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Molecularly Targeted Therapy: Past, Present and Future

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Abstract

Cancer is a heterogeneous disease with diverse underlying molecular causes and equally diverse clinical profiles. It has long been a goal to develop cancer therapies that would be distinct and appropriate for each patient. The recent concept of "oncogene addiction" has been very helpful in guiding this path "from the bench to the bed". The most significant advance in the treatment of cancer in the past few decades has been the introduction of molecularly targeted therapies, such as imatinib for chronic myelogenous leukemia and gefitinib for lung cancer. In addition, many new promising agents have been developed in the laboratory and are now entering clinical testing. Thus, our current challenge is to better understand how to utilize these agents in clinical practice and to better understand the mechanisms of drug resistance that may be encountered. Advances in these areas would allow for more targeted and effective treatment options for cancer patients. This review describes the development of molecularly targeted therapies from the past achievements to current efforts, the insights learned by this effort, the problems encountered in the clinic, and the potential for novel development of next-generation kinase inhibitors.

Keywords: Molecularly targeted therapy; Tyrosine kinase inhibitors; Imatinib; Gefitinib; Crizotinib; Drug resistance

Abbreviations: MTT: Molecularly Targeted Therapy; SMW: Small Molecular Weight; TKIs: Tyrosine Kinase Inhibitors; RTKs: Receptor Tyrosine Kinases; PI3-K: Phosphoinositide-3 Kinases; CML: Chronic Myelogenous Leukemia; GIST: Gastrointestinal Stromal Tumors; NSCLC: Non-Small Cell Lung Carcinoma; EGFR: Epidermal Growth Factor Receptor; HER2: Human-EGFR2; VEGFR: Vascular Endothelial Growth Factor Receptor; ALK: Anaplastic Lymphoma Kinase; mTOR: mammalian Target of Rapamycin; IGF-R: Insulin-like Growth Factor Receptor; MEK: Mitogen Activated protein kinase-kinase; PDGFR: Platelet-Derived Growth Factor Receptor; RCC: Renal Cell Carcinoma

Introduction

The concept of individualized cancer therapy has recently become a reality in clinical medicine, supported by the development of drugs targeting molecules specific to certain cancers. This approach has also been termed "molecularly targeted therapy" and has provided hope for the development of treatments that may be more effective than conventional therapies [1]. Currently, molecularly targeted therapies utilize either monoclonal antibodies or small molecular weight (SMW) tyrosine kinase inhibitors (TKIs) [2]. This strategy of targeting molecules particular to each cancer stems from our understanding that cancer cells display abnormal activation of intracellular signaling networks that are different from those operating in non-neoplastic cells. Cancer cells usually become more dependent on the activity of a specific molecule, since during the process of multistep carcinogenesis, genes which may play a similar physiological role tend to be turned off [3]. Consequently, cancer cells typically become "addicted" to a specific molecule for their growth and viability, which, in most cases, is an oncogene product. Furthermore, each tumor becomes "addicted" to a different signaling protein/pathway [3]. This concept of "oncogene addiction" allows for the possibility of therapy by inhibiting the activity of the critical molecule, and thus theoretically could selectively eradicate cancer cells while sparing normal cells [4]. This concept may explain why a subset of tumors harboring specific activating mutations or amplification of oncogenes sometimes display impressive clinical responses to agents targeting their aberrant gene products.

The efficacy of imatinib in chronic myelogenous leukemia (CML) is an outstanding example. However, this is an exceptional case because it is genetically simple neoplasm caused by a single aberrant gene product that is absent in non-neoplastic cells (the BCR-ABL1 fusion protein). In contrast, in the majority of common malignant tumors, aberrations of multiple different genes are responsible for carcinogenesis, and theoretically, a multitude of different therapeutic agents would be required [5].

In this sense, the major challenge at present for further drug discovery in molecularly targeted therapy is the elucidation of specific molecular mechanisms underlying the various forms of cancer found in the clinic. Therefore, research aimed at developing this goal is mainly geared toward the following three principles: i) the identification and validation of the "addicting gene product" of tumors as the therapeutic target, ii) stratification of the patients most likely to benefit from agents and iii) clarification of the mechanism of resistance to the therapies.

First, (i) target identification. Although the search for individual addicting genes was the initial goal in developing targeted therapies, many agents designed to target a single molecule have been found to have many other potential targets [6]. Indeed, remarkable clinical responses have often been noted by single molecule inhibitors in many cancers, even in those having genetically complex aberrations [1]. Therefore, many molecularly targeted inhibitors that are currently used as clinical drugs or in preclinical trials may display a broader selectivity than initially envisioned.

Second, (ii) patient stratification. Demonstrating the dependence of a cancer on a particular target may not only lead to the development of

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new inhibitors, but may accelerate the selection of patients most likely to benefit from these agents in clinical trials. One powerful strategy for pursuing i) and ii) is the use of phosphoproteomics to investigate the protein(s) with deregulated activities in cancer [1,7].

Lastly, iii) drug resistance is common feature observed in many patients undergoing treatment, and combating resistance is one of the most important issues that needs to be overcome when considering a new target and/or developing a new inhibitor.

In this new era of molecular targeted therapies, treatment options are basically dependent on the accurate molecular and genetic profiling of the cancer cells. Hence, the development of effective targeted therapeutics requires precise knowledge of the genes and pathways underlying the pathobiological profiles of each cancer.

Kinase as a Target

Overview

What are the appropriate targets for cancer therapy? The typical targets of molecular targeted therapies have been protein and lipid kinases. Among more than 500 protein kinases and about 20 lipid-modifying kinases encoded by the human genome, more than 100 protein kinases are encoded by cancer-related genes [1].

Protein and lipid kinases fulfill essential roles in signaling pathways that regulate many basic physiological functions and they are relatively amenable to modulation as drug targets [8]. As these kinases regulate diverse normal cellular processes, their deregulation can lead to abnormal cell growth and a variety of pathologies, including immunological and endocrine disorders, and malignant tumors [9]. Since the end of the 1990s, genetic analyses have identified a plethora of gene amplifications and mutations and consequent deregulated kinase activities that are linked to specific human cancers [1,7]. Amplification, mutation or chromosomal rearrangement of these kinase genes may provide important clues for drug discovery. In particular, aberrant signalings resulting from dysregulated receptor tyrosine kinases (RTKs) play a role in many cancers and thus are attractive and prominent therapeutic targets. In addition, lipid-modulated protein kinases, such as phosphoinositide-3 kinases (PI3-K), and Ras-Raf-MAPK have been promising therapeutic targets, as they or their signal pathways are frequently mutated in cancer and they function as the downstream effectors of the RTKs (Figure 1) [10].

One way to effectively block RTK signaling is by inhibition of RTK catalytic activity with SMW kinase inhibitors or monoclonal antibodies directed against its extracellular domains. Imatinib (Novartis Pharmaceuticals) is a SMW-TKI of RTK that is used not only to treat CML caused by the *BCR-ABL1* fusion, but also for gastrointestinal stromal tumors (GIST) harboring mutated *c-KIT*. The SMW-TKIs, gefitinib (AstraZeneca Pharmaceuticals) and erlotinib (OSI Pharmaceuticals) are used to treat non-small cell lung carcinoma (NSCLC) associated with mutated epidermal growth factor receptor (*EGFR*) [2,11]. Trastuzumab (Genentech Pharmaceuticals) is a monoclonal antibody against human EGFR2 (HER2), which is frequently overexpressed in breast carcinomas. So far, drugs targeting the EGFR, vascular endothelial growth factor (VEGF) and anaplastic lymphoma kinase (ALK) have been approved by the U.S. Food and Drug Administration (US-FDA) for the treatment of advanced NSCLC [2,12]. Although 11 SMW-TKIs have been approved for clinical use, these target only a few kinase types, including RTKs (EGFR, ERBB2, etc), non-receptor TKs (Abl etc,), and the Ser/Thr-specific kinase, mammalian target of rapamycin (mTOR) (Table 1). The success of these agents has triggered the development of potential successors, including broad-spectrum inhibitors that might be useful against TKI-resistant mutants. Although activating mutations in kinases have been identified in many cancers, it will require much additional effort to understand the dependence, i.e. addiction of tumor growth on a particular kinase or its pathway(s). Unfortunately, advanced stage cancers usually harbor many complex genetic aberrations, making the identification of the kinase(s) responsible for cell proliferation in each tumor difficult [1]. This complexity has hindered the identification of drug targets and our ability to predict the response of different cancers against particular therapeutic agents [13].

The discovery of activating mutations in the kinase domain of the *EGFR* has revolutionized the treatment of NSCLC by allowing more effective patient stratification (principle ii). This eminent milestone in NSCLC therapy was established by using the first-generation *EGFR*-TKIs, gefitinib and erlotinib. Gefitinib was approved for use in NSCLC before the critical role of *EGFR* mutations was understood, but has since been developed as a sophisticated therapy that exploits our knowledge of the relationships between *EGFR* mutations and drug sensitivity, and thus has heralded the beginning of a new era of molecular targeted therapy in cancer [12,14]. However, while activating mutations in protein kinases can increase their sensitivity to TKIs in some cases, they can also increase resistance in other cases, further complicating the development of TKIs [15].

A large number of kinase inhibitors have been considered for targeted cancer therapy, both for their potential efficacy and for minimizing possible side effects [1]. The initial focus of drug development was set on the agents specific for a single kinase target, following the examples of imatinib and gefitinib. Targeting single molecules would also be expected to reduce potential side effects. However, the reality is that the vast majority of human malignancies harbor very complex aberrations in their genomes and signaling networks, and thus inhibiting a single target alone might not be therapeutically effective. Furthermore, even though blocking the activity of a single target has been shown to prevent tumor progression in the short-term, it is common for the cancer to eventually progress over the longer term. This acquired resistance is

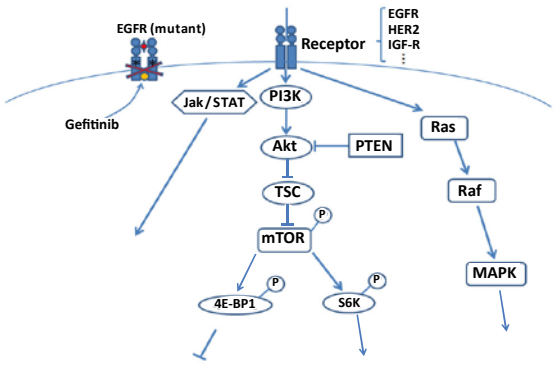

Figure 1: Schematic representation of the signalling pathway from the growth factor receptor. Arrows represent activation and bars represent inhibition. Abbreviations: PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K, ribosomal p70 S6 kinase; STAT, signal transducer and activator of transcription; MAPK, mitogen activating protein kinase.

Agent / Compound	company	Target	Reference
Antibody			
trastuzumab	Genentech	HER2: breast, gastric cancer	29,32,77
cetuximab/IMC-C225	ImClone/Bristol-Myers Squibb	EGFR: SCCHN,[1] Colorectal cancer	29
panitumumab	Amgen	EGFR: Colorectal cancer with wild-type *KRAS*	30
bevacizumab	Genentech	VEGF: Colorectal carcinoma NSCLC[2]	29
rituximab	Genentech	CD20: B-cell lymphomas,	47,48
pertuzumab	Genentech	HER2: Breast cancer	84
Tyrosine kinase Inhibitors			
Reversible inhibitors			
imatinib	Novartis	c-KIT, PDGFR: CML[3], GIST[4]	1,5,11,21
gefitinib	AstraZeneca	EGFR: NSCLC	1,25,26,27
erlotiinib	OSI	EGFR: NSCLC	2,26
lapatinib	Glaxo SmithKline	EGFR/HER-2: breast cancer	1,78
crizonitib (PF2341066)	Pfizer	ALK: NSCLC MET: Gastric cancer, RCC[5]	13,38,39, 40
TAE684	ARIAD	ALK: NSCLC	39
SU11274	Sugen	c-MET	12
AP26113	ARIAD	ALK (mutant)/EGFR: NSCLC	37,38,85
CH542802	Chugai	ALK (mutant): NSCLC	37,38,85
NVP-TAE684	ARIAD	ALK (mutant): NSCLC	86
Irreversible ihibitors			
neratinib / HKI272	Wyeth	EGFR/ERBB2/Pan-ErbB: NSCLC, breast cancer	1,26,74
PF002998904 (dacomitinib)	Pfizer	EGFR/HER2/HER4	1,27,74
BIBW-2992 (Afatinib)	Boehringer Ingelheim	EGFR/HER2 NSCLC, SCCHN breast cancer	1,64
Multi-targeted Inhibitors			
sunitinib	Pfizer	VEGFR, PDGFR, c-Kit, RET, Flt3,BCR-ABL, SRC: RCC, GIST	12,29
sorafenib	Onyx and Bayer	Raf, VEGFR-2/3, PDGFR-B: GIST	11,29,43
dasatinib	Bristol-Myers Squibb	SRC, ABL, c-KIT: CML	11
nilotinib	Novartis	c-KIT, PDGFR-A/B: CML	11
XL184 / BMS-907351	Exelexis/Bristol Squibb Myers	RET, KIT, MET, VEGFR, FLT3, TIE2, PDGFR: NSCLC, Endocrine cancer; Glioblastoma	12,27
EXEL880 (Foretinib)	Exelixis/Glaxo SmithKline	Met/VEGFR2	12,27
ARQ197 (tivantinib)	ArQule	Met/Focal Adhesion Kinase: NSCLC	12,27
EXEL-7647	Exelixis	EGFR/HER2/VEGFR: Breast cancer	79
Other agents			
temsirolimus /CCI-779	Wyeth	mTOR: RCC, pancreas NEC	45,46
everolimus /RAD-001	Novartis	mTOR: RCC	45,46
flavopiridol	Sanofi Aventis	cdk[6]: CLL[7]	1,49
AZD5363	AstraZeneca	Akt	53
Akti-1/2	-	Akt	55
triciribine phosphate	-	Akt	56
perifosine	Aeterna Zentaris	Akt/mTOR	54
NVP-BEZ235	Novartis	PI3-K/mTOR	1,51
GSK2126458	Glaxo SmithKline	PI3-K/mTOR	1,51
PF-04691502	Pfeizer	PI3-K/mTOR	1,51
GDC-0941	Genentech	PI3-K/mTOR	1,51
geldanamycin	-	HSP90	76,82,83
tanaspimycin/17-AAG	Bristol-Myers Squibb	HSP90	26,27,76
T-DM1	Genentech	HER2	59,81,82

1) SCCHN, squamous cell carcinoma of the head and neck; 2) NSCLC, non-small cell lung carcinoma; 3) CML, chronic myelogenous leukemia; 4) GIST, gastrointestinal stromal tumors; 5) RCC,renal cell carcinoma; 6) cdk, cyclin dependenk kinase; 7) CLL, chronic lymphocytic leukemia.

Table 1: Representative molecularly targeted inhibitors.

a major issue in the development of molecularly targeted therapies, and agents that act against multiple targets may have more promise in avoiding the development of resistance [16]. A combined regimen of several single-target agents or a single multi-targeted inhibitor of several pathways both provide greater opportunity for potential benefit. Such combinations could lower the effective dose of each agent while retaining comparable or enhanced activity, and thus may reduce toxicity [12].

In general, evidence from recent clinical research into kinase inhibitors suggests that when no specific pathway driving tumor proliferation can be identified, inhibition of multiple targets produces greater benefit over single target inhibition [17].

Biochemistry of kinase inhibitors

All protein kinases share a conserved catalytic kinase domain containing the ATP binding site, which is the primary target of most SMW kinase inhibitors [18]. Kinase inhibitors can be broadly classified as ATP-competitive or non-ATP-competitive.

Most kinase inhibitors are ATP-competitive, i.e. interacting with the ATP binding site, as the structures of protein kinases in the active ATP-bound state are similar [18]. ATP-competitive inhibitors can be designed efficiently using recombinant, activated catalytic domains of kinases, regardless of selectivity. A recent trend has been the development of irreversible inhibitors which bind covalently to the target, and the majority of these are the ATP-binding site-directed inhibitors [19]. The critical issue for future development of the agents is the targeting of the ATP binding site in a manner that is selective for the protein of interest.

In contrast to the ATP-bound active states, the structure of kinases in their inactive states are relatively diverse, thus the inactive conformation can be exploited to design selective inhibitors. Accordingly, non-ATP-competitive kinase inhibitors generally exhibit higher selectivity than ATP-site-directed inhibitors, since they exploit structural features that are unique to each protein kinase [20], for example, regulatory domains that modulate the activity of the catalytic domain [18]. Such non-ATP-competitive inhibitors targeting allosteric sites have been designed for SRC, insulin-like growth factor-I receptor (IGF-IR), mitogen activated protein kinase-kinase (MEK), mTOR, ABL, and AKT [1]. However, compared to ATP-pocket binders, non-ATP-competitive inhibitors are relatively difficult to design and to find by screening.

Targeting Agents

At present, about 150 kinase-targeted drugs are in the clinical stage and many more are in preclinical development [1]. Many of these kinase inhibitors target RTKs or downstream signal transduction molecules such as second messenger-dependent (lipid and protein) kinases and nuclear proteins.

SMW-TKIs, such as imatinib and gefitinib, have been highly evaluated and have received marketing approval, and many other candidates are in clinical trials. Although c-MET (the receptors for hepatocyte growth factor) and IGF-IR are also frequently genetically altered in advanced cancers, TKIs of these receptors have not yet entered the market.

Next, we describe representative kinase inhibitors and other agents that have been successfully used in the clinic.

Imatinib

The commercial success of imatinib, the first kinase inhibitor to enter the clinic, has garnered considerable attention not only from hematologists, but also from gastroenterologists. Imatinib targets the inactive conformation of the ABL1 kinase, which is constitutively activated in more than 95% of CML due to a chromosomal translocation, i.e. the Philadelphia chromosome (Ph) [21]. Since its introduction, the rapid development of TKIs has changed the mode of cancer therapy. In addition, imatinib is used as a RTK inhibitor for the treatment of gastrointestinal stromal tumors (GIST) that harbor mutant *c-KIT* (*CD117*). GIST is characterized by gain-of-function mutations in the KIT or platelet-derived growth factor receptor (PDGFR) genes, leading to their constitutive activation [11].

Treatments with imatinib confers benefit in up to 80% of patients with advanced GIST, while the remaining 20% exhibit primary resistance or are intolerant to treatment. Moreover, 50% eventually show progressive disease within the first 2 years of therapy due to the emergence of acquired resistance [11]. The mechanisms hypothesized to be responsible for secondary resistance include the acquisition of new *KIT/PDGFR* mutations, loss of c-KIT expression, activation of an alternative RTK, activation of alternative downstream signaling pathway and genomic amplification of *KIT* [11]. For example, so-called "gatekeeper" mutations in the TKI-domain/ATP-binding pocket, such as KIT V654A and T670I, have recently been demonstrated to result in poor or no response to imatinib treatment [22,23].

To treat cases that have developed resistance against imatinib, new generation inhibitors are being pursued, and indeed the vast majority of drugs under development are multi-kinase inhibitors. Sorafenib (Onyx and Bayer Pharmaceuticals) inhibits imatinib-resistant T607I or PDGFR T681I mutants [11]. Dasatinib (BMS-354825, Bristol-Myers Squibb Pharmaceuticals) is an ATP-competitive dual SRC/ABL inhibitor that potently inhibits wild-type KIT, as well as the juxtamembrane domain mutant (D816Y and D816V) and loop mutant isoforms [11]. Dasatinib is indicated in patients with any phase of CML or Ph+ acute lymphoblastic leukemia who are resistant or intolerant to imatinib. Nilotinib (Novartis) is a second-generation TKI targeting c-KIT, PDGFR-A and PDGFR-B and is indicated for patients with CML who are resistant or intolerant to imatinib [11]. Recent studies have reported that dasatinib and nilotinib show efficacy as first-line therapies, and both have been approved in the US-FDA for newly diagnosed, chronic-phase CML [24].

EGFR-inhibitors

The success with imatinib has stimulated an interest in EGFR as a target for anticancer therapies by both SMW TKIs and monoclonal antibodies. Before gefitinib was approved for use as a second- and third-line therapy, patients with advanced NSCLC who had not responded to first-line platinum-based chemotherapy had limited treatment options and only modest survival [25]. Initially, 10% of all NSCLC patients experienced partial radiographic responses to gefitinib. This 10% response rate contrasts unfavorably with the 90% response rate to imatinib in CML [17]. However, the therapeutic strategy was refined to a more appropriate selection of patients and the response rate increased dramatically. Patients who were more likely to respond to gefitinib were female, of Asian ethnicity, had a minimal smoking history and adenocarcinoma histology [26]. Retrospective molecular analysis eventually disclosed an association between somatic mutations in *EGFR* and sensitivity to gefitinib [26]. Somatic mutations in *EGFR* are found in 10 to 40% of NSCLC patients and those with sensitizing somatic mutations treated with EGFR-TKIs have an initial clinical response of 55 to 82%, compared with a response rate of 20 to 30% with conventional platinum-based chemotherapy [27]. Therefore, in addition to CML and GIST, this clear correlation between the sensitivity and mutations in NSCLC represents another example of "oncogene addiction".

A larger-scale study examining gefitinib or carboplatin/paclitaxel as therapies for adenocarcinoma demonstrated that gefitinib was significantly more effective than conventional chemotherapy as a first-line therapy in patients with *EGFR* mutations, whereas conventional chemotherapy was superior in patients without *EGFR* mutations [13].

Some *EGFR* mutations have been clarified to modulate differential responses to TKIs, either enhanced sensitivity or resistance to TKIs [28]. There are also unique subgroups of patients with previously

uncharacterized mutations who benefit from SMW-TKIs, therefore careful selection based on the patient's genetic profile is essential [28].

The clinical efficacy of these EGFR-TKIs are often hampered in late-stage NSCLC, as the advanced disease typically harbors secondary genetic aberrations and/or complicated cross-talk among several intracellular signal transduction pathways. As a result, late-stage NSCLC cells can acquire drug resistance and escape from targeted therapy [1].

Monoclonal antibodies are another approach to molecularly targeted therapy. Cetuximab (ImClone Systems/ Bristol-Myers Squibb) is currently approved for the treatment of advanced colorectal cancer and squamous cell carcinoma of the head and neck (SCCHN) [29]. Panitumumab (Amgen Incorporation) was shown to be beneficial in metastatic colorectal carcinomas that do not have mutations in the *K-RAS* [30].

Trastuzumab and Lapatinib

Approximately 25% of human breast cancers overexpress the HER2 (ErbB2) protein, which confers a more aggressive phenotype and is associated with poor prognosis [31]. For this group of cancers, trastuzumab (Genentech), a humanized antibody targeting HER2 has provided a significantly improved outcome and has changed the therapeutic landscape for breast carcinomas [32]. Trastuzumab exerts its antitumor activity through a combination of antibody-dependent cellular cytotoxic and antiangiogenic effects, downregulation of HER2 receptors, disruption of downstream proliferative pathways, and inhibition of cell-cycle progression [32].

Trastuzumab therapy is indicated based on clear-cut criteria using immunohistochemical analysis (IHC) of paraffin tumor sections. Tumors exhibiting overexpression of HER2 protein with intensities of 3+ in IHC are indicative for trastuzumab therapy. In cases where IHC positivity is of 2+ intensity, additional detection of *HER2* amplification by fluorescence *in situ* hybridization analysis (FISH) is required. Tumors exhibiting 1+ or negative staining are not indicative for trastuzumab therapy [33].

In addition to breast carcinomas, a combination of trastuzumab plus conventional chemotherapy has been evaluated in a clinical trial (ToGA test) as the first-line treatment in patients with HER2-positive advanced cancers of the stomach and gastro-esophageal junction (GEJ) [29]. The results of these studies were promising, and a protocol was developed using IHC/FISH analyses in histopathology to select the patients eligible for combined trastuzumab/conventional chemotherapy. This regimen has become a new standard option for the treatment for these cancers [29].

Crizonitib

Anaplastic lymphoma kinase (ALK) was initially identified as part of an oncogenic fusion with nucleophosmin (NPM) in anaplastic large cell lymphoma (ALCL) [34]. Subsequently, rearrangements involving the *ALK* gene have also been found in NSCLC, inflammatory myofibroblastic tumors, and other cancers [13]. In NSCLC, oncogenic fusion partners of *ALK* include *echinoderm microtubule-associated protein-like 4* (*EML4*), *kinesin family member 5B* (*KIF5B*) and *TRK-fused gene* (*TFG)* [13,35,36].

The prevalence of various *ALK* fusion genes has been reported as ranging from 3 to 7% of all NSCLC cases, and the *ALK* fusion is largely exclusive of *EGFR* or *KRAS* [13,37]. The *EML4–ALK* fusion gene was identified and confirmed to be tumorigenic in NSCLC in 2007 [35]. However, the precise mechanism by which the EML4-ALK fusion protein activates intracellular signaling in tumor cells has not been fully elucidated.

Based on the experience with EGFR-TKIs, a multi-target RTK inhibitor crizotinib (PF-02341066, Pfizer Pharmaceuticals), which had been under early Phase I clinical development as a c-MET inhibitor, was rapidly tested in a clinical trial against *ALK* fusion gene-positive NSCLC and achieved success [13,38].

Crizotinib acts by binding to the ATP binding pocket of ALK, thereby competitively preventing ATP binding [13,39]. In contrast, crizotinib inhibits c-Met activity by binding outside of the ATP-binding site, and impairs kinase activation allosterically [40]. Although *ALK* status does not influence the response to platinum-based doublet chemotherapy, crizonitib showed an objective response rate of 56% in NSCLC cases harboring an *ALK* fusion gene [13]. Therefore, for this chemotherapy, *ALK* status should be determined before beginning treatment.

However, there are already reports of emerging crizotinib resistance in spite of its short period of clinical use [13,41]. Another ALK inhibitor, TAE684 (ARIAD Pharmaceuticals), has been considered as a second-generation inhibitor to treat crizotinib-resistance tumors [39].

As a c-MET inhibitor, crizonitib was designed to treat patients with tumors that harbor *MET* amplification (non-Barrett's GEJ cancer, gastric cancer,) or *MET* mutations (NSCLC in smokers, SCCHN, papillary renal cell carcinoma [RCC]). Some other c-MET-TKIs have been tested against cancers harboring mutated *MET*, and differential effects have been reported: the c-MET-specific TKI SU11274 (Sugen Incorporation) inhibits the activity of the wild-type and several MET mutants (H1094Y and M1250T), but does not inhibit other mutants (L1195V and Y1230H) [12].

Bevacizumab

Another important process underlying tumor growth is vascular proliferation. Consistently, increased expression of VEGF has been found in most human tumors [29], and accordingly VEGF/VEGFR inhibitors have been developed for the treatment of cancers that are dependent on angiogenesis [42]. Recently evaluated anti-VEGF therapeutics include the monoclonal antibody bevacizumab (Genentech Pharmaceuticals), and the multi-target TKIs, sunitinib and sorafenib [29].

Bevacizumab is a recombinant humanized monoclonal antibody that targets VEGF and significantly enhances the antitumor efficacy of some standard chemotherapy regimens in colorectal, lung, and breast cancers [29]. One peculiar adverse event reported for bevacizumab is extensive hemorrhage from the tumor, which is not surprising given its pharmacological property. As a result, application of bevacizumab in NSCLC is limited to non-squamous cell carcinomas that are not adjacent to large blood vessels and in patients who do not have a history of hemoptysis.

Sunitinib is an oral multitargeted TKI of VEGFR, PDGFR, c-Kit, RET, and Flt3 as well as BCR-ABL and SRC, and has been approved by the US-FDA for the treatment of advanced RCC and imatinib-resistant GIST [12,29]. Clinical trials of sunitinib as a second-line treatment for gastric and GEJ cancers are also underway. Sorafenib is a potent inhibitor of Raf and several RTKs, including VEGFR-2/3 and PDGFR-B [29]. In tumor xenograft models, sorafenib effectively inhibited tumor growth and angiogenesis [43]. A clinical trial was

performed using sorafenib combined with capecitabine and cisplatin as a first-line therapy in patients with advanced gastric and GEJ cancers [29].

Other Agents

In addition to Tyr kinases, Ser/Thr kinases have also been receiving much attention, as they account for 99% of all kinases [44]. There are two Ser/Thr-specific kinase inhibitors approved to date and both are immunosuppressant macrolide rapamycin derivatives (rapalogs): temsirolimus (Wyeth Pharmaceuticals) and everolimus (Novartis). These agents block the activity of mTOR, an atypical protein kinase that is the major downstream mediator of the PI3-K/Akt pathway [45]. Temsirolimus and everolimus were approved for the treatment of RCC and pancreatic neuroendocrine carcinomas and are currently being tested in clinical Phase II/III studies in various cancers as single agents or in combination with other drugs [45,46] (Table 1).

Another well-known molecularly targeted therapeutic agent is rituximab (Genentech). Rituximab is a chimeric monoclonal antibody directed against the CD20 antigen on B-lymphocytes. It was licensed for the treatment of non-Hodgkin B-cell lymphomas (NHL) as well as B-cell-induced autoimmune diseases [47,48]. By directly targeting CD20 on B-lymphocytes, rituximab exerts an anti-tumor activity through antibody-dependent cellular cytotoxicity and complement-mediated lysis [47,48]. Other protein kinase inhibitors that are in advanced stages of clinical trials include the cyclin-dependent kinase inhibitor flavopiridol (Alvocidib, HMR-1275, Sanofi Aventis Incorporation) for the treatment of chronic lymphocytic leukemia and a variety of solid tumors [49]. Inhibitors targeting PI3-K include NVP-BEZ235 (BEZ235, Novartis), GSK2126458 (Glaxo SmithKline Pharmaceuticals), PF-04691502 (Pfeizer), GDC-0941 (Genentech) and PI103, but none of these has yet to enter the clinic [50-52]. Several promising Akt-specific inhibitors have recently been introduced. AZD5363 (AstraZeneca), a novel pyrrolopyrimidine-derived compound, inhibited all Akt isoforms and inhibited phosphorylation of Akt substrates in cells [53]. Perifosine (Aeterna Zentaris Pharmaceuticals), an allosteric inhibitor, efficiently reduces the levels of Akt, and blocks Akt membrane localization in breast and ovarian cancer cells [54]. A selective ATP non-competitive inhibitor, Akti-1/2 (naphthyridinone, Merck Biosciences) blocks Akt activity [55]. Triciribine phosphate (VioQuest Pharmaceuticals), which inhibits Akt phosphorylation and recruitment to the plasma membrane, has been evaluated in clinical trials [56].

Resistance

Mechanisms of resistance

Despite encouraging clinical results, resistance to chemotherapeutic agents represents a consistent obstacle in the use of molecularly targeted therapies, since a substantial proportion of patients become refractory to these treatments. The efficacy of TKIs is limited either by primary (*de novo*) or acquired (secondary) resistance after therapy, and a number of preclinical and clinical studies are focused on elucidating the mechanisms of resistance.

Primary resistance is typically caused by the presence of a co-existing mutation, such as mutations in other RTK(s), *KRAS*, *PIK3CA* or *PTEN*, which can interfere with the efficacy of TKIs [27,57,58].

In contrast, in acquired resistance, "sensitive" tumors initially respond to TKIs, but invariably develop progressive disease. As the underlying mechanism of this acquired resistance, treatment with targeted agents inhibits the "addicted" pathway on one side, but may favor the development of new addiction pathways to escape from the selective pressure on another side. More detailed descriptions of known mechanisms of acquired resistance are as follows [1,5,12,15].

i) Altered trafficking of the receptor.

ii) Alterations in the binding site of the agents, due to masking of epitopes, secretion of truncated RTK (as seen with HER2 p95), and/or mutations in the kinase domain. Single amino acid mutations in the catalytic domains of some kinases can abrogate compound efficacy, as has been documented for BCR-ABL1, EGFR, c-KIT, and PDGFR.

iii) Compensation by overexpression of alternative ligands or receptors: upregulation of alternative kinase pathways such as c-MET or IGF-IR to EGFR-TKIs is frequently encountered.

iv) Functional dimerization/interaction with other receptors (i.e., HER2 with IGF-IR or c-MET).

v) Loss of downstream regulators (PTEN, etc) and/or autonomous activation of downstream effectors (PI3K-Akt, Ras-Raf-MAPK cascades, etc.). This type of compensatory RTK signaling can enable cancer cells to override the effects of agents that selectively target a single molecule.

In rare instances, inhibition of one effector can lead to activation of upstream pathways by interfering with feedback inhibition. For example, mTOR inhibition can lead to activation of an upstream RTK (IGF-IR) and/or Akt, which activates the cascade the inhibitor was intended to suppress, and can lead to resistance over the long term [57]. In such cases, additional targeting of these activated pathways may be required to overcome resistance [57].

Complicating the matter ever further is the intrinsic heterogeneity of cancers. In particular, cancers in the advanced stage often harbor multiple mutations, chromosomal aberrations and display genomic instability, which can generate a number of abnormal profiles such as those listed above that cause drug resistance [1]. Studies of clinical human tumor samples have indicated the existence of crosstalk between different signal transduction cascades and redundant signaling pathways that function downstream of receptors [12].

A current focus of drug discovery in oncology is the development of agents that have multiple effects as they may be able to suppress the many overlapping biological pathways activated in cancer cells. In this sense, therapeutic intervention by broad spectrum target inhibition may be ideal, and could be achieved either by combining several selective kinase inhibitors or by using a single multi-kinase inhibitor. However, therapies where there is unequal potency against different targets may be limited in their efficacy and result in unsatisfactory inhibition and consequent side effects [1].

Combating resistance

Since resistance occurs inevitably with molecularly targeted therapies, similar to conventional therapies, several strategies have been carried out to overcome this problem.

First, even when resistance to a particular agent is found, it may still be worthwhile to restart therapy after cessation, and this has been proposed in the case of EGFR-TKIs-resistant NSCLC. This strategy is based on the observation that in many cases with this kind of carcinoma, symptoms improve and tumor size decreases after restarting TKIs, suggesting that some tumor cells remain sensitive to EGFR-TKIs. This effect has also been noted in imatinib-resistant GIST and trastuzumab-

resistant *HER2*-amplified breast cancer, even after disease progression is documented radiographically, i.e., the patients are continued on these targeted agents [12,26,59,60]. These notions suggest that one strategy is not to abandon the use of TKIs in patients who have previously responded to the agent, but now show resistance, and the treatment should resume, after some interval, with the same agent and with the same dose that had been used before discontinuation.

Second, some investigators have reported that, even after patients with NSCLC have shown resistance to either gefitinib or erlotinib, they may still exhibit responses to the other drug [28,61], e.g. in a patient with erlotinib-refractory lung adenocarcinoma harboring a novel E884K mutation (exon 22), a striking response to gefitinib was observed [28]. This case exemplifies the idea that some *EGFR* mutations can modulate differential responses to gefitinib and erlotinib. However, another study reports that tumors exhibiting resistance to one EGFR-TKI have resistance to both [62].

At any rate, a unique subgroup of patients exists whose tumors contain previously uncharacterized genetic aberrations and who still benefit from individualized targeted therapy. Hence, continuous genetic, biochemical and functional assays are desired to design the better therapeutic strategy by better understanding of the alteration of cell signaling caused by these mutations [28].

Third, a large body of studies highlights the idea that cancer cells undergo a continuous adaptive switch to different signaling pathways as a survival strategy. Therefore, better success in treating these cancers may be achieved by using sequential and/or combination treatments with targeted agents, to suppress the various possible addicting signal pathways as they emerge. For this purpose, multitargeted kinase inhibitors are being explored, such as sunitinib and sorafenib, etc.

Similar to the third option, a fourth option is to switch the targeting agent by synchronizing the agent with each switch of addiction. When cancer cells exhibit resistance to one RTK, it is worthwhile to employ inhibitors of signaling molecules downstream of this RTK to block signals that escape RTK inhibition [26]. Those would include inhibitors of PI3-K, Akt and mTOR, etc. However, treatment with the PI3-K inhibitor XL-147 (Exelixis Pharmaceuticals) was reported to cause upregulation and phosphorylation of several other RTKs, including HER3, IGF-IR, and fibroblast growth factor receptors 2 and 3 (FGFR2/3) [63].

Fifth, for a number of targets, overcoming drug resistance has led to the development of "second-generation" kinase inhibitors that can form covalent bonds with the target, thereby increasing their effectiveness. For example, in attempts to overcome resistance to the EGFR-TKIs, the inhibitor BIBW 2992 (afatinib; Boehringer Ingelheim Pharmaceuticals), that binds covalently to the ATP-binding site of EGFR, was developed [1,64].

Lastly, other approaches to counter drug resistance include the use of agents that target molecules not directly involved in the signaling cascade of interest: HSP90 inhibitors are being studied for use in EGFR-TKI-resistant tumors, since quite a few RTKs as well as their downstream effectors are client proteins of HSP90 and presumably dependent on its chaperone function [26]. In addition, miRNAs, which can selectively inhibit gene expression, may offer an opportunity to reduce resistance by targeting signaling pathways that are often perturbed in cancer, either as a single miRNA or as a pool directed against multiple tumor-promoting targets [65]. Along these lines, the potential of miRNAs or antagomirs to sensitize resistant cells to common targeting agents is also being evaluated. Indeed, forced overexpression of miR-126 has been reported to sensitize NSCLC cells to EGFR-TKI [66].

Next, we introduce specific cases of drug resistance and the countermeasures that have been taken, with particular a focus on kinase inhibitors commonly used in cancer treatment.

Resistance against specific agents

EGFR inhibitors: Although patients harboring sensitizing somatic mutations of EGFR have an initial response of 55 to 82% to TKIs, they ultimately develop progressive disease [27,67]. Progression by acquired resistance is predominantly associated with the development of additional secondary mutations in *EGFR*, e.g., T790M in exon 20 has been found in approximately 50% of the cases exhibiting resistance [15,68] and a rare mutation conferring resistance, E884K in exon 22, has also been reported [69].

A second mechanism of acquired resistance is the activation of parallel pathways, in which the key downstream targets of EGFR are activated independently and function as compensatory pathways [27]. Amplification of the *MET* gene is a typical example of primary and acquired resistance and accounts for roughly 20% of the cases exhibitng resistance to EGFR-TKIs [70]. Promising data in early phase trials have been obtained with the c-MET inhibitors XL184, XL 880 (Exelixis) and ARQ197 (tivantinib, ArQule Pharmaceuticals), which restored sensitivity in NSCLC cells that had acquired resistance to gefitinib as a result of *MET* amplification [12,27].

Primary resistance to EGFR-TKIs is typically caused by somatic mutations of other genes, the best described of which is *KRAS* [27]. *KRAS* mutations are present in 15 to 30% of lung cancers and have been shown to confer resistance not only to EGFR-TKIs, but also to anti-EGFR antibodies [27,71]. Other genetic aberrations include *PTEN* deletions and silencing of the PTEN promoter through hypermethylation in 50% of NSCLC cases, Akt overexpression in 60% to 70% of the cases and *PI3-K* mutations in 2% to 3% of the NSCLC cases [27,72]. Consistently, inhibition of the PI3-K/Akt pathway restores gefitinib sensitivity in resistant cell lines [69].

Another mechanism of primary resistance that has been described is insertion mutations, such as D761-R774 in exon 20 of *EGFR*, which account for less than 5% of all known mutations [27]. Other mechanisms include increased levels of MAPK, IGF-IR, BCL-2 and/or VEGF/VEGFR proteins. EGFR-independent activation of PI3-K via signaling from other RTKs also confer primary resistance, albeit at a lower incidence [27].

Two other proteins in the HER family, HER2 and HER3, also play an important role in conferring resistance in lung cancer. EGFR is able to undergo either homo- or heterodimerization with receptors, resulting in autophosphorylation and activation of the intracellular signaling proteins. Thus, compensatory activation by EGFR/HER2 and EGFR/HER3 may also underlie acquired resistance against EGFR-TKIs [27,73].

Strategies aimed at overcoming resistance conferred by heterodimer formation, exon 20 insertion or T790M mutation include the use of irreversible inhibitors of EGFR. Agents currently being assessed in both preclinical and clinical studies are irreversible inhibitors of pan-ErbB, HKI-272 (neratinib, Wyeth) and dacomitinib (PF00299804, Pfizer), and a dual EGFR/HER2 inhibitor BIBW2992 [1,26,74]. These agents covalently bind to a cysteine residue in EGFR near amino acid position 797, and this feature allows them to overcome T790M-induced resistance [27].

In addition, these mutants of EGFR tend to render them less stable, and as a result they are more dependent on their interaction with HSP90 [75]. Thus, HSP90 inhibitor geldanamycin and its first-generation derivative, Tanespimycin (17-AAG, Bristol-Myers Squibb Pharmaceuticals) are being developed to promote the degradation of EGFR [27,76], and they exhibit greater *in vitro* activity against cell lines having an *EGFR*-T790M mutation compared to wild-type EGFR [26].

Trastuzumab and lapatinib: Resistance of HER2-overexpressing breast cancers to trastuzumab is quite high (66%–88%) when it is used as a monotherapy [77]. Proteins responsible for this resistance include Akt, PTEN, and non-HER family RTKs [58]. In particular, the switch of oncogenic addiction from HER2 to a different RTK is a critical issue in trastuzumab resistance.

In trastuzumab-resistant breast cancer cells (ex. UACC812/LR), *FGFR2* is highly amplified and is accompanied by reduced expression of HER2 [12]. In another study, trastuzumab was shown to upregulate c-MET expression, which, in turn, activates HER3, resulting in trastuzumab resistance [58]. Lapatinib was developed as a dual EGFR/HER2-TKI (Glaxo SmithKline) and has been approved for advanced or metastatic chemorefractory breast cancers overexpressing HER2. However, treatment with lapatinib still triggers activation of a compensatory cascade, similar to trastuzumab [78]. Next generation agents include a novel dual EGFR/HER2 and VEGFR inhibitor, EXEL-7647 (Exelixis), which inhibits almost all breast carcinoma cells that exhibit lapatinib resistance [79]. HKI-272 (neratinib) and BIBW 2992 (afatinib) are inhibitors of the EGFR/HER family of kinases, and were mentioned previously as agents effective against EGFR-TKI-resistant NSCLC. These agents are also potentially effective for HER2-positive and trastuzumab-resistant metastatic breast cancer [26,74].

Dysregulation of the mTOR pathway is also associated with the development of resistance to trastuzumab. Therefore, the addition of mTOR inhibitors to conventional agents has the potential to overcome primary (innate) or acquired resistance. For example, Everolimus (Novartis), an mTOR inhibitor with demonstrated preclinical activity against breast cancer cell lines, has been shown to reverse Akt-induced resistance to trastuzumab [80].

T-DM1 (Genentech, Roche) represents a novel approach to drug delivery. This agent consists of trastuzumab conjugated to an anti-microtubule agent (DM1, emtansine, maytansine) [81,82] via a stable [N-maleimidomethyl] cyclohexane-1-carboxylate (MCC) linker, which enables the efficient delivery of DM1 to HER2-overexpressing cancer cells [59]. In preclinical models, potent antitumor activity was observed with T-DM1, including against trastuzumab- and lapatinib-resistant cells [81,82].

Inhibition of the chaperone function of HSP90 can be achieved using inhibitors of its ATPase activity such as geldanamycin and tanespimycin. This inhibition results in the ubiquitination and subsequent degradation of a number of client proteins, including ErbB2 as well as p95ErbB2 [83], and accordingly these agents have demonstrated robust antitumor activity in preclinical models of HER2-positive breast cancer [82].

Pertuzumab (Genentech) is a humanized MAb that binds to a different site in the extracellular domain of HER2 from trastuzumab, and that blocks receptor dimerization [84]. In tumor xenograft models, the addition of pertuzumab to trastuzumab synergistically enhanced tumor inhibition, even after progression, compared with trastuzumab alone [84].

Crizonitib: A considerable number of *EML4–ALK*-positive NSCLC cases show resistance to crizotinib [13], which is largely due to a point mutation in *ALK*. The amino acid substitutions C1156Y, and L1196M, as well as S1206R and G1269S in ALK render NSCLC cells completely insensitive to crizotinib [37,41]. Another mutation, F1174L, was identified in *RANBP2–ALK*-positive inflammatory myofibroblastic tumors and confers poor sensitivity to crizonitib [13].

The mechanism of resistance conferred by most of these mutations can be rationalized based on a structural analysis of ALK. For example, the L1196M mutation sterically hinders crizotinib binding, while S1206R and G1269 eliminate the crizotinib-binding site [37]. An understanding of these so-called "gatekeeper" mutations has led to the development of many second-generation ALK inhibitors designed to overcome resistance, especially that conferred by L1196M, and many have entered early clinical development. Two SMW competitive inhibitors of the TK domain of ALK are AP26113 (ARIAD) and CH542802 (Chugai Pharmaceutical), which are reported to have more potent inhibitory activity against both wild-type and L1196M-ALK compared to crizotinib [37,85]. NVP-TAE684 (ARIAD) was also highly active against crizotinib-resistant cancer cells, and this drug has limited molecular interactions with the gatekeeper residues L1196 and G1269S, which may explain its ability to inhibit ALK having these two mutations [86].

EML4-ALK is also a client protein of HSP90, and this association can be disrupted by an HSP90 inhibitor [87]. HSP90 inhibition was shown to lead to the rapid degradation of EML4-ALK and induction of cell death in both crizotinib-sensitive and crizotinib-resistant (L1196M mutant, etc.) ALK-dependent cells [38,87]. Indeed, geldanamycin and tanespimycin have demonstrated preliminary clinical efficacy in patients with ALK-rearranged NSCLC [38].

Concluding Remarks

The application of molecularly targeted therapies has been based on the identification of the specific addicting target(s) on which the tumor depends, and this will surely be the focus into the future. However, in most cases, the specific addicting target causing the disease is not clear and selecting the most appropriate patient group, that is patient stratification, is a complex task except in a defined subset of cancers harboring aberrations of well-known genes. Therefore, the most critical current issue in the development of molecularly targeted therapies is clarifying the molecular targets of inhibitors to better understand both the on- and off-target drug pharmacology and resulting side effects.

Our understanding of the selectivity profile of protein/lipid kinase inhibitors and their adverse side effects are only now beginning to emerge [1]. Many of the approved single-targeted inhibitors simultaneously impair other targets, which can lead both to unexpected positive results and to increased toxicity and side effects [88]. In contrast to high molecular weight agents that extravasate from leaky capillary and tumor vessels, but accumulate selectively in tumor tissues, SMW agents extravasate easily from any vessel and circulate, and thus have a higher potential for unexpected side effects [89]. This potential for adverse side effects is accordingly greater with multi-targeted agents [89]. Therefore, while SMW multi-targeted agents may be preferable from a therapeutic viewpoint, they also carry increased risk of toxicity and side effects.

At present, the established kinase inhibitors are able to cover only 15% of the entire kinome, at most [1]. Moreover, many of the kinase inhibitors in clinical trials are not very specific and have not achieved

their anticipated results. This situation may be improved with the new generation of kinase inhibitors currently under development, as they have a better selectivity and may be applied to a genetically better defined subset of the patient population. Towards this goal, efforts using genome-wide screening are ongoing, and integrating this information with targeted agent-responsive phenotypes may pave the way for the discovery of new targets. The challenges are to improve these types of analyses, better assess the effects of the drugs on their targets, and lastly to use the molecular profiles obtained to guide the use of these drugs in the clinic. Since treatment options should be determined by the molecular and genetic tumor profiles of each patient, the paradigm of molecularly targeted therapy is practically to be established on a validated test to provide a uniform, sensitive, specific and easy protocol for detecting the critical molecular alteration. Recent advances in high-throughput technologies may provide such assays and allow more comprehensive identification of molecular markers and more informed application of various combinations of drugs in new clinical trials.

Declaration of Interest

All authors read and approved the final manuscript and report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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