Journal of Gynecology Research
Volume 4 | Issue 1
ISSN: 2454-3284

Review Article Open Access

Mechanisms of Resistance to Kinase Inhibitors and Strategies to Prevent the Development of Drug Resistance

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Citation: Kobayashi H, Iwai K, Niiro E, Morioka S, Yamada Y, et al. (2018) Mechanisms of Resistance to Kinase Inhibitors and Strategies to Prevent the Development of Drug Resistance. J Gynecol Res 4(1): 103

Received Date: February 12, 2018 **Accepted Date:** April 09, 2018 **Published Date:** April 11, 2018

Abstract

Targeting mutant proteins and associated signaling pathways of driver oncogenes by small molecule kinase inhibitors (KIs) are a promising strategy of cancer therapy. However, despite the initial success of treatment, KIs often become ineffective as intrinsic and acquired resistance. This article reviews the English-language literature to explore the underlying mechanisms of drug resistance and to present a challenge for developing drugs to overcome resistance. Mechanisms of acquired resistance include 1) the selection of pre-existing subclones with other mutations, 2) the emergence of secondary mutations in the target kinase domain, 3) upregulation of kinases both within the same kinase family and their related kinase families, as well as activation of alternative bypass pathways, 4) epithelial-mesenchymal transition, 5) overexpression of pro-survival Bcl-2 family proteins and 6) drug efflux mechanisms. Currently available methods are to obtain tumor biopsy samples at recurrence or progression if the tumor lesion is accessible to a biopsy and to use the second- and third-generation KIs based on the individual need of each patient. Furthermore, recent computational challenges provide design principles to prevent the development of drug resistance. In conclusion, we provide an overview of the postulated resistance mechanisms and highlight the future direction of computational structure-based design of new potent KIs.

Keywords: Acquired Resistance; Anticancer Therapy; Drug Design; Intrinsic Mutations; Kinase Inhibitors

Introduction

Cancer is one of the leading causes of death worldwide. It is a multifactorial disease involving specific genetic background, in combination with epigenetic marks and environmental exposures (chronic exposure to various environmental stresses, e.g., cigarette smoking, ultraviolet light, or air pollution) and lifestyle risk factors (improper diet). These risk factors lead to widespread accumulation of genetic or epigenetic changes in some important genes that might accelerate the process of carcinogenesis and disease progression [1]. Target genes are known as modulators of cell proliferation, cell cycle regulators, or anti-apoptotic signaling that governs the cell growth, angiogenesis, invasion and metastasis. During cancer development and progression, tumors promote cell growth by enhancing somatic gain-of-function mutations and amplifications of oncogenes and their receptors (e.g., EGFR [epidermal growth factor receptor], ERBB2 [HER2], FGFR [fibroblast-like growth factor receptor], PDGFR [platelet derived growth factor receptor], IGF1R [insulin like growth factor-1 receptor], MET [MET proto-oncogene, receptor tyrosine kinase], ALK [anaplastic lymphoma kinase receptor tyrosine kinase], VEGFR [vascular epidermal growth factor receptor], FLT3 [FMS-like tyrosine kinase 3], BRAF [B-Raf proto-oncogene, serine/threonine kinase], MYC [MYC proto-oncogene, bHLH transcription factor], SRC [SRC proto-oncogene, non-receptor tyrosine kinase], and BCR-ABL [BCR, RhoGEF and GTPase activating protein-ABL proto-oncogene 1, non-receptor tyrosine kinase]) and their modulators (FAK [Focal adhesion kinase], BCL2 (BCL2, apoptosis regulator), or PARP [poly (ADP-ribose) polymerase]) [2]. The discovery of actionable clonal molecular alterations in driver oncogenes can develop novel molecularly targeted therapeutics. Key regulators of driver oncogenic signaling pathways and specific modulators targeting apoptotic pathways, which can limit off-target tissue toxicity, have been investigated for anticancer therapy [3]. Numerous therapeutics including monoclonal antibodies and small molecule inhibitors directed toward targeted proteins or critical oncogenic drivers have been developed as potential anticancer candidates [4,5].

We review the clinical and preclinical data available for targeted therapy of small molecule kinase inhibitors (KIs). The second scope of this review involves the current knowledge base with respect to mechanisms of resistance to treatment. We finally focus on how to prevent the development of acquired resistance.

Materials and Methods

This study aimed to summarize the current status of small molecule KIs as anticancer therapy. Systematic review of the literature using electronic search in the PubMed databases (http://www.ncbi.nlm.nih.gov/pubmed). A search of the relevant literature published until June 2017 was performed. All English-language relevant articles were searched using the keywords 'intrinsic resistance', 'acquired resistance', 'anticancer therapy', 'small molecule', 'kinase inhibitors', or 'mechanism' in the titles or abstracts of articles. Relevant studies were also selected through reference tracking and a hand search.

The following studies met the inclusion criteria: i) clinical studies that evaluated cancer patients treated with KIs and compared them with patients who used placebo; ii) randomized clinical trials, retrospective or prospective cohort studies, and case-control studies; and iii) preclinical experiments that evaluated the functions of KIs *in vitro* and *in vivo*. If the data were from the review articles, only the highest-quality regular article written in the review was included. The following types of articles were excluded: letters, conference abstracts, and comments.

To minimize selection bias, screening of the studies was independently performed by two of the co-authors (KI and EN) after agreeing on the selection criteria. Any disagreement between the two authors was resolved by consulting a third author.

Results

A total of 4401 studies ('intrinsic resistance' and 'kinase inhibitors', n=429; 'acquired resistance' and 'kinase inhibitors', n=1630; and 'mechanism' and 'resistance' and 'kinase inhibitors', n=2342) were initially identified, and 3898 were excluded owing to the publication type and duplication. After reviewing titles and abstracts, an additional 279 articles were excluded. 41 additional articles from the references were added to this review. The full texts of 265 studies were then thoroughly reviewed.

Selective small molecule inhibitors as anticancer agents

Intensive efforts have been developed to inhibit kinase activity of oncogenic proteins in many of human cancers. Clinically available small molecule KIs, designed to target a variety of cancer patients with activating mutations, amplification or rearrangement of oncogenes, have become an important therapeutic paradigm [6]. KIs are commonly used via oral administration and allowed to absorb efficiently. Recent studies provide ample evidence of the success in targeting the oncogenic drivers with these inhibitors [2,5]. KIs have been proven to provide progression-free survival benefit in kinase-mutated cancer cells [7].

Receptor tyrosine kinases (RTKs), non-RTKs and additional TK-independent mechanisms via phosphorylation of their tyrosine, serine and threonine residues serve as important regulators of signaling pathways associated with cellular proliferation, invasion and metastatic spread [5]. It is estimated that there are greater than 500 different protein kinases in humans. Currently, at least 20 KIs are approved and remain the cornerstone for treatment in many cancers (Table 1). Almost all substrate can be activated via their ATP binding pockets [5]. Most KIs directly dock into the ATP-binding site of kinase molecules and inhibit the catalytic activity of the kinase domains. The selectivity of inhibitors depends on the size, shape and configuration of kinase's ATP-binding site. Based on structural and evolutional conservation in the ATP-binding pocket, the initial class of inhibitors exhibit noncovalent interactions with the ATP-binding pocket, but the second-generation inhibitors covalently bind in the ATP pocket due to their high binding affinity and selectivity.

Drug	Target tumors	Main target kinases
Afatinib	EGFR mutated NSCLC	EGFR, HER2, HER4
Alectinib	ALK+ non-small cell lung cancer	ALK
Axitinib	RCC	VEGFR
Bosutinib	CML	Bcr-Abl, SRC
Cabozantinib	MTC	HGFR, RET, VEGFR
Ceritinib	Crizotinib-resistant ALK lung cancer	ALK, IGF-1R
Crizotinib	NSCLC	ALK, HGFR
Dasatinib	CML, ALL	SRC, Bcr-abl, c-KIT, PDGFR
Erlotinib	NSCLC, pancreatic cancer	EGFR
Gefitinib	NSCLC	EGFR
Imatinib	CML, chronic eosinophilic leukemia, ALL, GIST	Bcr-abl, c-KIT, and PDGFR
Lapatinib	Breast cancer	EGFR, HER2
Lenvatinib	Thyroid cancer	VEGFR, FGFR
Nilotinib	CML	Bcr-abl c-KIT, and PDGFR

Drug	Target tumors	Main target kinases
Osimertinib	T790M+ EGFR-mutated lung cancer	Mutated EGFR
Pazopanib	Soft tissue sarcoma, RCC	VEGFR, PRGFR, c-KIT
Regorafenib	Colorectal cancer	VEGFR, FGFR, PDGFR, KIT
Ruxolitinib	Myelofibrosis	JAK
Sorafenib	RCC, hepatocellular carcinoma	BRAF, VEGFR, PDGFR
Sunitinib	GIST, RCC, pancreatic neuroendocrine tumor	VEGFR, PDGFR, c-KIT
Vandetanib	MTC	RET, EGFR, VEGFR
Vemurafenib	Metastatic melanoma	BRAF

Table 1: Small molecule inhibitors developed as anticancer therapeutics

We initially review recent advances in the treatment strategies to selectively block hyperactive oncogene function in cancer therapy. Chronic myeloid leukemia (CML) is caused by the Philadelphia (Ph) chromosome translocation, BCR-ABL on the chromosome 22q. For example, the availability of new therapies, such as the TKI imatinib mesylate, Gleevec, has shown promise in the paradigm shift that encompasses the identification of optimal and front line treatments for CML [8]. Imatinib mesylate was first introduced into clinical practice in 1998 [9]. Since the proof-of-concept clinical trial evaluated the efficacy of imatinib, this inhibitor was approved by the Food and Drug Administration (FDA) for treatment of CML in 2001. However, many patients developed acquired resistance to imatinib. The second-generation TKIs, dasatinib, nilotinib, and ponatinib, have been approved for overcoming the resistance [9]. Several groups have subsequently undergone successful treatments with the second-generation TKIs in various settings of leukemia. Furthermore, FLT3 (FMS-like tyrosine kinase 3), a member of RTK family, is typically associated with an aggressive clinical course of acute myeloid leukemia (AML) [10]. FLT3 gene is mutated in 30% of AML and represents one of the most attractive targets [10]. Among several FLT3 inhibitors, such as crenolanib, quizartinib and midostaurin, midostaurin is an effective and reliable treatment for AML [11]. The advent of KIs is changing the paradigm of therapy for a variety of leukemia, but their clinical efficacy is limited due to the generation of drug resistance.

In addition, the development of targeted small molecule inhibitors has revolutionized the therapeutic approach to solid tumors in the choice of treatment modalities. Drugs targeting EGFR have clinical advantages and appear generally well tolerated in clinical practice in the treatment of non-small cell lung cancer (NSCLC), breast cancer, pancreas cancer, or colorectal cancer [12]. The most promising new treatment option for lung cancer is the use of EGFR-TKIs. The first-generation EGFR-TKIs, gefitinib and erlotinib, are the hit-lead-candidates based on databases to target EGFR, leading to the inhibition of both mutant and, to a lesser extent, wild-type EGFR. They are orally effective kinases that are used in the treatment of EGFR-mutant lung cancer [13]. Gefitinib and erlotinib bind reversibly to the kinase domain of the receptor. Osimertinib is approved for later-line use in the treatment of NSCLC patients with T790M mutation of EGFR [14]. Crizotinib is a first-generation oral multi-TKI that targets ALK-positive NSCLC driven by the EML4 (echinoderm microtubule associated protein like 4)-ALK fusion protein [15] and was approved for the treatment of advanced-stage NSCLC patients with ALK rearrangement [16]. Although crizotinib may be an effective treatment, many patients developed resistance within a year [15]. A second-generation ALK inhibitor, alectinib, can be used in NSCLC patient with the L1196M mutation who progressed on crizotinib [17,18]. Lapatinib is an orally effective, dual TKI of HER2 and EGFR for the treatment of HER2 positive metastatic breast cancer patients who were heavily pretreated [5,13]. Lapatinib downregulates PI3K (phosphoinositide-3-kinase)/AKT and MAPK (mitogen activated kinase-like protein) pathways and is currently used for metastatic HER2 positive breast cancer who developed resistance to the monoclonal antibody trastuzumab, Herceptin [19]. Some clinical trials showed the efficacy of lapatinib in HER2-positive aggressive cancer, and a combined treatment with lapatinib and chemotherapy has radically changed the prognosis of disease [19]. Unfortunately, efficacy is limited by therapeutic resistance to lapatinib. A multi-TKI, sorafenib, inhibits a variety of pro-angiogenic oncogenes, including proto-oncogenes VEGFR, FGFR, PDGFR and KIT (KIT proto-oncogene receptor tyrosine kinase, C-Kit). Sorafenib is a direct inhibitor of MAPK/ERK pathway and is used to treat chemorefractory renal cell carcinoma (RCC), thyroid cancer and hepatocellular cancer [20]. Sunitinib is also used as first-line treatment for the patients with advanced RCC. Other inhibitors of VEGFR1/2/3 kinases, axitinib (an oral second-generation TKI and a potent VEGFR inhibitor, [21], cabozantinib (an inhibitor of kinases including MET and VEGFRs) [22], lenvatinib (TKI targeting VEGFR1-3, FGFR1-4, PDGFR-b, RET and c-KIT) [23], and pazopanib (TKI targeting VEGFR1-3, PDGFR-a and b, and c-KIT) [24], are progressively approved for the treatment of advanced RCC [25]. Axitinib is used for the treatment of patients with metastatic RCC who developed resistance to the prior first-line treatment [26]. Regorafenib, an inhibitor of multi-kinases including VEGFRs, PDGF, FGFR, or BRAF, was shown to prolong survival as a later treatment for patients with heavily pretreated metastatic colorectal cancer [27]. Olaparib is the first oral, small molecule, poly (ADP-ribose) polymerase (PARP) inhibitor being approved in 2014 by FDA for the treatment of BRCA1/BRCA2 mutations-positive ovarian cancer [28]. PARP inhibitors impair single-strand DNA break repair and often accumulate double-strand DNA breaks, which remain unrepaired in homologous recombination-deficient (HRD) BRCA1/BRCA2 mutant cells. PARP inhibitors induce synthetic lethality in germline and somatic BRCA1/BRCA2-deficient tumor cells, leading to targeted tumor cell death. Rucaparib and niraparib are also potent

PARP inhibitors and clearly effective in both deleterious BRCA mutations associated ovarian cancers and ovarian cancers with HRD or loss of heterozygosity (LOH) [29]. Five drugs (vemurafenib, dabrafenib, trametinib, cometinib, and talimogene laherparepvec) were approved by FDA for the treatment of advanced or metastatic melanoma, including combination therapies of both small molecule kinase inhibitors and immune checkpoint inhibitors [30]. BRAF-TKIs, such as vemurafenib and dabrafenib, have been proven to provide survival benefit in patients with unresectable or metastatic melanoma with the BRAF V600E mutation/variant. The most frequent genetic alteration of the BRAF gene is a valine to glutamic acid (V600E) substitution [31]. BRAF inhibitors also uncover new cancer treatment strategies in melanoma or hepatocellular carcinoma [12]. Selective inhibitors to anti-apoptotic BCL-2 family members, including BCL-2, BCL-XL (BCL2-like 1), BFL-1 (BCL2 related protein A1), BCL-W (BCL2 like 2), and MCL-1 (MCL1, BCL2 family apoptosis regulator), have prompted cancer cells to undergo cell death [2].

Despite the advances in KI therapies, only some inhibitors have been proven to be successful in randomized phase III trials. In this setting, axitinib (an oral second-generation TKI and a potent VEGFR inhibitor) [21], midostaurin (a first-generation FLT3 TKI) [32], dacomitinib (a second-generation, irreversible EGFR TKI) [33], ceritinib (a next-generation ALK inhibitor) [34], alectinib [35], olaparib [36], afatinib (an irreversible ErbB family blocker) [37], Gefitinib [38], Cabozantinib [22], Dabrafenib plus Trametinib (BRAF inhibitor plus MEK inhibitor) [39], and mTOR inhibitors [40] have demonstrated an improvement in progression-free survival in a randomized Phase III trial. Afatinib improved overall survival in patients with EGFR Del19 mutations, but other inhibitors failed to be effective in increasing overall survival [37]. One of the reasons is the development of resistance, but also because of lack of specificity, off-target effects, or targeting of passenger mutations. Further prospective randomized studies are needed to demonstrate their feasibility, safety and effectiveness.

Intrinsic resistance to KIs

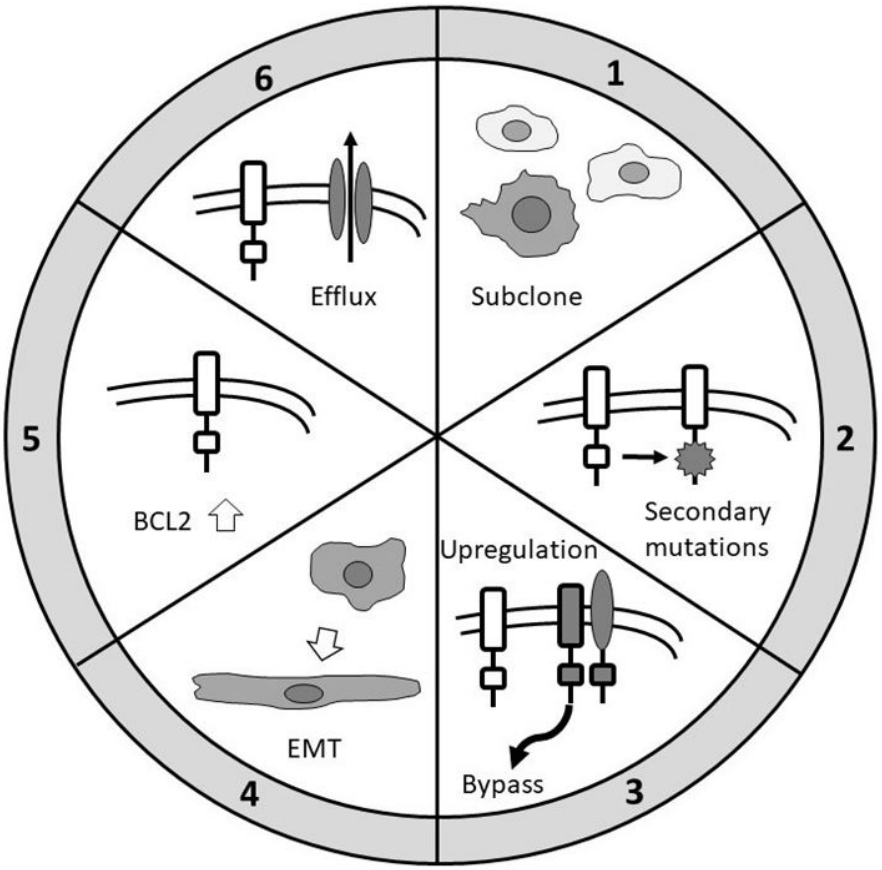
Resistance can generally be categorized as either primary (de novo) or acquired. We initially summarize some of the underlying mechanisms of intrinsic resistance that explain treatment failure. Evidence of extensive intra- and inter-lesional heterogeneity of de novo mutations and oncogene amplifications may be associated with clonal complexity as the mechanisms of intrinsic resistance [41]. Intrinsic resistance usually results in an immediate inefficacy of KIs.

One example is represented by the presence of non-sensitive EGFR mutations such as the exon 20 insertion mutations [42]. 25–30% of patients are intrinsically resistant to EGFR-TKIs [43]. The mutational status of BRAF might be a genetic cause of intrinsic resistance to TKIs therapy [31]. Patients with metastatic melanomas harboring the BRAF V600E mutation can benefit from the TKIs therapy [44]. There is evidence that some patients have other less common BRAF mutations in positions other than V600. These non-V600 position BRAF mutations show impaired kinase activity, exhibiting signs of intrinsic drug resistance [31].

Another example involved in intrinsic resistance is represented by the ability of tumors to activate de novo signalings, such as IGF1 [45] and NF-κB (nuclear factor-kappaB) [46]. IGF1 rendered a variety of cancer cells resistant to chemotherapy [47]. The role of IGF1 and its receptor in promoting resistance to chemotherapy is well established [48]. IGF1 intrinsically induces chemoresistance through the activation of the ERα (estrogen receptor-alpha) in breast cancer. Furthermore, the transcription factor NF-κB plays a critical role in cell-survival and anti-apoptotic signalings, and promotes chemoresistance in tumors [49]. For example, an intrinsic resistance of Linsitinib, an orally bioavailable IGF1R inhibitor, is predominantly mediated through activating the NF-κB pathway [46]. We also provide evidence that patients harboring deletion of BCL2L11 (BCL2 like 11), a proapoptotic Bcl-2 family member, are intrinsically resistant to EGFR-TKIs, because EGFR-TKIs induces apoptosis in EGFR-mutant NSCLC through upregulation of BCL2L11. This study provides the limited insight into the molecular mechanisms controlling intrinsic resistance.

Acquired resistant to KIs

The second aim of the present review is to give an overview about potential mechanisms by which cancer cells acquire resistance to KIs, and discuss technical options for preventing the emergence of resistance. Drug resistance exists in all types of the tyrosine, serine, or threonine KIs [12]. Acquired drug resistance has emerged as a common theme and become a major clinical challenge for the therapy of many cancers [3,7,8]. Targeted therapies significantly improve outcomes, but duration of remission is short-lived [19], suggesting that resistance occurs quickly or frequently. Recent research articles have elucidated the underlying molecular mechanisms of acquired resistance and have shed light on strategies for overcoming or avoiding resistance to targeted therapies [50]. Identification of the mechanism driving resistance via repeat tissue biopsy if clinically feasible and selection of a new agent in an individual cancer patient can potentially overcome acquired resistance to treatment [7]. The development of rationally designed, second- or third-generation KIs would show a better response to treatment in patients with acquired resistance to primary inhibitors. The new treatment strategies restore sensitivity in some of the KIs-resistant cells, but, virtually all patients will develop acquired resistance to these treatments again. The result is a vicious cycle of a personalized therapy. There are at least six potential mechanisms of acquired resistance, including 1) the result of a selection of pre-existing subclones with other mutations, 2) the occurrence of secondary mutations in the target kinase domains, 3) upregulation of TKs both within the same TK family and other TK families, as well as activation of alternative bypass pathways, 4) Phenotypic and morphological changes to acquire mesenchymal cell type characteristics (epithelial mesenchymal transition), 5) overexpression of pro-survival Bcl-2 family proteins and 6) drug efflux mechanisms that reduce overall intracellular drug concentrations (Figure 1).



This figure summarizes hypothesized mechanisms of acquired resistance to small molecule kinase inhibitors. Different mechanisms have been identified, including 1) selection of pre-existing subclones, 2) secondary mutations, 3) upregulation of the kinase family and activation of alternative bypass pathways, 4) EMT, 5) overexpression of pro-survival Bcl-2 and 6) drug efflux.
Figure 1: Major mechanisms for resistant to therapy

Selection of subclones with other mutations

The first example of acquired resistance is represented by a selection of pre-existing clones with other mutations. Many types of cancers are composed of multiple subclones harboring genetic and phenotypic heterogeneity. Subclones with drug-resistant mutations likely coexist within the same tumor. Relapse in some cases is generated from subclones that pre-exist at low levels in the untreated primary tumor at diagnosis may progress under different therapeutic pressures such as chemotherapy. Next-generation sequencing technologies have facilitated intensive exploration of intrinsic gene mutations [51]. For example, the use of deep sequencers has demonstrated that resistant clones are very often already present at diagnosis of AML [51]. Furthermore, selection of subclones with mutations that prevent the binding of KIs to the ATP binding pocket of targeted kinases is a previously described phenomenon in CML patients with acquired resistance to imatinib [52]. BRAF gene amplification can be acquired in subclones as the evolutionary selection in BRAFV600E-mutant cancer [53]. Selection of clonal diversity by therapeutic pressure leads to acquired resistance in cancer.

Secondary mutations in the target kinase domains

Second, somatic genomic alterations such as secondary mutations, point mutations and copy number alterations may be associated with evolutionary stages during the acquisition of KI resistance. The frequent mode of acquired resistance in the kinase domains induces a conformational change and subsequently alter binding ability of the inhibitor [7]. Secondary mutations are common in the KI-resistant patients. For example, secondary mutations in the EGFR kinase domain leads to acquired resistance of lung cancer to gefitinib or erlotinib. Drug resistance induced by EGFR-TKIs often confers the emergence of acquired T790M somatic mutation that is the most common mechanism of acquired resistance to first-generation EGFR-TKIs. EGFR T790M mutations enables conversion of an inactive form of the RTK to a constitutively active form and confers resistance through steric interference with EGFR-TKIs binding, thereby reducing the affinity of binding of the TKIs to the target kinase. After identifying the resistance mechanisms, new drugs that can target secondary mutations have been developed to improve the poor prognosis [12]. The acquisition of secondary mutations demonstrates the diversity and plasticity of cancer cells as well as the evolution of tumor heterogeneity that inhibits KI binding while allows ATP binding and restores kinase activity [54].

The T315I mutation in residues within the inactive conformation of BCR-ABL1 kinase domain would affect the conformation

and structural topology of the ATP binding pocket, resulting in acquired resistance to imatinib. Imatinib binds the inactive conformation of the BCR-ABL kinase, but second-generation TKIs, such as dasatinib and nilotinib, can bind both the inactive and active forms. Therefore, second-generation TKIs have become available in the CML patients who developed acquired resistance to imatinib as the front-line therapy [55]. Cancer therapy with a third-generation TKI, ponatinib, is an exciting advancement in the treatment of CML with T315I mutation [56].

BRAF gene mutations observed in melanoma following BRAF inhibitor exposure has been identified as secondary genetic changes, which has been linked to the development of resistance. The BRAFV600E mutation is a prediction marker of the response to TKI therapy in melanoma patients. Vemurafenib is indicated for treatment of patients with the BRAFV600E mutation for the treatment of metastatic melanomas, but not those with wild-type BRAF melanoma. BRAF inhibitors also paradoxically activate the MAPK signalling pathway (see **Activation of alternative bypass pathways** section).

Although the crizotinib therapy is successful during an initial treatment in advanced-stage NSCLC patients with ALK rearrangement, most cases ultimately developed acquired resistance to this inhibitor. L1196M and G1269A mutations were considered to be resistant to crizotinib treatment [16]. The conformational change of ALK induced by L1196M suggests that binding free energies determined by direct interaction between the inhibitor and L1196M ALKs are lower than those of inhibitor-wild ALKs [57]. Secondary mutations located in the ATP-binding pocket and amplification of the ALK fusion gene can be an underlying mechanism for acquired resistance [58]. The emergence of acquired secondary kinase domain mutations upon ALK inhibitor exposure is the dominant mechanism of resistance [59].

Upregulation of kinase molecules both within the same kinase family and other kinase families, as well as activation of alternative bypass pathways

Third, alternate mechanism of acquired resistance might be involved in a network activation of the same downstream pathways regulated by other kinases that cannot be inhibited by the target inhibitors. Activation of novel bypass pathways that has been established as a potential alternative mechanism for acquired resistance to inhibitors [7]. One example is presented by gene amplification of ALK and EGFR that leads to resistance to ALK inhibitors for the treatment of advanced or metastatic cancers [58]. To overcome the resistance, combined treatment with ALK inhibitors and afatinib, a KI targeting both wild-type and mutated EGFR, causes synergistic growth inhibition in ALK inhibitor-resistant cancer models.

Another example is represented by ERBB2 (HER2) gene amplification [19]. Since cancer cells harboring mutations and amplifications of EGFR and HER2 are aggressive subtype with a poor prognosis, these oncogenes have been the therapeutic choices. Acquired resistance to EGFR inhibition was associated with overexpression of HER2 family member, which is sufficient to bypass dependency on EGFR [60]. Increased HER2 expression was also followed by constitutive activation of downstream MAPK pathways. IGF1R activation acted as a bypass signaling pathway in EGFR-TKI-resistant NSCLC [61]. Therefore, acquired resistance to EGFR inhibitors results in upregulation of kinase molecules both within the ERBB receptor family (e.g., ERBB2/3) and other kinase families (e.g., IGF1R, MET, FGFR2, PDGFR, FAK, SRC family kinases).

Also, acquired resistance limits durable responses to lapatinib for breast cancer patients with HER2 amplification [19]. The underlying mechanisms involved in this acquired resistance was not a secondary mutation within the targeted kinase domains in HER2-positive breast cancer. Instead, resistance to lapatinib can be activated by the RAS/RAF/MEK/ERK and the PTEN/PI3K/AKT pathways that act as a pro-survival molecules [19]. Furthermore, resistance can occur by reactivation of the MAPK pathway as pro-survival strategy following EGFR or BRAF blockade, demonstrating that changes in gene amplifications of other kinase families and secondary activation of the MAPK pathway molecules have been linked to resistance [62,63]. A reactivation of ERK/AKT may occur by a cancellation of a feedback inhibition of the kinases following the inhibitors treatments [64]. BRAF inhibitor resistant melanomas were also associated with reactivation of AKT and subsequent PI3K activation [65]. Other resistance mechanism to BRAF inhibitors may be the loss-of-function PTEN mutations [66]. PTEN is a negative regulator of PI3K pathway. Loss of PTEN function promotes an activation of the PI3K/AKT pathway, which in turn leads to cellular survival through upregulation of Bcl-2 that blocks the apoptotic death [66].

Resistant cells acquired features of EGFR-, KRAS- and BRAF-mutations after treatment with their KIs [67]. Constitutively activated kinases stimulate downstream signaling pathways such as MAPK and PI3K/AKT, leading to a continued increase in cell proliferation and the generation of an anti-apoptotic state. Cancer cells induce adaptive network responses that bypass the inhibiting effects of initial KI treatments. Therapeutic failures are likely due to acquisition of the successful survival strategies through multiple signaling pathways as mutational or non-mutational mechanisms.

Phenotypic and morphological changes to acquire mesenchymal cell type characteristics (epithelial mesenchymal transition)

Fourth, several case reports demonstrated that cancer patients who was resistant to EGFR-TKIs had undergone phenotypic and morphological transformation or epithelial-mesenchymal transition (EMT) [68]. Long-term treatments of HER2-positive breast cancer cells with KIs induces an upregulation of the FGFR1-ERK1/2-Twist signaling functions [69] and nuclear accumulation of

β-Catenin [70]. Cancer cells resistant to the KIs also produce TGF-β1 (transforming growth factor beta 1) and promotes EMT [77]. These results demonstrated that EMT is one of the mechanisms involved in the development of tolerance towards the KIs [69,71].

Overexpression of pro-survival BCL2 family proteins

The fifth is acquired resistance due to overexpression of pro-survival Bcl-2. Approximately half of the patients with acquired resistance to EGFR-TKIs were induced by T790M mutations, but it is unlikely that secondary mutations alone is accounted for resistance to gefitinib [72]. Cancer cells treated with KIs increased protein levels of anti-apoptotic proteins of the BCL2 family, demonstrating that the acquired resistance may occur based on the upregulation of Bcl-2 and Bcl-xL proteins [73]. An inhibitor of the anti-apoptotic factors BCLcl-2 and Bcl-xL restored sensitivity in KI-resistant cancer cells. Furthermore, the Src inhibitor, dasatinib, also induces apoptosis in T790M-mutant cells through suppression of the expression of Bcl-xL [72]. Thus, secondary resistance to KIs is encountered often in cases with overexpression of pro-survival Bcl-2 family proteins [74]. Little is known about how pro-survival Bcl-2 family proteins are overexpressed during drug therapy.

Drug efflux and other mechanisms that reduce overall intracellular drug concentrations

Finally, the multidrug resistance (MDR) often limits therapeutic efficacy and clinical success by pumping the drugs outside from cancer cells and decreasing intracellular concentrations of drugs. The overexpression of ATP-binding cassette (ABC) transporters is one of the recognized MDR mechanisms [75]. ABC transporters interact with several KIs and alters the pharmacokinetic profiles [8,76]. For example, a multikinase inhibitor, regorafenib, and a BRAF inhibitor, vemurafenib, are substrates for ABC transporters [77]. However, drug efflux may have very little effect on KI efficacy.

Molecular modeling approaches for drug design

Identifying new drug target for anticancer therapy remains a major clinical challenge [78]. It is efficient to identify the differentially expressed genes or protein-set essential for cancer cell proliferation but absent in normal cells. A PCR-based cDNA subtraction of the normal cell genome from essential genes of cancer cells can offer new opportunities to assess cancer cell targets. Targeting these candidate proteins is a promising strategy for cancer treatment. It is urgently needed to develop drugs to selectively inhibit only the target proteins.

Based on the molecular structure of specific targets of interest, the integration of available protein crystal structures, omics data, and bioinformatics infrastructure can identify attractive targets, which will aid drug design for cancer treatment and explain the high levels of potency and selectivity [78-81]. After the targets optimization, several novel derivatives have been designed and synthesized based on a combination approach of the proteomics data analysis and homology modeling including X-ray crystallographic studies. Computer-assisted drug discovery and structure-based drug design, including the in silico virtual screening of different chemical databases, provide opportunity to select the best possible inhibitors [79,82]. Several large libraries contain databases of popular compound libraries for virtual screening and the high-resolution crystal structure of a known cryptic pocket. Cryptic pockets often found at protein-protein interaction (PPI) interfaces are considered to be candidate binding sites on targets that only become apparent when drugs specifically bind. These pockets can be used to design small molecule inhibitors [83]. A combination of docking and molecular dynamics simulations as structure-based 3D molecular screening technologies can describe methods for correctly identifying small molecule inhibitors that bind in the cryptic pockets [84]. Free-energy calculations and pocket optimization simulations can provide small molecule inhibitors with low-energy pocket-containing conformations [80,83,85]. Hydrophobicity is the leading force in PPI. If cryptic sites are less hydrophobic and more flexible, the pocket allows the binding of a wide variety of nonspecific ligands. Molecular docking and binding free energy simulations were performed to validate the credibility of the computational approach results [86]. Electrostatic van der Waals interactions are the main driving forces stabilizing the complexes and identify key residues within hot spots in its active form of kinase domains [87,88]. The model framework is computationally applied to find binding hot spots within the cryptic pockets. This proof-of-concept study can design potent and selective inhibitors that specifically bind kinase domains in the low micromolar range and compete for ligand binding [89]. More recently, the aspect of binding via hydrogen-bonds or van der Waals-forces may be classic drug development. Both 2nd and 3rd generation EGFR inhibitors, afatinib and osimertinib, bind covalently via disulfide-bonds using cysteine [14,36]. This was considered too toxic over decades but now becomes more and more common practice.

Many small molecules ATP-competitive inhibitors occupy the allosteric pocket of the kinase binding sites and block kinase activities by rigidly binding to the ATP-binding pocket of specific targets of interest. Molecular docking simulations reveal that small molecule inhibitors or peptides compactly insert into the ATP binding pocket of kinase domains [8]. For example, docking analysis used the crystal structures of dasatinib complexes with its target molecules, such as ABL, SRC and CSK (C-terminal Src kinase) [86]. Although hot spots in protein-protein interface between KI and the ATP pocket are predicted by calculating binding energy, the interface between ligand and receptor tends to fluctuate upon binding due to conformational flexibility [90]. The strict binding to the ATP-binding pocket may result in the emergence of acquired somatic mutations in kinase domains [91]. To prevent acquired resistance, it is worthy to design the small molecule inhibitors that do not deeply insert into the ATP binding pocket, with making the rigid and compact binding [90].

A drug design principle to prevent resistance

Here we report a successful example to avoid the emergence of secondary mutations, which leads to prevent the development of acquired resistance. One example to prevent acquired resistance is presented by the design of covalent KIs. The initial class of KIs exhibit noncovalent interactions with the ATP-binding pocket, but covalent targeting-KIs as second-generation inhibitors have been developed because of their high binding affinity and selectivity [92]. This provides a strategy for overcoming the acquired resistance of non-covalent KIs. Three irreversible KIs, such as afatinib, osimertinib and ibrutinib, were approved by the FDA [93]. Afatinib is used to treat metastatic NSCLC patients with EGFR exon 19 deletions or exon 21 (L858R) substitution mutations. Osimertinib is used for metastatic NSCLC patients with EGFR T790M mutation. Ibrutinib, the BTK (Bruton tyrosine kinase) inhibitor, is used for treatment of B-cell malignancies such as mantle cell lymphoma, chronic lymphocytic leukemia, and Waldenström macroglobulinemia Despite the development of covalent modifications, EGFR-mutated NSCLC patients treated with afatinib acquired T790M mutation at the time of progression, playing a vicious spiral of new technology and acquired resistance [94].

Another example is presented by a modification of small molecule inhibitors that do not deeply insert into the ligand binding pocket. Unfortunately, there has been no example of TKIs. We will provide structure-guided design of selective inhibitors for urokinase-type plasminogen activator receptor (uPAR). uPAR is a glycosyl-phosphatidylinositol (GPI)-anchored cell surface receptor that promotes extracellular matrix degradation and integrin signaling [95]. uPAR is increased in many human cancers and promotes an array of cellular processes that include adhesion, cell spreading, invasion and metastasis. Blocking the interaction of uPAR and its ligand uPA is a promising mechanism for anticancer therapy [96]. uPAR is also able to function through a bidirectional crosstalk with RTKs such as EGFR. Several uPAR binding peptides have been designed and formed [96]. The ab initio molecular simulations have clarified that some amino acid residues of amino-terminal fragment (ATF) of uPA play important roles in the specific binding between uPA and uPAR [97-100]. A synthetic peptide (residues 17-34, uPA_{17-34}) within the ATF of uPA competitively inhibits uPA binding to uPAR and is considered to be a strong uPAR inhibitor [101]. The uPA_{17-34} can deeply insert into the uPA binding pocket of uPAR like a drill sleeve and effectively inhibit cancer invasion *in vitro*.

We have been manufacturing small-molecule uPAR inhibitors by way of computer-assisted drug discovery and structure-based drug design [96-100]. We found that selective uPAR inhibitors recognize amino acid residues Glu36, Glu134 and Glu135 of uPAR as key anchors to cover the pocket [99]. These amino acid residues are located on the surface of uPA binding pocket and have a profound impact on the binding of uPA to uPAR. The target inhibitors have been generated from ab initio molecular simulations that define a Grand Canonical Monte Carlo (GCMC) Hamiltonian [102]. A series of inhibitors that can bridge the key anchors of uPA-binding pockets were designed and synthesized [96]. Among the synthetic peptides, H1 peptide (Gly-Lys-(Gly)n-Lys-Gly) is a promising candidate for the suppression of cancer invasion *in vitro* [96]. Small molecule H1 peptide can recognize three amino acid residues of uPAR as key anchors to cover the uPA binding pocket of uPAR, but does not insert into the pocket.

We describe the long-term effects of the small molecules, uPA_{17-34} and H1, on human ovarian cancer cell line SKOV3 [96]. These two peptides can effectively inhibit SKOV3 ovarian cancer cell invasion *in vitro*. To generate a peptide-resistant subclone, SKOV3 cells were cultured over 3 months with stepwise increases in uPA_{17-34} or H1 concentration. The peptide concentrations used ranged from 1 nM to 10 μM. Cells persistently treated with 1.0 μM of each peptide were obtained 3 months after the initial drug exposure, named as SKOV3/uPA_{17-34} or SKOV3/H1. These two subclones did not affect cell proliferation, compared to the untreated parental SKOV3 cells. SKOV3/uPA_{17-34} confers resistance to uPA_{17-34}, but not H1. Acquired resistance to uPA_{17-34} peptide remains a common obstacle, demonstrating the plastic nature of cancers. The most common mutations in the SKOV3/uPA_{17-34} are a L139R point mutation in the uPA binding pocket of uPAR (unpublished data). In contrast, we found improved success in inhibiting SKOV3/H1 invasion using uPA_{17-34} or H1, suggesting no acquired resistance. Therefore, H1 peptide may avoid the development of acquired resistance to targeted therapies compared to uPA_{17-34} peptide.

The diversity and unpredictability of resistance mechanisms presents a challenge for developing robust drugs to overcome resistance. It would be critical to develop new therapeutic inhibitors that bind specific residues as key anchors to cover the ATP binding pocket of kinase domains, but does not penetrate into the pocket. This finding may give new insight into mechanisms of drug resistance, including the emergence of secondary mutations in the target kinase domains.

Discussion

This review focuses on the current status of the development of KIs, and diverse molecular mechanisms that confer intrinsic and acquired resistance to these inhibitors, and finally options to prevent acquired resistance. Currently, at least 20 KIs were approved for treatment of a variety of advanced or metastatic cancers (Table 1). We summarize some of the underlying mechanisms of intrinsic and acquired resistance. Possible mechanisms explaining the intrinsic resistance include clonal complexity, existence of subclones resistant to the drug, or emergence of alternative compensatory pathway [31,41,44-46]. Intrinsic resistance to TKIs is one of the major obstacles, possibly due to the heterogeneity of primary (de novo) mutations associated with clonal complexity. The main mechanisms of acquired resistance identified to date may include secondary mutations and an oncogene kinase switch from a pre-existing oncogenic RTK dependency to another RTK [6]. Cancer cells also acquired the amplification and activation of the bypass pathway. For example, the EGFR T790M mutation can be detected in 50% of NSCLC cases, and MET amplification

accounts for 20% of patients with EGFR-mutant TKI-resistant NSCLC [103]. Other important reasons are lack of specificity, off-target effects, or targeting of passenger mutations [6]. However, there is no exact information which mechanism is more frequent. Acquired resistance may severely compromise future treatment options and is the most common cause of death [6]. Therefore, most of KIs failed to improve overall survival [37]. Currently available methods are to use the second- and third-generation KIs to tailor treatment regimens based on the individual need of each patient. One of intriguing future approaches may be the use of a minimally invasive liquid biopsy such as circulating exosomal proteins and cell-free DNA to improve patient selection for optimal treatment.

Finally, we provide the design, synthesis, and preclinical characterization of a second-generation uPAR inhibitor. We present an example, including lessons learned from uPAR inhibition, of recent computational studies on molecular mechanisms and design principles to avoid drug resistance [95-102]. Next-generation KIs can be developed in imitation of this technique. From a logical point-of-view, KIs that do not deeply insert into the ATP binding pocket may prevent the emergence of resistance mutations. We outline future directions and perspectives to overcome this issue.

Conclusion

In conclusion, we discuss the recent advances in computational structure-based design of new potent KIs, postulated resistance mechanisms and drug design to prevent emergence of drug resistance.

Conflict of Interest Statement

The authors declare that this review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgement

The present study was supported by grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan to the Department of Obstetrics and Gynecology, Nara Medical University (to H.K.).

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