockade. By contrast with mutation-driven resistance, identifying a compensatory bypass track requires both genomic and proteomic analyses, and it might identify ways to pharmacologically circumvent resistance by using drug combinations [15].

Herein, we first discuss the basics of feedback regulatory loops embedded in kinase cascades, as well as their involvement in resistance to some PKIs. Next, we systematically review classes of PKIs and four generic mechanisms underlying tumor tolerance. Later, we provide specific examples by relating to some of the clinically more relevant PKIs and their oncogenic kinases. Our discussion culminates with strategies to re-sensitize PKI-resistant tumors. The reader is referred to several recent reviews that highlight structural aspects [16], as well as clinical implications [15,17].

2. A primer to feedback regulation and adaptation of kinase cascades

To exemplify how critical drug-induced rewiring might be in clinical settings, one might refer to a Phase I trial of rapamycin, an inhibitor of mTOR, in patients with recurrent glioblastoma [18]. Assuming that loss of PTEN sensitizes tumors to the inhibitor of mTOR, 14 patients who lacked expression of PTEN were treated with rapamycin. This led to paradoxical AKT activation in seven patients. Unfortunately, AKT activation was associated with shorter time-to-progression during post-surgical rapamycin therapy. Presumably, AKT activation in 50% of patients was due to loss of negative feedback allowing mTOR to backward inhibit AKT. This negative feedback might involve another kinase, probably a SRC family member, such that combined inhibition of mTOR and SRC family kinases can prevent rapamycin-induced feedback activation of AKT and elicit tumor regression. In an analogous way, it was found that AKT inhibition induces the expression and phosphorylation of multiple RTKs, including HER3, IGF1 receptor, and insulin receptor [19]. This is in part due to mTOR inhibition, specifically inhibition of the mTORC1 complex. Thus, PI3K–AKT inhibitors might activate (rather than inhibit) RTK signaling, thereby attenuate antitumor activity [20].

These two examples underscore the importance of detailed mapping of feedback regulatory loops, and they also clarify that feedback mechanisms fall into two classes: transcription-mediated delayed loops, and the more rapid, transcription-independent loops involving posttranslational modifications, such as phosphorylation and ubiquitinylation [21]. Additional transcription-independent negative feedback programs include ERK phosphorylation of SOS and RAF, which downregulates their activity, mTOR-mediated destabilization of IRS1, an adaptor protein, through S6K activation [22], and ubiquitinylation of active RTKs by an E3 ubiquitin ligase called CBL. This latter modification sorts active receptors to endocytosis and degradation, but it is partly defective in cancer cells [23,24]. A similar global alteration of the other class of negative feedback, transcription-mediated loops, has been reported: EGF induces numerous negative feedback components including proteins that can downregulate ERK (e.g., several dual specificity phosphatases, DUSPs, that downregulate the MAPK cascade) and ERK transcriptional programs (e.g., JUNB, ATF3, FOSL, ID1 and KLF2), but several regulators were found to be underexpressed in tumors [25]. In summary, although modified in tumors, negative feedback programs must be taken into account when intercepting a kinase cascade using PKIs. Emergence of feedback mechanisms, as well as switching to bypass tracks, might preempt PKIs unless both the primary target and the compensatory loop are simultaneously intercepted.

3. Classes and common features of kinase inhibitors

All protein kinases share a structurally conserved catalytic domain, and accordingly the majority of PKIs display some common structures. Moreover, structure–activity relationships (SAR) for many of the approved drugs have been extensively studied

Table 1
Kinase inhibitors approved for application in oncology (August 2015).

Name (trade name)	Company	Year	Known target	Indication	Main mechanism of resistance
Afatinib (Gilotrif)	Boehringer	2013	EGFR	NSCLC	T790M-EGFR amplification
Axitinib (Inlyta)	Pfizer	2012	VEGFR1/2/3	RCC, pancreatic cancer	Increased glucose metabolism
Bosutinib (Bosulif)	Wyeth	2012	BRC-Abl, Src, Lyn and Hck	CML	T315I mutation-CML
Cabozantinib (Cometriq)	Exelixis	2010	RET, Met, VEGFR1/2/3, Kit, TrkB, Flt3, Axl, Tie2	Metastatic medullary thyroid cancer, Prostate cancer	MET activation
Ceritinib (Zykadia)	Novartis	2014	ALK, IGF-1R, InsR, ROS1	ALK-positive NSCLC, after crizotinib resistance	ALK mutations
Crizotinib (Xalkori)	Pfizer	2011	ALK, Met and Ros	ALK-positive NSCLC	ALK mutation, EGFR signaling
Dabrafenib (Tafinlar)	GSK	2013	B-Raf	Melanoma	MAPK reactivation
Dasatinib (Sprycel)	Bristol-Meyers Squibb	2006	BRC-Abl, Src, Lck, Yes, Fyn, Kit, EphA2 and PDGFR	CML, ALL	T315I mutation-CML
Erlotinib (Tarceva)	OSI	2004	EGFR	NSCLC and pancreatic cancer	T790M mutation-NSCLC
Everolimus (Afinitor)	Novartis	2009	FKBP12/mTOR	Progressive neuroendocrine tumor of pancreatic origin, RCC, subependymal giant cell astrocytoma, breast cancer	TCS2 mutation
Gefitinib (Iressa)	Astra Zeneca	2003	EGFR	NSCLC	T790M mutation-NSCLC
Ibrutinib (Imbruvica)	Pharmacyclics and J&J	2013	Bruton's kinase	Mantle cell lymphoma, CLL, Waldenstrom's macroglobulinemia	BTK mutation
Imatinib (Gleevec)	Novartis	2001	BCR-Abl, Kit and PDGFR	CML, ALL aggressive systemic mastocytosis, GIST	T315I mutation-CML
Lapatinib (Tykerb)	SmithKline	2007	EGFR and HER2	Breast cancer	ER activation
Lenvatinib (Lenvima)	Easai	2015	VEGFRs/FGFRs/PDGFR/Kit/RET	Thyroid cancer	HGF pathway
Nilotinib (Tasigna)	Novartis	2007	BCR-Abl, PDGFR	CML	T315I mutation-CML
Nintedanib (Ofev)	Boehringer Ingelheim	2014	VEGFR, FGFR, PDGFR	Idiopathic pulmonary fibrosis	?
Palbociclib (Ibrance)	Park Davis	2015	CDK4/6	Breast cancer	?
Pazopanib (Votrient)	GSK	2009	VEGFR1/2/3, PDGFR, FGFR1/3, Kit, Lck, Fms and Itk	RCC, soft tissue sarcoma	Angiogenic switch, EMT
Ponatinib (Iclusig)	Ariad	2012	BCR-Abl, BCR-Abl T315I, VEGFR, PDGFR, FGFR, Eph, Src family kinases, Kit, RET, Tie2 and Flt3	CML, Ph chromosome positive ALL	BCR-ABL compound mutations
Regorafenib (Stivarga)	Bayer	2012	VEGFR1/2/3, BCR-Abl, B-Raf, B-Raf^{V600E}, Kit, PDGFR, RET, FGFR1/2, Tie2 and Eph2A	CRC, GIST	?
Ruxolitinib (Jakafi)	Incyte	2011	JAK1/2	Myelofibrosis and PV	JAK2 mutation
Sirolimus (Rapamune)	Wyeth	1999	FKBP12/mTOR	Renal transplant	?
Sorafenib (Nexavar)	Bayer	2005	C-Raf, B-Raf, B-Raf^{V600E}, Kit, Flt3, RET, VEGFR1/2/3 and PDGFR	Hepatocellular carcinoma, RCC, DTC	EMT
Sunitinib (Sutent)	Pfizer	2006	PDGFR, VEGFR1/2/3, Kit, Flt3, CSF-1R and RET	RCC, GIST, pancreatic neuroendocrine tumors	KIT mutation
Temsirolimus (Torisel)	Wyeth	2007	FKBP12/mTOR	RCC	mTOR2 constitutive activation
Tofacitinib (Xeljanz)	Pfizer	2012	JAK3	Rheumatoid arthritis	?
Trametinib (Mekinist)	GSK	2013	MEK1/2	Melanoma	MAPK reactivation (dabrafenib/trametinib)
Vandetanib (Caprelsa)	IPR Pharms	2011	EGFR, VEGFRs, RET, Brk, Tie2, EphRs and Src family kinases	Medullary thyroid cancer	RET mutations (in vitro)
Vemurafenib (Zelboraf)	Hoffmann La Roche	2011	A/B/C-Raf and B-Raf^{V600E}	Melanoma with BRAF^{V600E} mutation	MAPK-reactivation by NRAS and MEK1 mut

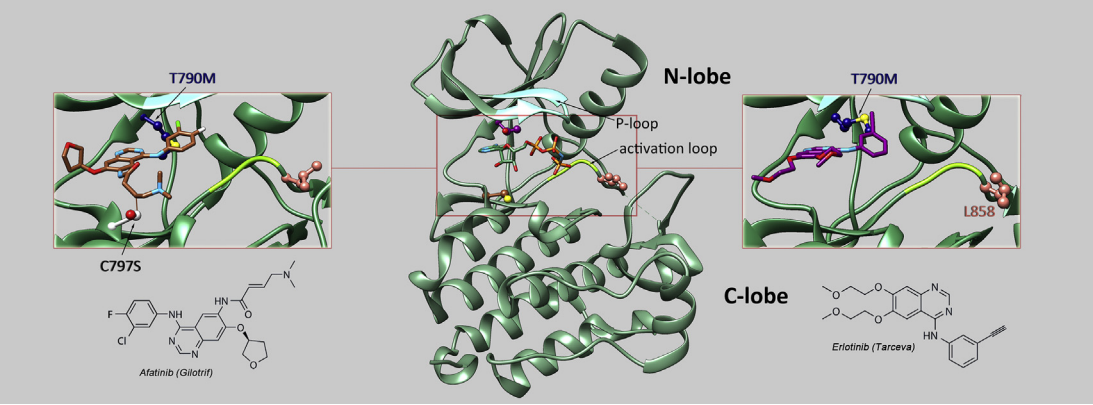

Fig. 1. Protein kinase domains and kinase inhibitors. The 3D structure of a typical tyrosine-specific kinase domain, of EGFR, is shown in the middle panel, along with an ATP analog, AMP-PNP. The P-loop and the activation loop are colored in light blue and in light green, respectively. Leucine 858 (L858), which is frequently mutated in lung cancer, is highlighted (in pink). Similarly labeled is threonine 790 (purple), which is replaced by a methionine in some advanced lung tumors and confers resistance to first generation kinase inhibitors. The squared area is magnified in the side panels and shows the structure of the T790M mutant in a complex with either a first generation inhibitor, erlotinib (right), or with a second generation inhibitor, afatinib (left). The latter structure shows a replacement of cysteine 797 with a serine (red). This mutation prevents covalent binding of afatinib to EGFR and might confer resistance to second and third generation EGFR inhibitors. The chemical structures of both erlotinib and afatinib are shown.

using crystal structures and other approaches [6,26]. Typically, a protein kinase domain folds into a bilobular structure that defines a deep ATP binding cleft, in between the two lobes (Fig. 1). The segment connecting the lobes, the 'hinge', forms hydrogen bonds with the adenine group of ATP, whereas the ribose and the triphosphate groups bind with a hydrophobic site close to the substrate-binding site. Kinase activation is dictated by an activation loop and a conserved triad of aminoacids, Asp-Phe-Gly (DFG): when the substrate-binding site is blocked by the activation loop (DFG-out) catalysis is arrested. By contrast, the activation loop permits enzymatic activity on assuming a catalytically competent conformation (DGF-in). The majority of PKIs are ATP mimetic drugs that form hydrogen bonds with the hinge region, but do not exploit the ribose-binding site. Notably, only two approved PKIs, afatinib and ibrutinib, form covalent bonds with a cysteine proximal to the ATP-binding site, thus establish irreversible kinase inhibition. The reversible PKIs fall into four groups, defined on the basis of their binding sites and conformation of the inhibited kinase domain [27]:

Type I inhibitors. This type of inhibitors constitutes the majority of ATP-competitive drugs, all recognize the active conformation of the kinase (DFG-in).
Type II inhibitors. By contrast, this type of inhibitors recognize the inactive conformation of the kinase (DFG-out), in which the aspartate protrudes outward of the ATP-binding site.
Type III (allosteric) inhibitors. These compounds avoid the ATP-binding site and recognize an adjacent allosteric site. Hence, type III inhibitors disrupt the interaction between ATP and its pocket, and they generally exhibit higher degree of target selectivity. However, despite promising attributes and clinical tests of several candidates, only one PKI of type III, trametinib, has been approved for clinical application.
Type IV inhibitors. These compounds bind with a site remote from the ATP-binding site [28].

Another basis for classification of PKIs is their target specificity. With a few exceptions most of the approved PKIs inhibit tyrosine kinases, rather than serine/threonine-specific kinases [29]. And while the majority of compounds are defined as mono-specific, some inhibit with similar potency more than one kinase. For example, lapatinib inhibits both EGFR and HER2, and sorafenib was initially developed as a RAF-specific inhibitor, but emerged as a blocker of several other kinases, including FLT-3, KIT, RET, and the receptors for PDGF and VEGF (see Fig. 2). In pharmacology, increasing target specificity and minimizing off-target effects represent main determinants of drug efficacy [16]. However, specificity of kinase inhibitors presents an open issue [9]. The genomic heterogeneity of cancer has led to recent interest in polypharmacology and drugs targeting several oncogenic kinases. For example, an insect RET-driven model of multiple endocrine neoplasia type 2 found that inhibition of RET along with other kinases (i.e., RAF, SRC and the ribosomal S6 kinase; S6K) was required for optimal animal survival, implying that compounds with a maximal therapeutic index might inhibit several, rather than a single protein kinase [30]. As we discuss below, the concept of multi-target specificity might be especially appealing for treatment of melanoma, lung and other tumors characterized by multiple mutations and dynamic evolvement of resistance to TKIs.

4. General mechanisms of acquired resistance to kinase inhibitors

Some cancer patients whose tumors harbor an oncogenic mutation might not respond to the respective PKI due to poorly understood mechanisms of primary (also called intrinsic) resistance. Unfortunately, within less than 20 months almost all patients who initially responded to PKIs develop acquired resistance, commonly defined as tumor progression following an initial response. Molecular machineries enabling tolerance to PKIs have lately been under intense investigation, as they profoundly limit clinical application of PKIs. In general, two classes of mechanisms might confer resistance. As detailed below, the first class refers to alterations occurring at the level of drug or host (patient), rather than within the malignant tissue, while the other class brings together all adaptive alterations taking place in the cancerous tissue (see Fig. 3).

Pharmacological resistance: Any mechanism that prevents a PKI from reaching its intracellular target might be categorized under pharmacological resistance. For example, pharmacokinetics effects that deplete a drug, prevent uptake or metabolize it into less active fragments. Also included are drug-drug interactions and patient-specific variables that inhibit drug delivery to cancer cells, which might still retain drug sensitivity. For example, a study that compared current smokers and non-smokers found that smokers achieved significantly less erlotinib exposure following a single dose of the drug, consistent with accelerated metabolic clearance in current smokers [31]. Alternatively, acquired resistance might be driven by central nervous system- (CNS) based mechanisms, such

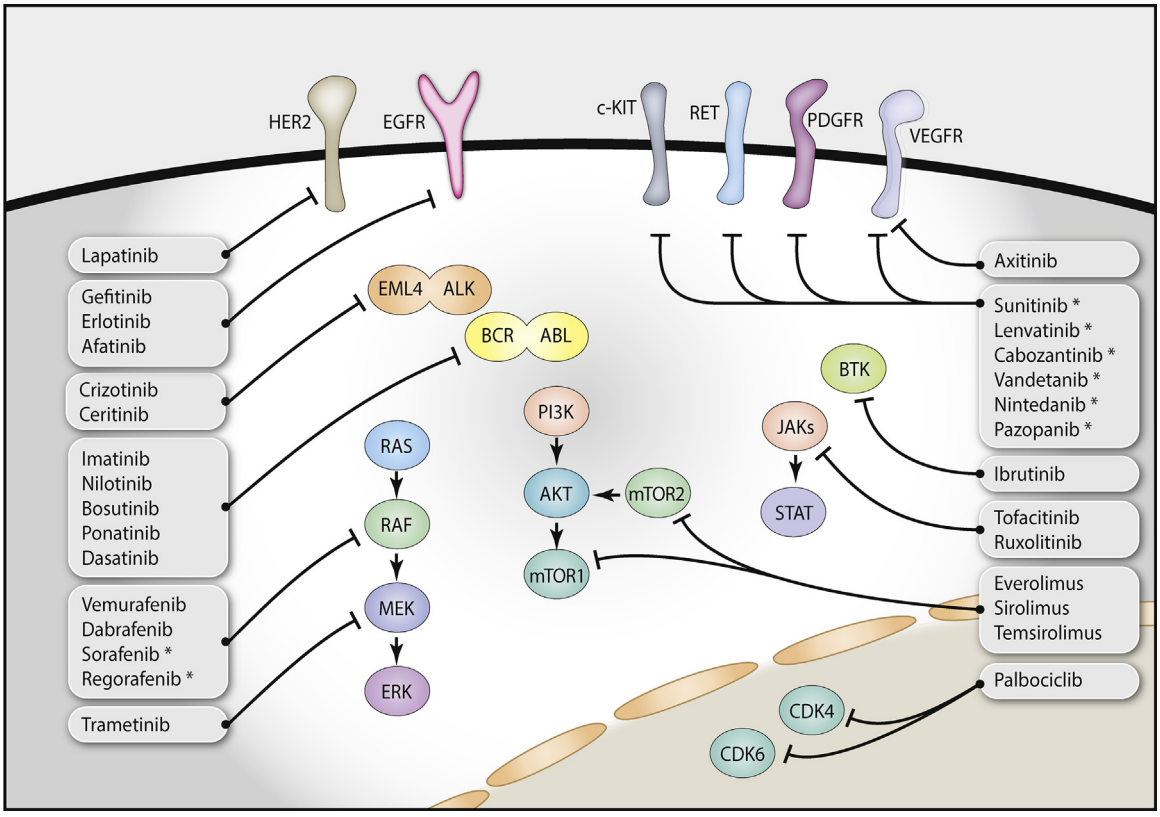

Fig. 2. Schematic representation of kinase cascades and respective, clinically approved kinase inhibitors. A cancer cell is schematically depicted with the nucleus in the lower right side. All clinically approved kinase inhibitors are identified by their names and by the respective pharmacological targets. Note that kinase inhibitors might be mono-specific, or they might arrest several kinases with similar potency. Asterisks label inhibitors having more than one target.

as failure of drug delivery across the blood brain barrier (BBB). For instance, it has been reported that crizotinib is ineffective on brain metastases derived from lung tumors expressing rearranged EML4-ALK fusions [32]. Overall, this type of acquired resistance is deeply connected to PKI-specific pharmacokinetics parameters and it goes beyond the scope of this review.

Biological resistance: This group of molecular and cellular mechanisms represents tumor adaptation while under drug treatment [15,17], and will be our main focus. Whether or not adaptation occurs at a single cell level (de novo alterations) or at the population level (pre-existing variation) is currently debated and might differ among the four general mechanisms we review below (see Fig. 3). The scenario assuming pre-existing, population-wide diversity argues that the majority of human cancers display diverse patterns of genomic instability ranging from single-nucleotide mutations to large-scale changes in chromosome structures. This diversity provides the substrate for Darwinian-type selection within tumors exposed to a PKI [33]. Thus, the cytotoxic effect of a PKI translates to a strong selective pressure for cells that acquired resistance through mutations within the kinase domain, or through amplification of the oncogene. Other mechanisms involve activation of alternative pathways of cell survival, which use either a parallel (bypass) track or an active target downstream of the blocked kinase. What follows is a concise description of each general mechanism of biological resistance to PKIs.

Intrinsic mutations in drug targets: Secondary somatic alterations within the target oncogene itself were first described in patients with CML treated with imatinib [34]. Interestingly, sequencing data support a clonal selection model of preexisting BCR-ABL mutations that confer imatinib resistance [35,36]. BCR-ABL mutations fall into two groups: those that alter amino acids that directly contact imatinib and those presumably preventing BCR-ABL from achieving the inactive conformational state required for imatinib binding. Among contact point mutations, the most frequently mutated is a gatekeeper 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. Importantly, although the corresponding mutated residues in kinases like BCR-ABL (T315I) and EGFR (T790M) are in close proximity to PKIs, they typically do not avoid ATP binding. Consequently, gatekeeper mutations cause little or no change in kinase activity, but they might confer resistance to specific inhibitors.

Gene copy alterations: Amplification of the gene corresponding to the PKI's target represents another somatic resistance mechanism in relapsing tumors. Examples include amplification of *BCR-ABL* in imatinib-resistant CML [34] and amplification of the PKI-resistant gatekeeper mutant of EGFR, namely EGFR-T790M. Notably, gene amplification was observed in a model of acquired resistance to an irreversible EGFR inhibitor, PF00299804 [37]. Interestingly, resistance to PF00299804 arises, at least in part, through selection of a pre-existing EGFR-T790M-amplified clone both in vitro and in a xenograft model in vivo.

Pathway alterations: A variety of both genomic and non-genomic mechanisms recover signaling, despite PKI-mediated arrest of an oncogenic protein kinase [38]. One subtype of this category places a constitutively active effector downstream of the blocked kinase. For example, genetic and histologic analyses of tumor biopsies from 37 patients with drug-resistant non-small cell lung cancers (NSCLCs) carrying EGFR mutations identified some resistant cancers expressing mutant forms of the *PIK3CA* gene [39]. Additional components of the EGFR pathway, such as BRAF [39,40]

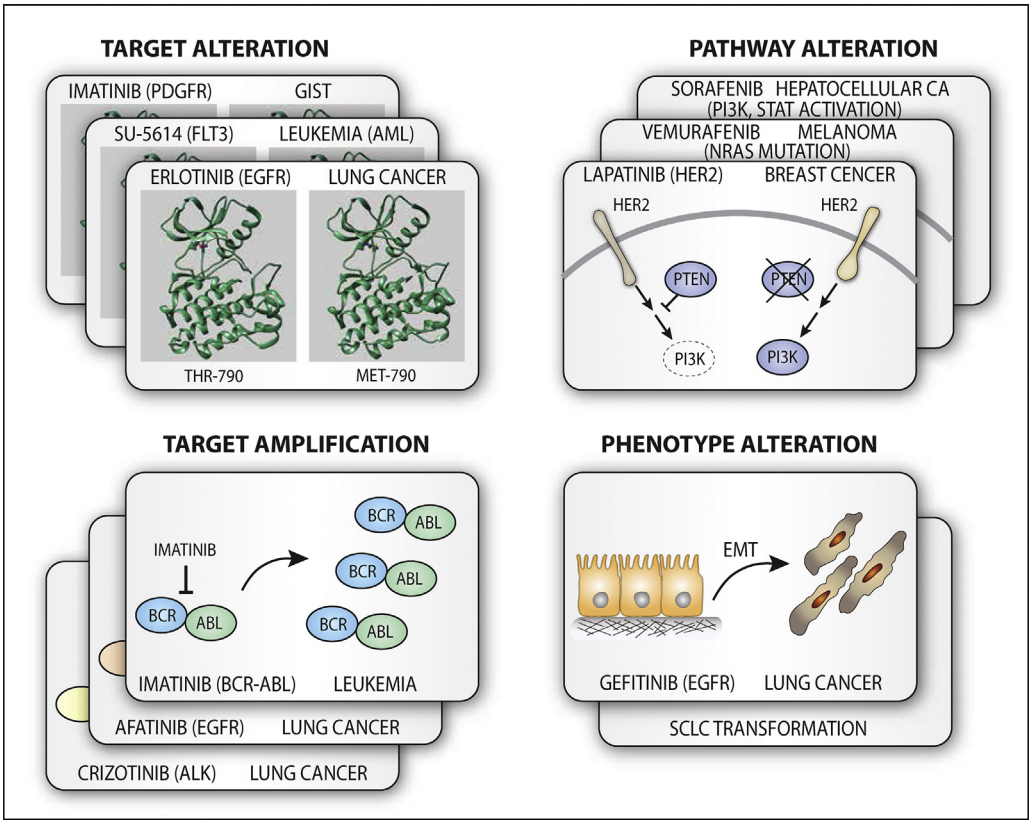

Fig. 3. Mechanisms of biological resistance to kinase inhibitors. Schematic representation of four major classes of alterations leading to resistance to PKIs, along with specific examples (see text for details). Target alterations refer to mutations within the extinguished kinase, which weaken inhibition by the kinase inhibitor (e.g., a replacement of the gatekeeper threonine 790 of EGFR with a bulky methionine). Pathway alterations bypass the targeted kinase by permitting signal transfer in a mode independent from the extinguished kinase (e.g., loss of PTEN in breast cancer, which confers resistance to a HER2-specific PKI, lapatinib, and recovers activation of PI3K). Amplification of the gene encoding the targeted kinase, for example BCR-ABL, might confer resistance of leukemia to the respective inhibitor, imatinib. The last mechanism is the least understood, as it involves gross phenotypic alterations of treated cancer cells. For instance, acquisition of a motile, fibroblast-like phenotype by lung epithelial tumors following treatment with an EGFR-specific inhibitor.

and MAPK1 [41], might also compensate for an inactive form of EGFR.

Phenotype alterations: This pathway-independent mechanism of resistance is the least understood and also the less common mode. The aforementioned survey of 37 NSCLC patients [39] identified 5 cases of morphological transition to small cell lung cancer (SCLC), as well as two cases of epithelila-to-mesenchymal transition. Notably, EMT has been associated with acquisition of stem/progenitor cellular characteristics [42] and it was shown that leukemic stem cells are resistant to imatinib [35,36]. These observations raise the intriguing possibility that resistance to PKIs might be the outcome of dedifferentiation to tissue-specific stem cells [43].

5. Lung cancer: resistance to EGFR and ALK inhibitors

Lung cancer remains the leading cause of cancer-related mortality worldwide. Approximately 85% of patients are diagnosed with NSCLC and most present with advanced disease that is not amenable to curative therapy. For these patients, platinum-based combination chemotherapy offers only modest prolongation in survival [44]. The discovery in 2004 of mutant forms of EGFR in NSCLC identified groups of patients who are sensitive to PKIs like gefitinib and erlotinib [45–47]. Remarkably, earlier application of both gefitinib and erlotinib in large clinical trials that recruited unselected patients with lung cancer showed only minimal clinical activity (response rate < 10%) [48,49]. However, a key observation made by these early trials was rare responses, which were often dramatic and unusually durable. These observations are understandable in hindsight, because later studies discovered an association between EGFR activating mutations and PKI-responsive patients with NSCLC [45,46]. This ability to select patients on the basis of genomic changes introduced the era of targeted therapy in advanced lung cancer, shifting treatment from chemotherapy to molecularly tailored treatments.

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. However several rare mutations, such as exon 20 insertions, have been reported [50]. Notably, amplification of the mutated allele occurs quite often in EGFR-driven NSCLC [51]. Later biochemical and crystallographic studies demonstrated that mutant receptors possess higher affinity for the inhibitors, gefitinib and erlotinib, compared to the wild-type receptor [52,53]. Hence, inhibition of mutant kinases is achieved at lower drug concentrations compared to the wild type form of EGFR. More recent genomic analyses of NSCLC identified other potential driver oncogenes and targets for PKIs. They include *ROS1* rearrangements, *RET* fusions, *MET* amplification and activating mutations in *BRAF*, *HER2* and *KRAS*, in frequencies exceeding 1% (see Fig. 4). Rearrangement of the *ALK* gene provided a second biomarker linked to an approved use of a targeted agent in patients with advanced NSCLC. The first identifies fused gene, *EML4* (echinoderm microtubule-associated protein-like 4)-*ALK* [54], is the predominant *ALK* fusion in NSCLC, but additional fusions exist [54,55]. Crizotinib (PF-02341066), is a type I small molecule compound, which is highly specific to MET but also active against ALK and ROS1. Crizotinib achieved

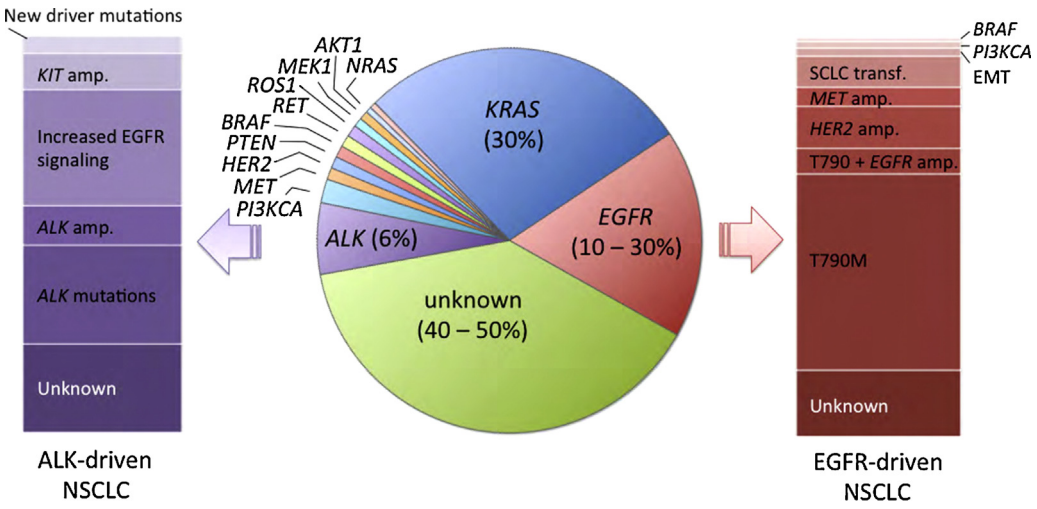

Fig. 4. Driver oncogenes and resistance of NSCLC to kinase inhibitors. The pie chart presents the currently known driver oncogenes of NSCLC and their incidence. The left column depicts mechanisms thought to confer resistance of ALK-mutated tumors to crizotinib. Similarly, mechanisms underlying resistance to erlotinib and gefitinib in EGFR-mutant NSCLC are presented in the right panel. Note that frequencies are approximate, and data are compiled from multiple clinical sources. The abbreviations used are: transf for transformation, and amp for gene amplification.

remarkable clinical effects when applied in phase I and II clinical trials in selected cohorts of patients with *ALK* rearrangements [56,57]. These trials led to the 2011 accelerated approval of crizotinib for treatment of *ALK*-positive advanced NSCLC.

Similar to EGFR inhibitors, acquired resistance ultimately limits the clinical benefit of crizotinib and studies using next-generation inhibitors are ongoing. Moreover, patients often relapse due to CNS progression, while they are treated with crizotinib. Acquisition of crizotinib resistance due to ALK mutations and copy number gain have been implicated in approximately 30–45% of crizotinib-refractory patients [58,59]. A common ALK mutation is the L1196M mutation, which is similar to gatekeeper mutations observed in BCR-ABL (T315I) and in mutant EGFRs of NSCLC (T790M) [60,61]. However, unlike EGFR or BCR-ABL mutations, L1196M comprises only a fraction of a broad range of resistance mutations distributed throughout the kinase domain of ALK (e.g., C1156Y, L1152R, G1202R, S1206Y, F1174C, D1203N, G1269A and G1123S/D) [62]. As already mentioned, acquired resistance to crizotinib can also be mediated by an amplification of the rearranged *ALK* gene, and this is often accompanied by an *ALK* mutation [59]. A common ALK bypass track occurring in about 30–35% of crizotinib-refractory patients, entails activation of EGFR signaling. This can be achieved by activating mutations or amplification of EGFR [63,64], or by a paracrine loop through secretion of EGFR or HER3 ligands (e.g., EGF, TGF-alpha, HB-EGF or NRG) [65]. Furthermore, SRC [66] and amplification of other RTK genes (e.g., *KIT* and *IGF-1R*) have also been reported [67]. The second-generation ALK inhibitors ceritinib and alectinib have been developed and approved for use in crizotinib-relapsed *ALK*-fusion-positive NSCLC patients [68,69]. Importantly, both ceritinib and alectinib have demonstrated activity in brain metastases of crizotinib-relapsed patients. However, resistance to both of these inhibitors has emerged due to secondary mutations, such as G1202R (in response to ceritinib) and two other mutations (V1180L and I1171T; in response to alectinib). Next-generation ALK inhibitors designed to overcome ALK resistance mutations and brain metastasis (e.g., PF-06462922) have the potential to treat ALK-driven cancer in the refractory setting [70].

In analogy to crizotinib, despite initial dramatic activity of erlotinib and gefitinib, EGFR-specific PKIs, all patients acquire resistance within approximately one year [71,72], and the most common mechanism of resistance involves a gatekeeper mutation, T790M [10–12]. Importantly, the T790M mutation occurs *in-cis* with the primary EGFR mutation. More rare secondary mutations, D761Y and L747S, also mediate gefitinib/erlotinib resistance. As already discussed in this review, the T790 substitution is often accompanied by amplification of the *EGFR* gene, a phenomenon that mediates resistance to both first and second generation EGFR-kinase inhibitors [37]. Amplification of the gene encoding another RTK, MET, occurs in 5–10% of cases of acquired resistance [73,74]. Activation of MET through its own ligand, HGF, might also confer resistance [75]. MET activation is thought to promote rerouting of the signaling cascade via formation of a MET-HER3 complex, which in turn restores the PI3K-to-AKT pro-survival pathway [76]. The oncogenic role of MET in NSCLC and preexistence of MET-amplified tumor cell clones, prior to anti-EGFR treatment, suggest that MET has a role to play in both primary and acquired resistance to anti-EGFR inhibitors [74,77]. A role for the MET-HER3-PI3K module in acquired resistance is supported by several lines of evidence: activation of PI3K and the downstream kinase, AKT, by an ectopically expressed active mutant of PI3K conferred PKI resistance in a cellular model, and downregulation of PTEN, which normally inhibits PI3K signals, was found to be sufficient for emergence of resistance in another model [78,79].

MET amplification was the first described bypass track mechanism. Similarly, *HER2* gene amplification has been implicated in resistance to PKIs. *HER2* was found to be amplified in 12% of tumors with acquired resistance versus only 1% of untreated lung adenocarcinomas [80]. Notably, *HER2* amplification and EGFR T790M are mutually exclusive. Activation of other RTK genes, such as *AXL*, *IGF1R* and *FGFR*, has also been reported to circumvent EGFR inhibition by PKIs [81–83]. In addition, mutations activating intracellular kinases, such as BRAF, MAPK1 and PI3KCA, as well as loss of PTEN, have been reported as mechanisms of acquired resistance to EGFR inhibitors in preclinical and clinical models [39,84–87]. Lastly, phenotypic changes to either small-cell lung cancer or to NSCLC with evidence of epithelia-to-mesenchimal transition (EMT) have been observed at the time of acquired resistance [39,40,88,89], but how does this phenotypic switch mediate resistance is currently unclear.

6. Chronic myelogenous leukemia: resistance to BCR-ABL inhibitors

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder that is a consequence of a somatic mutation affecting

hematopoietic stem cells. This mutation results in a translocation between chromosomes 9 and 22, termed the Ph chromosome [t(9;22)(q34;q11)]. The translocation juxtaposes a portion of the Abelson leukemia (*ABL*) gene from chromosome 9 and the breakpoint cluster region (*BCR*) gene of chromosome 22, and encodes a BCR-ABL fusion protein. Nearly 90% of patients who are diagnosed with CML have chronic-phase disease, meaning elevated white-cell counts with circulating immature precursors. After approximately 4–5 years, untreated CML progresses to the more aggressive accelerated and blast phases, characterized by increasing numbers of leukemic blasts in the blood and bone marrow. The median survival of patients in the blast phase is less than 6 months. The BCR-ABL fusion protein, which contains the active tyrosine kinase region of ABL, drives a constitutive proliferative signal [90,91]. Development of the first BCR-ABL inhibitor, imatinib (STI-571 or Gleevec®), represents a milestone in the history of molecular targeted cancer therapy [92]. Imatinib is a type II ATP-competitive inhibitor of the ABL kinase. Despite remarkable clinical results, intrinsic target alterations, such as mutations in the kinase domain and overexpression of the BCR-ABL protein, remain the primary causes for relapse [93,94], particularly in advanced stages of the disease [95]. Preventing target recognition by imatinib seems to be a common feature among the multiple resistance conferring point mutations so far identified [96]. In particular, the T315I mutation is among the most frequent and it produces the highest magnitude of resistance to imatinib [34]. Based on computational modeling and crystal structure analysis, this substitution is predicted to reduce drug affinity in two ways: by preventing formation of a hydrogen bond with imatinib [97], and by creating a steric hindrance for drug binding [34]. Other mutations often cluster within the P-loop and destabilize the conformation required for imatinib binding [36]. Overexpression of the BCR-ABL protein, due to gene amplification, was first observed in vitro when resistant CML cell lines were exposed to increasing doses of imatinib [98,99]. However clinical relevance of overexpression is limited to a small fraction of patients [100,101]. Although intrinsic alterations cover the majority of relapses, BCR-ABL independent (or bypass track) mechanisms, such as upregulation of SRC family kinases, have been linked to imatinib treatment failure [102].

Second generation BCR-ABL inhibitors have been developed, three of which (nilotininb, dasatinib and bosutinib) have been approved for the treatment of patients who developed imatininb resistance. These inhibitors are active against all of the common BCR-ABL mutants with the exception of T315I. Hence, it is possible that sequential treatment with PKIs might cause selection of this and other mutations [103–105]. Ponatinib, a third generation BCR-ABL inhibitor that exhibits potent T315I inhibitory activity, was approved in 2013 [106].

7. Breast cancer: resistance to lapatinib

Amplification of human epidermal growth factor receptor 2 (*HER2*, also called *ERBB2*) occurs in approximately 20% of breast cancers and is associated with shortened patient survival time [107]. HER2 is the closest kin of EGFR, but unlike other RTKs it binds with no known growth factor [108]. Instead, HER2 forms dimers with its family members, primarily with HER3 and EGFR. Combining HER2-targeted agents, such as an antibody called trastuzumab, with standard chemotherapy represents an effective therapeutic approach for patients with HER2-positive metastatic breast cancer. When combined with first-line chemotherapy, trastuzumab increases the time to progression and overall survival among patients with metastatic disease [109]. Lapatinib, a highly selective, small-molecule inhibitor of both HER2 and EGFR [110], is currently the only clinically approved PKI for the treatment of advanced stage HER2-positive breast cancer. Lapatinib is a type II ATP-competitive inhibitor that binds with the inactive conformation of its targets, hence prevents kinase activation [111]. Although a significant advancement in the treatment of breast cancer, the clinical efficacy of lapatinib has been limited by the development of acquired therapeutic resistance [112,113]. In contrast to other kinase inhibitors, for which intrinsic target alterations, mainly mutations within the ATP-binding pocket, represent a major escape route, HER2 mutations do not play a major role in lapatinib resistance [114]. Instead, several lines of evidence implicate resistance mediated by de-repression and/or activation of compensatory, HER2-independent survival pathways [115]. Consistent with this scenario, HER2 knockdown appears insufficient to revert lapatinib resistance in cell culture settings [116]. For example, lapatnib-induced inhibition of the PI3K/AKT pathway relieved FoxO3 repression, thus leading to increased transcription of the estrogen receptor and downstream signaling [117]. In line with this observation, genome-wide RNA interference screens identified the tumor suppressor PTEN as a modulator of lapatinib sensitivity in vitro and in vivo [118]. In addition, it was found that dominant activating mutations in *PIK3CA* (E545K and H1047R), which are prevalent in breast cancer, also confer resistance to lapatinib. Hence, deregulation of the PI3K pathway, either through loss-of-function mutations in PTEN or dominant activating mutations in *PIK3CA*, might act as an escape pathway of lapatinib-treated, HER2-overexpressing tumor cells. Another study identified AXL overexpression as a mechanism of resistance [119]. According to yet another model, ligand-mediated autocrine secretion of neuregulin (also called heregulin) and activation of the EGFR-HER2 axis can confer resistance to lapatinib [120]. In addition, according to Spector and colleagues, a truncated form of HER2 that is expressed in the nuclei of breast cancer cells, has a role to play in development of therapeutic resistance to HER2-specific PKIs [121].

8. Melanoma: resistance to BRAF inhibitors

Malignant melanoma is the most fatal type of skin cancer and its incidence continues to increase. Melanomas have the highest mutational load of all human tumors. Common mutations in melanoma include $BRAF^{V600E}$ (50–60% of all melanomas), $NRAS^{Q61L/R}$ (15–20%) and $RAC1^{P29S}$ (4–8%) [122]. The identification of *BRAF* mutations and their ability to destabilize the inactive kinase conformation [123,124] led to the development of two PKIs, which significantly improved prognosis of metastatic melanoma patients compared to chemotherapy. Vemurafenib was approved in 2011 for the treatment of metastatic melanoma and thyroid tumors, and this was followed by the approval of dabrafenib in 2013 [125–128]. Primary (intrinsic) and secondary (acquired) resistance, along with a remarkably diverse adaptive resistance significantly limit clinical benefit, such that complete response does not exceed 6% [129]. Approximately 15% of patients treated with BRAF inhibitors achieve no tumor regression, and this has been attributed to different mechanisms, such as RAC1 mutations [130], loss of PTEN [131] or NF1 [132], overexpression of cyclin D1 [133] and abundance of HGF [134]. Acquisition of secondary resistance, which normally occurs 2–18 months after treatment initiation, involves mainly reactivation of the ERK pathway [135,136].

Interestingly, out of the four general mechanisms of drug resistance depicted in Fig. 3, the major one, namely secondary mutations affecting gatekeeper and other residues of the extinguished kinase, appears irrelevant to BRAF as no secondary mutations have been detected and the primary one is persistent in all progressive tumors [137]. Another unusual feature appears to be multiplicity of resistance mechanisms that co-exist in the same tumor, 70% of which reactivate MAPK and only 22% of the secondary aberrations

affect the PI3K–PTEN–AKT pathway [137]. The following molecular mechanisms have been implicated in secondary resistance to BRAF inhibitors [reviewed in [138,139]]: gene amplification and activating *NRAS* mutations [140–142], activating MEK1/2 mutations [143], *BRAF* gene amplification [144] and elevated CRAF levels [145], overexpression of MAP3K8/COT, a kinase that directly activates MEK [146], and alternative splicing of BRAF's transcript [147]. In the latter mechanism, variant $BRAF^{V600E}$ transcripts lacking exons coding for a protein region encompassing the RAS-binding domain, lead to ERK reactivation via RAS-independent BRAF dimerization. Activation of RTKs [e.g., HER3/ERBB3, IGF-1 receptor and PDGF-receptor may also cause resistance by promoting alternative signaling pathways, notably the PI3K/AKT/mTOR pathway] [142,148].

9. Strategies circumventing resistance to protein kinase inhibitors

Precise identification of mechanisms underlying evasion of drug's therapeutic effects is the first step in implementing alternative, resistance-overcoming treatment strategies. Accordingly, an important question to be addressed when studying an unknown mechanism of resistance is whether or not a resistant cancer cell still relies on the original biochemical pathway, for example the ERK–MAPK pathway in metastatic melanomas that evolved secondary resistance to BRAF inhibitors. Another general mechanism to be considered at the outset entails involvement of up-regulated RTKs [20] or specific growth factors. Notably, several studies implicated elevated levels of autocrine or paracrine (stroma-derived) growth factors like VEGF, HGF and neuregulins in the acquisition of resistance to PKIs and to other drugs [134,149]. What follows is a summary of the currently emerging strategies that might circumvent drug resistance in cancer models and in clinical settings.

Overcoming stimulatory effects of secondary kinase domain mutations: Circumventing the action of secondary mutations translates to selecting next-generation kinase inhibitors. This might employ fragment-based drug discovery (FBDD), which involves screening of relatively small chemical compounds in order to discover leads against the kinase target. Identified leads are then optimized using medicinal chemistry, often with significant structural input, either from X-ray crystallography or from NMR spectroscopy [150]. Molecular docking studies might aid attempts to achieve increased potency and specificity of selected compounds, as reported for an EGFR inhibitor [151]. For example, second-generation EGFR inhibitors like neratinib, afatinib and dacomitinib, which bind to EGFR in an irreversible manner, aimed at providing therapeutic answers to T790M-mediated resistance [152,153]. The newer, third-generation irreversible inhibitors, specifically inhibit EGFR-T790M while sparing wild-type *EGFR* [154,155]. Like the previous generation, these compounds irreversibly (covalently) bind with the mutant receptor at cysteine 797. AZD9291, a third generation irreversible inhibitor, spares wild-type EGFR, and it is available orally. Pre-clinically, the drug potently inhibited signaling pathways, which translated into profound and sustained tumor regression in EGFR mutant tumor xenografts [155]. AZD9291 and another third generation inhibitor have received FDA "breakthrough therapy designation" and are currently undertaken for advanced clinical trials [156]. However, acquired resistance to these inhibitors might be anticipated, either due to amplification of the T790M mutated allele [37,157–159] or to emergence of a novel mutation at cysteine 797, which prevents binding of the irreversible drugs to EGFR [160]. Furthermore, third generation EGFR inhibitors seem also to share EGFR-independent mechanisms of acquired resistance with the old generation of drugs; amplifications of both MET and HER2 have been observed in biopsy specimens from patients progressing under AZD9291 treatment [161].

Immunological strategies to overcoming resistance to kinase inhibitors: Because mutations within exons encoding the kinase domain of EGFR, HER2 and other RTKs are frequent, but the respective extracellular domain rarely undergo mutagenesis, antibodies might be able to break repeated cycles of emerging resistance to PKIs. For example, a phase Ib study that enrolled NSCLC patients who acquired resistance to erlotinib/gefitinib, and combined afatinib and cetuximab, an anti-EGFR mAb, observed comparable but relatively high overall response rates (approximately 29%) in T790M-positive and in T790M-negative tumors [162]. Interestingly, applying cetuximab in combination with chemotherapy on an unselected cohort of NSCLC patients in a previous, randomized phase III trial that compared chemotherapy plus cetuximab with chemotherapy alone demonstrated improved overall survival for chemotherapy plus cetuximab in patients who had some degree of EGFR expression in their tumors. However, a similar trial failed to demonstrate an improvement in progression-free survival (reviewed in [163]). Pre-clinical studies indicate that when applied alone, cetuximab induces feedback regulatory loops that up-regulate both HER2 and HER3, but a combination of three mAbs, to EGFR, HER2 and HER3, blocked feedback and effectively inhibited tumors expressing the PKI-resistant, EGFR-T790M, in an animal model [164]. Similarly, a combination of three pairs of mAbs each directed against two non-overlapping epitopes of EGFR, HER2 and HER3, was able to downregulate the three receptors and strongly inhibit a broad range of drug-resistant tumor models [165]. In conclusion, combining a PKI and a mAb or applying mixtures of mAbs might overcome resistance to PKIs and preempt emergence of resistance-conferring mutations within kinase domains of oncogenic receptors.

Overcoming resistance by blocking rescue pathways: In theory, elucidating a resistance-conferring bypass pathway would identify a rational combination treatment able to concurrently block the extinguished kinase, as well as the default pathway, thereby gain prolongation of patient response. For example, the identification of dominant activating mutations in *PIK3CA* as potential lapatinib resistance mechanisms might lead to simultaneous treatment of breast cancer patients with a combination of lapatinib and NVP-BEZ235, a dual inhibitor of PI3K/mTOR [118]. Another example, which has recently reached clinical application, is based on the finding that resistance to therapy with BRAF kinase inhibitors is associated with reactivation of the MAPK pathway [135,166]. Hence complete inhibition of this pathway might be needed to induce cell death in BRAF V600 melanoma. This can be achieved by combining a BRAF inhibitor with a MEK inhibitor. In line with this prediction, a clinical trial that combined treatment with dabrafenib, a selective BRAF inhibitor, and trametinib, a selective MAPK kinase (MEK) inhibitor reported that progression-free survival was significantly improved by the combination of drugs [167,168]. Potentially, similar observations might yield effective drug combinations, including up-regulation of HER3 in the context of inhibited AKT [19], and up-regulation of MET in NSCLC tumors undergoing treatment with EGFR inhibitors [76].

10. Perspectives and future directions

The remarkably fast pace and unexpected path traveled by the field of personalized medical oncology over the last decade has inevitably instigated a similarly dynamic arena devoted to resolving mechanistic bases of acquired resistance to molecular targeted drugs. This new field holds enormous promise for cancer patients, for knowledge of cancer drug resistance gained through such efforts would enable delaying onset of resistance and also

enhancing efficacy of currently approved, as well as future cancer drugs. As with other young fields, some common features are slowly emerging and this review tried to delineate them. While advanced chemical synthesis will surely provide ever more effective compounds that overcome secondary mutations within kinase domains, re-mapping of signaling networks will likely uncover potential compensatory routes. Predictably, we will witness a significant expansion of the field of emergent (acquired) resistance to kinase inhibitors. This will entail a streamlined dialog between clinicians and biologists, along with utilization of novel methodologies. For example, single cell genomics and proteomics, methods able to resolve tumor heterogeneity at high granularity, hold great promise for the field. Likewise, genome wide loss-of-function screens (based on RNA interference and the crispr-cas9 system), when applied on tumor biopsies and animal models, might uncover dormant mechanisms of resistance long before they emerge in patients.

All currently approved kinase inhibitors are listed in alphabetical order, along with year of first clinical approval and main disease indication (abbreviated text). Also indicated are major mechanisms of resistance to each drug.

The 3D structure of a typical tyrosine-specific kinase domain, of EGFR, is shown in the middle panel, along with an ATP analog, AMP-PNP. The P-loop and the activation loop are colored in light blue and in light green, respectively. Leucine 858 (L858), which is frequently mutated in lung cancer, is highlighted (in pink). Similarly labeled is threonine 790 (purple), which is replaced by a methionine in some advanced lung tumors and confers resistance to first generation kinase inhibitors. The squared area is magnified in the side panels and shows the structure of the T790M mutant in a complex with either a first generation inhibitor, erlotinib (right), or with a second generation inhibitor, afatinib (left). The latter structure shows a replacement of cysteine 797 with a serine (red). This mutation prevents covalent binding of afatinib to EGFR and might confer resistance to second and third generation EGFR inhibitors. The chemical structures of both erlotinib and afatinib are shown.

A cancer cell is schematically depicted with the nucleus in the lower right side. All clinically approved kinase inhibitors are identified by their names and by the respective pharmacological targets. Note that kinase inhibitors might be mono-specific, or they might arrest several kinases with similar potency. Asterisks label inhibitors having more than one target.

Schematic representation of four major classes of alterations leading to resistance to PKIs, along with specific examples (see text for details). Target alterations refer to mutations within the extinguished kinase, which weaken inhibition by the kinase inhibitor (e.g., a replacement of the gatekeeper threonine 790 of EGFR with a bulky methionine). Pathway alterations bypass the targeted kinase by permitting signal transfer in a mode independent from the extinguished kinase (e.g., loss of PTEN in breast cancer, which confers resistance to a HER2-specific PKI, lapatinib, and recovers activation of PI3K). Amplification of the gene encoding the targeted kinase, for example BCR-ABL, might confer resistance of leukemia to the respective inhibitor, imatinib. The last mechanism is the least understood, as it involves gross phenotypic alterations of treated cancer cells. For instance, acquisition of a motile, fibroblast-like phenotype by lung epithelial tumors following treatment with an EGFR-specific inhibitor.

The pie chart presents the currently known driver oncogenes of NSCLC and their incidence. The left column depicts mechanisms thought to confer resistance of *ALK*-mutated tumors to crizotinib. Similarly, mechanisms underlying resistance to erlotinib and gefitinib in *EGFR*-mutant NSCLC are presented in the right panel. Note that frequencies are approximate, and data are compiled from multiple clinical sources. The abbreviations used are: transf for transformation, and amp for gene amplification.

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