Targeting cancer with small molecule kinase inhibitors

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Abstract | Deregulation of kinase activity has emerged as a major mechanism by which cancer cells evade normal physiological constraints on growth and survival. To date, 11 kinase inhibitors have received US Food and Drug Administration approval as cancer treatments, and there are considerable efforts to develop selective small molecule inhibitors for a host of other kinases that are implicated in cancer and other diseases. Herein we discuss the current challenges in the field, such as designing selective inhibitors and developing strategies to overcome resistance mutations. This Review provides a broad overview of some of the approaches currently used to discover and characterize new kinase inhibitors.

Kinases have become one of the most intensively pursued classes of drug target with approximately 30 distinct kinase targets being developed to the level of a Phase I clinical trial. The vast majority of these targets are being investigated for the treatment of cancer. However, deregulation of kinase function has been implicated in other disorders, including immunological, neurological, metabolic and infectious disease. This has generated considerable interest in the development of small molecule kinase inhibitors for the treatment of these disorders[1]. The current popularity of kinases as drug targets is driven by the convergence of several factors. First, with approximately 518 kinases encoded in the human genome, virtually every signal transduction process is wired through a phosphotransfer cascade, suggesting that inhibition of kinase activity can elicit a real physiological response. Accordingly, inhibition of the tyrosine kinase activity of the oncogenic BCR (breakpoint cluster region)–ABL1 fusion protein, formation of which is a causative transforming event in chronic myeloid leukaemia (CML), is a validated means for therapeutic intervention[2,3]. Second, despite a high degree of conservation in the ATP binding site, highly selective small molecules with favourable pharmaceutical properties can be developed[4]. Third, surprisingly, inhibition of kinase activity in normal cells can often be tolerated, presenting a therapeutic window for the selective killing of tumour cells. For example, dasatinib, a drug recently approved for the treatment of imatinib-resistant CML, potently inhibits all nine members of the Src family of kinases (and numerous other tyrosine kinases), yet exhibits a more favourable side effect profile than conventional cytotoxic chemotherapy[5,6]. Fourth, drugs such as imatinib have had high-profile success, exhibiting up to 80% response rates in chronic-phase CML patients[7]. However, despite these motivating factors, the field faces significant challenges from drug resistance, lack of inhibitor selectivity, lack of inhibitor efficacy and difficulty in drug target validation for particular disease settings. In this Review, we examine the approaches currently in use to design and validate the next generation of kinase inhibitors.

Kinases in cancer

Oncology drug discovery has benefited significantly from progress in understanding how to target kinases with small molecules relative to other disease indications. One reason for this is that many kinases have been found to be intimately involved in the processes leading to tumour cell proliferation and survival. First, there are kinase targets that have become impervious to normal regulatory mechanisms following genetic mutation or translocation (Supplementary information S1 (table) lists kinases that have been intensively investigated). These kinases have transforming capacity and are therefore considered to be oncogenic. The constitutive activity of this class of kinase target makes them essential for survival and/or proliferation of the cancer cell. This so-called oncogene addiction[8,9] renders the cancer cell exceptionally susceptible to the appropriate kinase inhibitor. The success of mutationally marked kinases as drug targets has motivated an intensive effort to survey the kinome across a broad range of tumour types for mutations. These studies have uncovered a large number of kinases bearing mutations, which are currently in the process of being functionally characterized[10,11]. Perhaps the most notable success from these efforts was the discovery that *PI3KCA*

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At a glance

- Small-molecule kinase inhibitors are being intensively pursued as new anticancer therapeutics. To date, approximately 80 inhibitors have been advanced to some stage of clinical evaluation.
- Understanding the structural basis of kinase inhibitor selectivity is crucial to the ultimate goal of developing selective inhibitors to target every member of the kinome. Most currently known kinase inhibitors target the ATP binding site with the kinase activation loop in the active (type 1) or inactive (type 2) conformation.
- New kinase inhibitors are primarily developed with a combination of methods, including high-throughput screening using biochemical or cellular assays, analogue synthesis, structure-guided design and fragment-based assembly strategies.
- The repertoire of kinases targeted by a given inhibitor can be determined by profiling its activity in binding and enzymatic assays against extensive panels of recombinant kinases, by profiling activity in cellular assays and by affinity approaches integrated with detection by mass spectrometry.
- Kinase inhibitor resistance resulting from selection for mutant alleles or upregulation of alternative signalling pathways is a recurrent theme in the clinic. Strategies for developing multiple inhibitors targeting different kinase sites and for discovering synergistic inhibitor combinations are urgently needed.

is frequently mutated in numerous cancers and that these mutations impart transforming potential *in vitro* as well as *in vivo*[12–14]. Similarly, the V600E mutation, located in the activation loop of BRAF, has been implicated in development of carcinomas of the skin, ovary, thyroid, colon and pancreas[15,16]. The V617F activating mutation in the auto-inhibitory pseudokinase domain of JAK2 is frequently found in polycythaemia vera, essential thrombocythaemia, and idiopathic myelofibrosis, and has stimulated the rapid progression of several JAK2 inhibitors into Phase I studies[17]. Recently, both somatic and germline mutations that activate anaplastic lymphoma kinase (ALK) have been discovered in neuroblastoma, a devastating childhood tumour for which new targeted therapies are urgently needed[18].

For a second class of kinase target, inhibition of the kinase results in a synthetic-lethal phenotype when paired with another non-lethal mutation in the particular pathology of the tumour cell[19]. Although not oncogenic and rarely mutated in cancer, these kinases are preferentially required for the survival and/or proliferation of cancer cells and may be located in key signalling pathways downstream of transforming oncogenes. Examples include MEK1 and MEK2 (also known as MAP2K1 and MAP2K2), which are located in the critical MAPK pathway[20], mTOR (also known as FRAP1), which is located in the PI3K–Akt signalling system[21], and the ribosomal S6 kinase (RSK), which is downstream of the fibroblast growth factor (FGF) in the context of FGF receptor (FGFR)-dependent proliferation[22]. Other examples of this class of targets include kinases that are required to sustain rapid proliferation and/or survival in the presence of abnormal ploidy, such as the cyclin-dependent kinases (CDKs), which regulate cell cycle transitions; the Aurora kinases, which are essential for accurately dividing chromosomes to each daughter cell; and the Polo-like kinases, which are important during both mitosis and cytokinesis[23]. It should be noted that the mechanistic basis for selective cytotoxicity of many inhibitors in this second class is poorly understood.

Synthetic lethal
Two genes have a synthetic-lethal phenotype if the individual gene deletions are not lethal but the combined mutation is. A synthetic-lethal interaction is proof of a genetic interaction.

A third class of kinase targets are expressed in the tumour or in surrounding tissues and are required for different stages of tumour formation and maintenance in the human host. For example, the neurotrophic growth factor receptor (NTRK2) is essential for allowing some cells to survive detachment and may also be required for tumour cell metastasis[24]. Additional examples include the vascular endothelial growth factor receptor (VEGFR) and the FGFR kinases, which are important in developing and sustaining tumour blood supply[25], as well as the M2 splice isoform of pyruvate kinase, which is required for the tumorigenic switch to aerobic glycolysis that occurs in cancer cells[26].

For several reasons, inhibiting mutationally activated kinases has resulted in the most dramatic clinical responses. As these kinases are marked by mutation they are therefore the most readily identified by DNA sequencing[10,27]. Furthermore, a subset of these mutated kinases are themselves transforming, making it much easier to engineer mechanistically relevant cellular assays that can be used during the course of inhibitor discovery and optimization. Kinase targets of the second and third classes are not directly transforming but instead are required for the survival, proliferation and/or tumorigenesis of cancer cells. As such, they can be highly context dependent and much more difficult to investigate in cell culture systems. The context-dependent role of kinases in tumorigenesis provides opportunities and challenges for therapeutic target selection. A recent example is that PTEN-deficient cancers depend on PI3Kβ to sustain activation of the PI3K pathway, whereas PI3Kα kinase activity appears to be required to sustain the proliferation of established tumours[28,29] Typically, kinases that can be targeted through a synthetic-lethal interaction specific to cancer cells must be investigated and validated by a largely empirical process. Likewise, kinase targets required for tumorigenesis are usually evaluated in animal tumour models that are often not accurate reflections of the disease evolution in humans, so there is a need for genetic tumour models that can more accurately recapitulate the different stages of tumour progression and can be used to investigate the impact of selective kinase inhibitors.

Kinase inhibitor binding sites

Protein kinases are defined by their ability to catalyse the transfer of the terminal phosphate of ATP to substrates that usually contain a serine, threonine or tyrosine residue. They typically share a conserved arrangement of secondary structure elements that are arranged into 12 subdomains that fold into a bi-lobed catalytic core structure with ATP binding in a deep cleft located between the lobes[30–32]. ATP binds in the cleft with the adenine ring forming hydrogen bonds with the kinase 'hinge' — the segment that connects the amino- and carboxy-terminal kinase domains. The ribose and triphosphate groups of ATP bind in a hydrophilic channel extending to the substrate binding site that features conserved residues that are essential to catalysis. All kinases have a conserved activation loop, which is important in regulating kinase activity and is marked by conserved DFG and APE motifs (which refer to one-letter amino acid abbreviations) at

Heterocycle
An organic compound with a ring structure containing non-carbon atoms such as sulphur, oxygen or nitrogen as part of the ring.

Pharmacophore
A molecular framework that carries the essential features responsible for the biological activity of a drug.

the start and end of the loop, respectively. The activation loop can assume a large number of conformations with the extremes being a conformer that is catalytically competent and usually phosphorylated, and an 'inactive' conformer in which the activation loop blocks the substrate binding site. Most kinase inhibitors discovered to date are ATP competitive and present one to three hydrogen bonds to the amino acids located in the hinge region of the target kinase, thereby mimicking the hydrogen bonds that are normally formed by the adenine ring of ATP[32,33] (FIG. 1). The majority of kinase inhibitors do not exploit the ribose binding site (an exception being AZD0530, a novel Src and Abl dual family kinase inhibitor[34]) or the triphosphate binding site of ATP.

Type 1 inhibitors. This type of inhibitor constitutes the majority of ATP-competitive inhibitors and recognizes the so-called active conformation of the kinase (FIG. 1a), a conformation otherwise conducive to phosphotransfer[33]. The preponderance of type 1 inhibitors may be a consequence of many compounds having been discovered using enzymatic assays in which kinases were introduced in their active conformation and because many kinase inhibitors have been synthesized to mimic ATP (and each other). Type 1 inhibitors typically consist of a heterocyclic ring system that occupies the purine binding site, where it serves as a scaffold for side chains that occupy the adjacent hydrophobic regions I and II (FIG. 2).

Type 2 inhibitors. By contrast, type 2 kinase inhibitors recognize the inactive conformation of the kinase (FIG. 1b). The conformation that is recognized by type 2 inhibitors is sometimes referred to as DFG-out owing to the rearrangement of this motif. Movement of the activation loop to the DFG-out conformation exposes an additional hydrophobic binding site directly adjacent to the ATP binding site. The original discovery that inhibitors such as imatinib and sorafenib bind in the type 2 conformation was serendipitous, but subsequent analysis of multiple type 2 kinase inhibitor co-crystal structures has revealed that all share a similar pharmacophore and exploit a conserved set of hydrogen bonds (FIG. 1b).

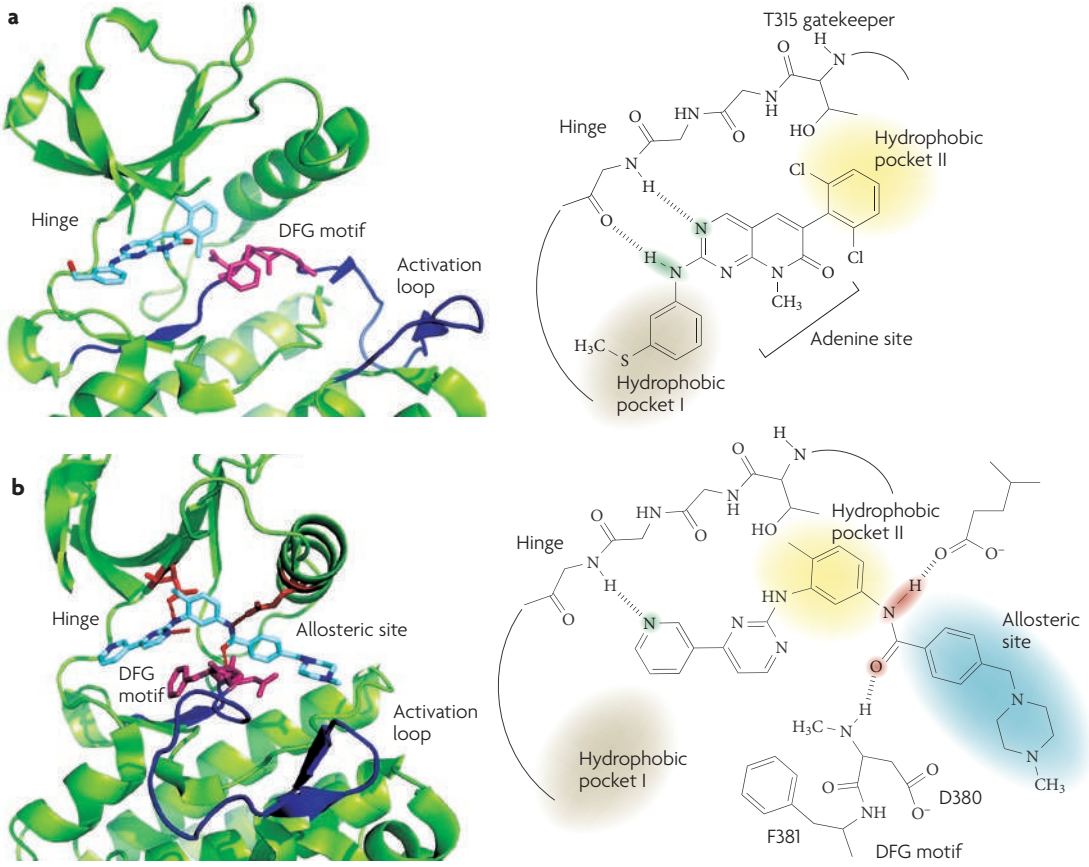


Figure 1 | **Kinase inhibitor binding modes.** Kinase inhibitor–protein interactions are depicted by ribbon structures (left) and chemical structures (right). The chemical structures depict hydrophobic regions I and II of ABL1 (shaded beige and yellow respectively) and hydrogen bonds between the kinase inhibitor (inhibitor atoms engaged in hydrogen bonds to hinge are highlighted in green or to allosteric site in red) and ABL1 are indicated by dashed lines. The DFG motif (pink), hinge and the activation loop of ABL1 are indicated in the ribbon representations. The kinase inhibitors are shown in light blue. **a** | ABL1 in complex with the type 1 ATP-competitive inhibitor PD166326 (Protein Data Bank (PDB) ID 1OPK)[104]. Shown here is the DFG-in conformation of the activation loop (dark blue). **b** | The DFG-out conformation of the activation loop of ABL1 (dark blue) with the type 2 inhibitor imatinib (PDB ID 1IEP)[105]. The allosteric pocket exposed in the DFG-out conformation is indicated by the blue shaded area (right).

Examples of type 2 inhibitors that are approved by the US Food and Drug Administration include the ABL1, KIT and platelet-derived growth factor receptor (PDGFR) inhibitors imatinib and nilotinib[35], and the KIT, PDGFR and Raf inhibitor sorafenib[36]. The inhibitor-stabilized conformational rearrangement of the activation loop observed in type 2 kinase inhibitor co-crystal structures demonstrates that the kinase active site is highly mobile and can remodel to accommodate a variety of inhibitors[33]. The ability to induce dramatic conformational change is not unique to type II kinase inhibitors, for example the type I inhibitor PIK-39 exhibits selectivity towards PI3Kγ over other PI3K isoforms by inducing a conformational rearrangement of the side chain of M804 to form a novel pocket at the entrance to the kinase active site in a manner similar to the DFG-out kinase conformation[37]. Although the methionine residue with which PIK-39 interacts is conserved among other PI3Ks, selectivity towards PI3Kγ is achieved as only this isoform permits an inhibitor-induced conformational rearrangement.

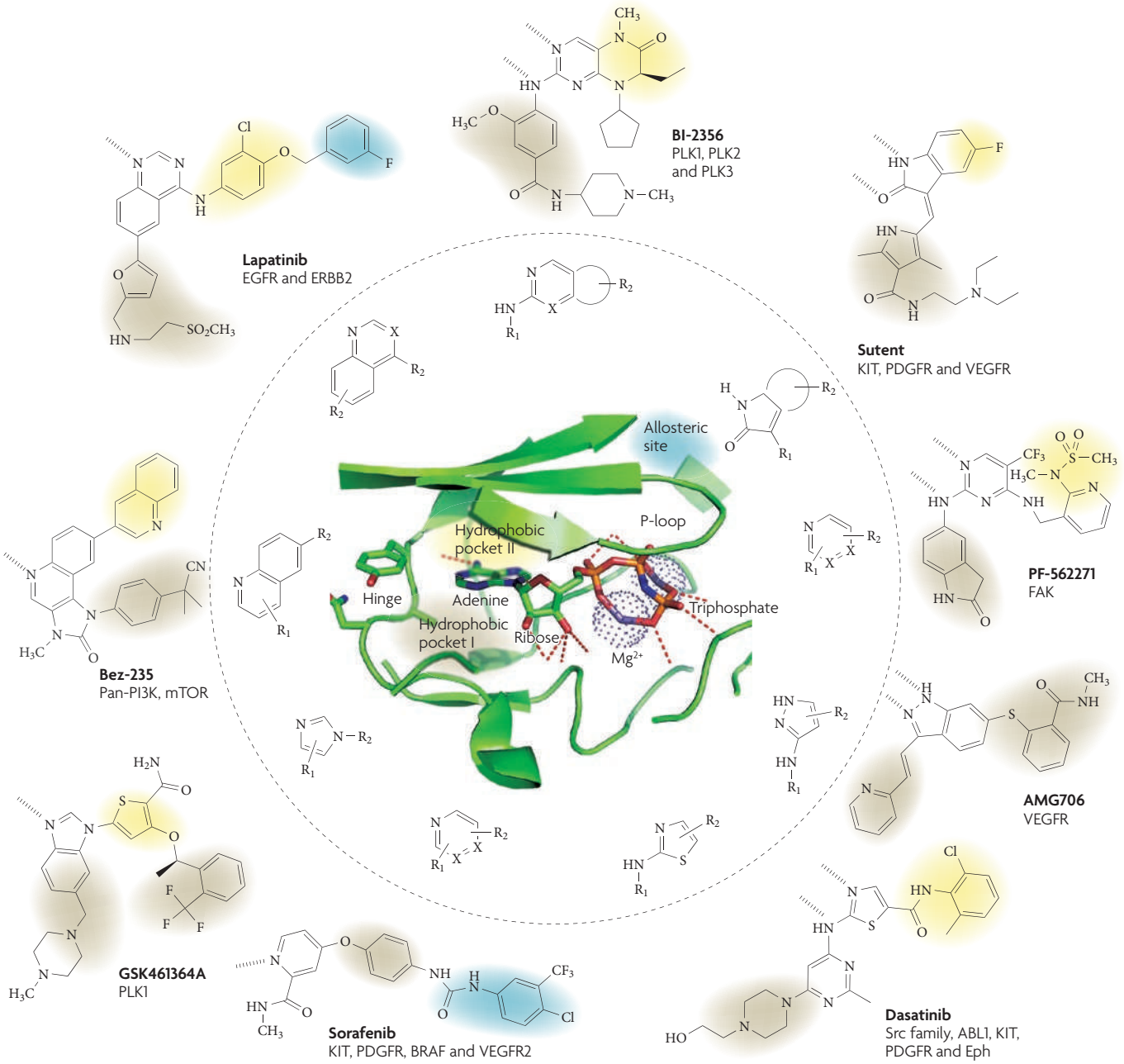


Figure 2 | **Diverse kinase inhibitors.** The ATP binding site of AKT1 complexed with ATP (Protein Data Bank (PDB) ID 1O6L) is depicted with key regions indicated and hydrogen bonds indicated by red dotted lines. The middle ring shows commonly used heterocyclic core scaffolds (X = C, N). The outer ring shows examples of structurally diverse type 1 inhibitors and their reported kinase targets. Hydrogen bonds are indicated by hashed lines on these structures. EGFR, epidermal growth factor receptor; Eph, ephrin receptor tyrosine kinases; FAK, focal adhesion kinase; PDGFR, platelet-derived growth factor; PLK, Polo-like kinase; VEGFR, vascular endothelial growth factor receptor.

Allosteric site
A site distinct from the enzyme active site that can bind a ligand that either positively or negatively regulates enzyme activity.

Nucleophilic
A group of atoms that is rich in electrons and that participates in a chemical reaction by donating electrons to an electron-poor electrophile (electron lover).

Electrophile
A group of atoms that is deficient in electrons and that participates in a chemical reaction by accepting electrons from an electron-rich nucleophile (nucleus lover).

Michael addition
Nucleophilic addition to an α,β-unsaturated carbonyl compound. It belongs to the larger class of conjugate additions.

Allosteric inhibitors. The third class of compounds binds outside the ATP-binding site — at an allosteric site — and modulates kinase activity in an allosteric manner. Inhibitors belonging to this category tend to exhibit the highest degree of kinase selectivity because they exploit binding sites and regulatory mechanisms that are unique to a particular kinase. The most well-characterized allosteric kinase inhibitor is CI-1040, which inhibits MEK1 and MEK2 by occupying a pocket adjacent to the ATP binding site[38] (FIG. 3a,b). Other examples include GNF2, which binds to the myristate binding site of BCR–ABL1 (REF. 39); the pleckstrin homology domain-dependent Akt inhibitor Akt-I-1 (REFS 40,41); and the IKK (inhibitor of nuclear factor-κB kinase) inhibitor BMS-345541 (REF. 42) (FIG. 3b). Allosteric activators of kinase activity have also been discovered, including RO0281675 (REF. 43) and numerous analogues[44] that activate glucokinase, as well as AICAR[45] and A-769662 (REF. 46), which activate AMP-activated protein kinase. More allosteric inhibitors are likely to be uncovered in the future as a greater emphasis is placed on cell-based assays in which kinases are interrogated in their native context. This approach presents the advantage of allowing compounds to be identified that may require an accessory protein for function. For example, the requirement of the mTOR inhibitor rapamycin for the intracellular protein FKBP1A would not have been discovered in a biochemical mTOR assay.

Covalent inhibitors. A fourth class of kinase inhibitors are capable of forming an irreversible, covalent bond to the kinase active site, most frequently by reacting with a nucleophilic cysteine residue[47,48] (FIG. 4). The clinically most advanced irreversible kinase inhibitors of the epidermal growth factor receptor (EGFR), HKI-272 (REF. 49) and CL-387785 (REF. 50), were developed to target a relatively rare cysteine residue located at the lip of the ATP binding site[51].

These compounds were rationally designed by appending an electrophile, which is reactive towards the electron-rich sulphur present in the cysteine residue, to the well-characterized EGFR-selective 4-anilinoquinazoline and 4-anilinoquinoline-3-carbonitrile scaffolds. Using the known structures of 4-anilinoquinazolines complexed with EGFR, it was possible to predict the optimal site for electrophile attachment. The resulting inhibitors undergo a Michael addition reaction in which the solvent-exposed cysteine residue that is present in EGFR (C773) or ERBB2 (C805) forms a covalent bond with the inhibitor, bestowing it with infinite affinity for

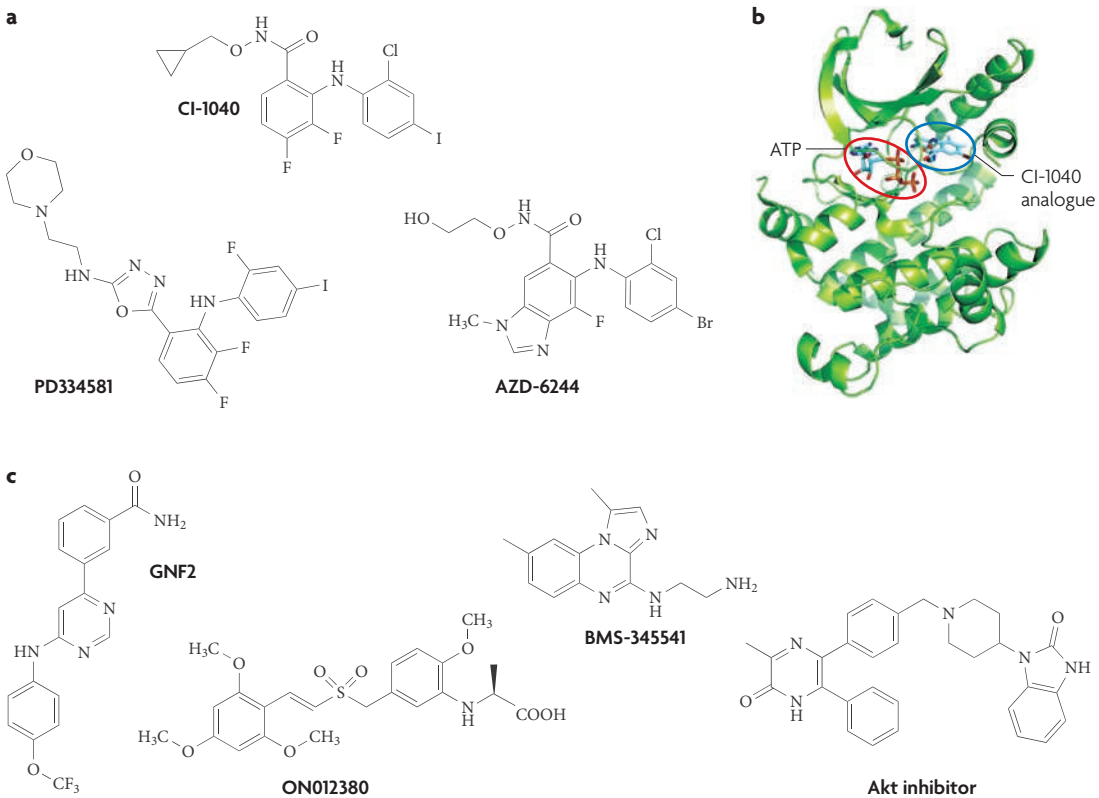


Figure 3 | **Allosteric kinase activity modulators. a** | The figure shows the chemical structures of MEK1 inhibitors. AZD-6244 and PD334581 are second-generation MEK1 inhibitors, currently under clinical development. **b** | CI-1040 (indicated by a blue circle) binds MEK1 (green ribbon, Protein Data Bank (PDB) ID 1S9J)[38] immediately adjacent to the ATP binding site (indicated by red circle). **c** | GNF2 binds the myristate binding site of BCR (breakpoint cluster region)–ABL1, which is located at the carboxyl terminal of the kinase domain. ON012380 is a non-ATP-competitive inhibitor of BCR–ABL1 that appears to be substrate site competitive. The binding site of the Akt inhibitors is unknown, but the pleckstrin homology domain of Akt is required for activity. The binding site for the inhibitor BMS-345541 on inhibitor of nuclear factor-κB kinase (IKK) is uncharacterized.

the ATP binding site (Supplementary information S2 (figure)). As a result, the inhibitor irreversibly blocks binding of ATP to the kinase, thereby rendering the kinase inactive. To date, five such EGFR kinase inhibitors are being evaluated in lung cancer clinical trials[48,52,53]. Irreversible kinase inhibitors have also been designed against vascular endothelial growth factor receptor 2 (VEGFR2)[54], the Tec family kinase BTK[55] and RSK[56] (BOX 1). Naturally occurring cytotoxic compounds have also evolved to irreversibly modify kinase cysteine residues in this manner. The resorcylic acid lactone polyketides — such as hypothemycin, which was isolated from the fungus *Hypomyces subiculosus* — contain a *cis*-enone that undergoes a Michael addition reaction with a cysteine that is located immediately before the conserved DFG motif of the kinase activation loop; hypothemycin was shown to covalently modify 18 out of the 19 kinases against which it was tested[57]. A bioinformatic analysis of the human kinome revealed that there are 46 kinases that have this particular cysteine[57] and there are approximately 200 different kinases that have a cysteine located in the vicinity of the ATP pocket (FIG. 4 and Supplementary information S3 (figure)), suggesting that a large number of additional kinases could be targeted by irreversible inhibitors. However, despite the large number of kinases that could be targeted for inhibition by this approach, many drug developers are concerned about the potential for toxicity of covalent inhibitors as a result of modification of unanticipated targets.

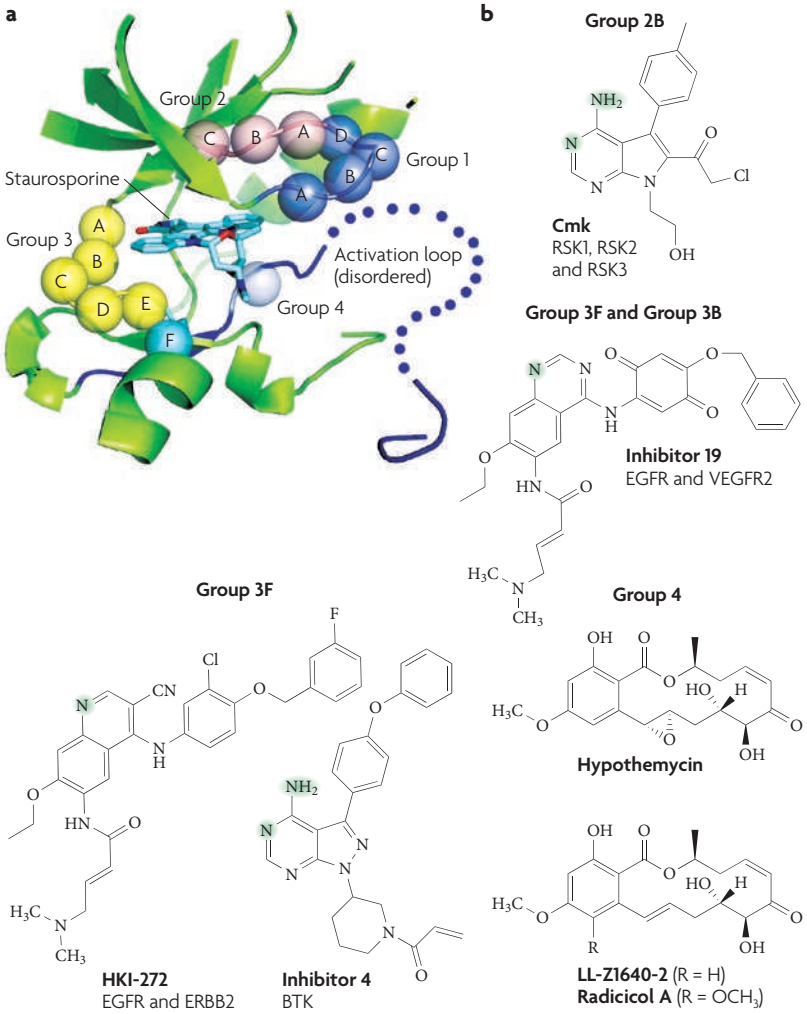


Figure 4 | **Representative irreversible inhibitors and locations of cysteines that are accessible for covalent modification across the kinome. a** | The ribbon structure depicts the cysteine-containing interleukin 2 tyrosine kinase (Protein Data Bank (PDB) ID 1SM2). A structure-guided bioinformatics analysis based on the available literature was conducted to identify all protein kinases with a cysteine residue that could potentially be targeted by an ATP binding site inhibitor revealed that more than 200 kinases (approximately 40% of the protein kinome) have such a cysteine. The kinases were classified according to the position of the cysteine residue in the active site. Group 1 kinases harbour such cysteines in the glycine-rich-loop (also known as the P-loop; shown in dark blue), which forms the 'roof' of the ATP binding site. In group 2 kinases, the cysteine is located within the three amino acids shown in pink directly after the glycine-rich loop. The cysteine in group 3 kinases (yellow) is located within the amino acids that constitute the hinge connecting the amino- and carboxy-terminal kinase lobes. These precede the DFG motif (pink), marking the start of the kinase activation loop (not visible in the X-ray structure because it is disordered) in which the cysteine residue that is modified in group 4 kinases (shown in dark blue) is located. **b** | Chemical structures of reported covalent inhibitors that react with the cysteine group are indicated. Green shading indicates inhibitor atoms involved in hydrogen bonding to the kinase hinge. The complete list of the kinases in the different groups is provided in Supplementary Information S3 (box). EGFR, epidermal growth factor receptor; RSK, ribosomal S6 kinase; VEGFR, vascular endothelial growth factor receptor.

Discovering new kinase inhibitors

As only a small fraction of the kinome can currently be targeted by a reasonably selective and potent inhibitor, there is an urgent need to develop strategies for efficient discovery and optimization of new inhibitors. Historically, many type 1 ATP-competitive inhibitors were discovered by performing high-throughput screening of compound collections. Unfortunately, this approach is becoming less effective, as it has now identified most of the scaffolds that are capable of serving as ATP-competitive ligands[58]. However, this method is still of use when a kinase with an unusual active site is screened or when allosteric inhibitors are specifically being sought.

New ATP site-targeted ligands are primarily developed using a combination of methods including analogue synthesis, structure-informed design and fragment-based assembly strategies (BOX 2).

Analogue synthesis. Efforts to identify inhibitors of a new kinase target often begin with a screen of archived inhibitors that were developed in previous kinase projects. For this reason, the analogue synthesis and isosteric replacement approaches are commonly initiated with a lead scaffold that was originally identified as an inhibitor of one kinase and later serendipitously found to be cross-reactive with a new kinase of interest. These efforts have resulted in the discovery and diversification of many scaffolds that are noteworthy in their ability to recognize the ATP-binding site, including quinazolines, pyrimidines, purines, imidazoles, pyrazoles, oxindoles and quinolines. Ideally, once such a scaffold is identified, it is co-crystallized with the kinase of interest in order to provide insight into the structural features that may be important for potency or selectivity. However, as inhibitor discovery efforts usually precede the determination of a crystal structure, homology models developed from one or more of the hundreds of other kinase–ligand co-crystal structures are often

Box 1 | Developing covalent inhibitors

The development of irreversible ribosomal S6 kinase-α1 (RSK1) inhibitors that can form a covalent bond with a cysteine in the P-loop, which forms the roof of the ATP binding site, provides a clear example of how selective irreversible inhibitors can be designed[47]. The starting scaffold for inhibitor development in this case was the relatively non-specific, low-potency tyrosine kinase inhibitor PP1 ($IC_{50,\ RSK2}$ = 1.2 μM). As RSK1, RSK2 and RSK4 share a unique cysteine residue in the P-loop, they can be alkylated by chloromethylketone- or fluoromethylketone-modified versions of PP1 (resulting in the generation of the compounds CMK and FMK respectively). FMK can potently inhibit RSK1 with an IC_{50} of 15 nM. Sequence alignments of 491 kinase domains reveal that seven other kinases also contain an identical cysteine residue: MSK1, MSK2, Polo-like kinase 1 (PLK1), PLK2, PLK3, NEK2 and MEKK1. However, these kinases are not inhibited by FMK, presumably because the presence of a larger residue at the gatekeeper — the amino acid that controls access to hydrophobic pocket II position of these kinases — sterically occludes binding of FMK in the active site. Although these compounds have not been explicitly demonstrated to be selective against 491 other kinases, exclusive labelling of RSK1 and RSK2 was observed in cellular extracts. These experiments suggest that scaffolds with moderate potency and selectivity can be converted to highly potent and selective inhibitors by the judicious introduction of an electrophile that can target unique cysteine residues.

used to predict a binding mode of the inhibitor to a three-dimensional homology model of the kinase. Analogues are then prepared to modulate potency, selectivity and pharmacological properties. Isosteric replacements are used to improve these properties while preserving the stereoelectronic contacts responsible for binding to the kinase. For example, numerous medicinal chemistry efforts have spawned second-generation inhibitors from the progenitor compound PD180970 (REF. 59), a pyrido[2,3-*d*]pyrimidine class compound that potently inhibits a variety of tyrosine kinases. These include PD173074, a selective inhibitor of FGFR1, FGFR3 and FGFR4 (REF. 60); BI-2356, a selective inhibitor of the Polo-like kinases[61]; and BI-D1870, an inhibitor of RSK[62]. All three compounds incorporate the pyrido[2,3-*d*]pyrimidine core motif (BOX 2) but derive selectivity from side chain substitutions. During the course of compound optimization, it is typically important to monitor structure–activity relationships in both biochemical and cellular kinase assays because the biochemical kinase assay will frequently not predict which compounds exhibit optimal cellular activity. Although cell permeability and intracellular accumulation can sometimes account for these differences, in other cases it appears that the physiologically relevant form of the kinase is not accurately reflected by the biochemical kinase assay, especially when only the kinase domain is used. For example, the insulin-like growth factor receptor 1 (IGF1R) inhibitor AEW541 displays almost identical potency for inhibition of IGF1R (IC_{50} = 150 nM) and insulin receptor (IC_{50} = 140 nM) in biochemical kinase assays; however, in cellular assays the compound is 25-fold more selective for IGF1R versus the insulin receptor ($EC_{50,\ IGFR}$ = 86 nM)[63].

Structure-informed design. In addition to guiding analogue synthesis, structure-informed design is used in diverse ways to facilitate inhibitor discovery and optimization. Perhaps the most frequent use is to determine the key interactions formed between the inhibitor and the active site and to learn where functionality may be introduced to modulate potency or selectivity. Alternatively, crystal structures can be used to design hybrid compounds in which a portion of one inhibitor is grafted onto a portion of another, guided by superimposition of the bound structures. This can be an especially effective way to create new type 2 inhibitors by generating molecular amalgams between known type 1 and 2 inhibitors[33,64]. Hybrid design can also involve grafting a selectivity determinant from one inhibitor series onto another. Crystal structures are also used for virtual ligand screening efforts, in which ligands are discovered using computational approaches. However, the enrichment factors that are derived from these efforts are not sufficient to make this approach generally applicable[65].

Fragment-based inhibitor discovery. Fragment-based inhibitor discovery involves identification of moieties that bind to different portions of the active site of the target kinase followed by covalent linkage of these moieties to create a new inhibitor template[66]. Fragments that bind to the active site of interest generally originate in a library and can be selected computationally or experimentally using NMR or crystallography. Typical fragments are of low molecular mass (<150 Da) and are selected to possess a combination of hydrophobic and polar functional groups that can bind with a high degree of atom efficiency (a measure of the affinity contributed per atom of the fragment). The major advantage of the fragment-based discovery approach is that a large chemical space of virtual structures can be explored without the actual synthesis of large numbers of compounds. An example of fragment-based screening is the discovery of the pyrazole-based CDK2 inhibitor AT7519, which is structurally distinct from the large number of known CDK2 inhibitors[67]. To date, only a limited number of kinase inhibitors have been developed using this approach, perhaps owing to a requirement for continual collaboration between synthetic chemists and structural biologists.

Kinase inhibitor selectivity

Discovering the full range of intracellular targets of a small molecule kinase inhibitor is a daunting challenge[4,68]. Acquiring this information is crucial to understanding the mechanistic basis for potential toxicities and to understanding which tumours may respond to a particular inhibitor. In addition to the 518 kinases encoded in the human genome, there are over 2,000 other nucleotide-dependent enzymes, including polymerases, chaperones, motor proteins, reductases and methyltransferases, that possess potential binding sites[69]. Ideally, the selectivity of a new kinase inhibitor is evaluated at the level of the protein, cell and whole organism. Initially, kinase inhibitors are typically evaluated at the protein level for their potential to inhibit kinase-catalysed phosphotransfer from ATP to a substrate protein or peptide. Currently, panels of more than 400 diverse kinase enzymatic or binding assays are available from a number of service providers. Owing to the relatively high costs associated with these commercial services, compounds are typically screened at a single concentration of 1 or 10 μM and then further evaluated in dose–response

Polyketide
Secondary metabolites from bacteria, fungi, plants and animals with diverse biological activities and pharmacological properties.

Isostere
A chemical group with similar size and electronic properties.

Homology model
A class of methods for constructing a three-dimensional structure of a target protein for which only the sequence is available, provided at least one empirical three-dimensional template structure with >30% sequence identity is available.

IC_{50}
Inhibitor concentration for half-maximum response.

EC_{50}
Effector concentration for half-maximum response.

Enrichment factor
The ratio of the number of biologically active compounds discovered using computationally based selection to the number found by random screening.

Chemical space
The space spanned by all possible (that is, energetically stable) stoichiometrical combinations of electrons and atomic nuclei and topologies (isomers) in molecules and also compounds in general.

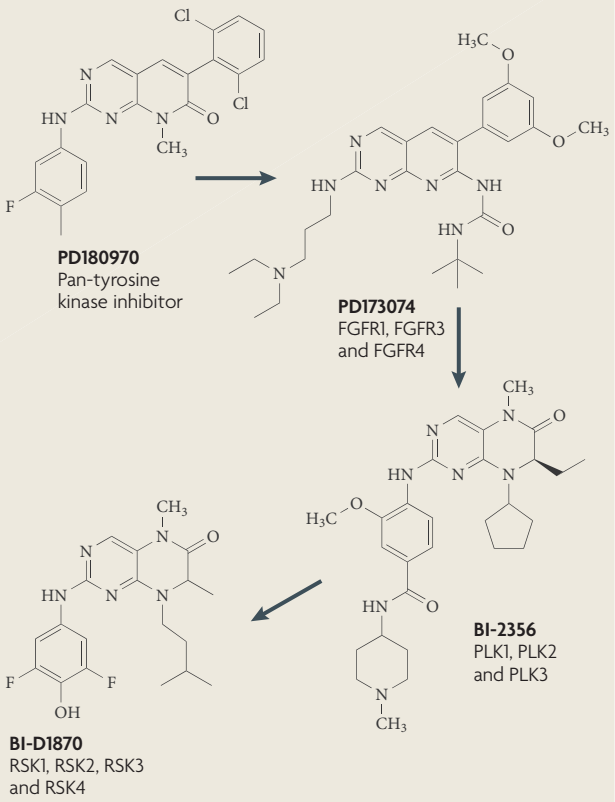
Box 2 | **Methods to create selective kinase inhibitors**

Synthesis of analogues and isosteric replacement

Pyrido[2,3-*d*]pyrimidines such as PD180970 (see the figure) were originally identified as inhibitors of the Src family kinases but were subsequently determined to be highly promiscuous tyrosine kinase inhibitors. Crystallization of PD180970 with SRC revealed that the phenylaminopyrimidine motif binds to the hinge and the dichlorophenyl ring is situated in hydrophobic motif II. Focused medicinal chemistry efforts to vary the aniline and dichlorophenyl substitutents resulted in the identification of functionality that maintained potency against fibroblast growth factor receptors (FGFRs) yet imparted a high degree of kinase selectivity[95]. Subsequent crystallography of PD173074 with FGFR1 in conjunction with examination of structure–activity relationships revealed that the 3,5-disposition of the methoxy groups appears to be a key selectivity determinant that is uniquely tolerated by FGFR[60]. A highly selective inhibitor of the PLKs, BI-2356 (REF. 61), was created by replacing the pyrimidone ring of PD180970 with a saturated six-membered ring containing a cyclized amino acid. Here the key selectivity determinants, as determined by crystallography and analogue synthesis, are distributed throughout the structure and include the orthomethoxy group, the *N*-methyl group and the ethyl-substituted chiral centre[96]. An inhibitor of RSKs, BI-D1870, was independently derived from the same scaffold as BI-2356 by varying the side-chain functional groups[62].

Structure-informed design

The large number of available inhibitor–kinase co-crystal structures makes it possible to graft structural features that are present in one structure onto another. For example, new type 2 inhibitors have been created by introducing a 3-trifluoromethylbenzamide group to occupy the allosteric binding pocket (FIG. 1). This approach allowed the creation of a new type 2 inhibitor with a completely different selectivity profile[64]. A second example is the development of a potent PI3Kδ or PI3Kγ inhibitor, PIK-294, through introduction of a 3-hydroxyphenyl group that occupies hydrophobic region II (the so-called affinity pocket for PI3Ks)[37].



studies against selected kinases of interest. Thus, despite the availability of these large panels, only limited selectivity data are available for many commonly used kinase inhibitors[68]. However, a series of papers examining the kinase specificity profiles of many clinical and preclinical stage kinase inhibitors have recently been published[70,71]. For example, binding assays were used to demonstrate that VX-680 (REF. 72), a pyrimidine-derived Aurora kinase inhibitor, also binds to the clinically important T315I mutant of BCR–ABL1, which is resistant to currently approved inhibitors[70]. Characterization of the cellular activity against T315I BCR–ABL1 and co-crystallography with an ABL1 mutant[73] supported this finding and have motivated subsequent clinical investigation.

A second approach consists of measuring the ability of a test compound to alter the melting temperature (T_m) of a kinase[74]. The larger the shift in T_m, the tighter the binding affinity of the inhibitor is to the kinase, as manifested by increased stabilization of kinase tertiary structure. This approach was recently used to discover that LY333531, a putatively highly selective bisindolylmaleimide protein kinase Cβ inhibitor that is being developed for diabetic retinopathy, also possessed potent inhibitory activity against Pim family kinases, which are potential leukaemia targets[74].

Cellular selectivity of kinase inhibitors can be evaluated either using panels of cell lines that are engineered to report on inhibition of a particular kinase or using unbiased proteomics approaches. Many such cell lines have been developed from the murine Ba/F3 cell line[75]. This pro-B-cell line normally requires the cytokine interleukin 3 (IL-3) to proliferate. Transformation by an oncogenic kinase, however, leads to IL-3-independent proliferation and survival such that the rate of cell growth and proliferation can be used as a read-out of intracellular kinase inhibition. Cytotoxicity that results from specific kinase inhibition can be discriminated from non-specific cytotoxicity by 'rescue' through the introduction of IL-3. Panels of kinase-transformed Ba/F3 cells have been created by using either naturally occurring oncogenic fusion proteins (for example, BCR–ABL1 and NPM–ALK) or by creating artificial fusions between kinase domains and the protein multimerization domain Tel. The Tel domain was used because it is found in several naturally occurring fusion proteins and it exhibits a strong ability to multimerize fused proteins[76]. Other cell lines have been generated through the use of reporter gene assays. Unbiased proteomics-based approaches for characterizing kinase inhibitor selectivity involve either traditional affinity chromatography[77] or competitive displacement assays. For example, a competitive displacement assay was established in which an affinity bead resin bearing seven different ATP-competitive ligands that displayed little selectivity was demonstrated to be able to capture hundreds of different kinases from HeLa cell extracts[78]. The inclusion in the cell lysate of a test inhibitor of interest that specifically binds its target reduces the amount of free target available for capture on the resin. This technology was recently used to make

Bisindolylmaleimide
Two fused indole rings bridged by a maleimide that can act as an ATP-competitive kinase inhibitor.

the impressive discovery that imatinib, perhaps one of the most extensively investigated kinase inhibitors, had a previously unsuspected additional receptor tyrosine kinase target: the discoidin domain receptor DDR1 (REFS 78,79).

Evaluation of kinase inhibitor selectivity on an organismal level remains a significant research challenge. Current efforts are usually limited to determining drug distribution to various organ compartments and monitoring a pharmacodynamic marker, such as a particular phosphorylation site. We know from studies using isolated cell lines that different lines can exhibit dramatically different responses to a given inhibitor, so it is clear that we need new methods to globally monitor the changes in phosphorylation that result from kinase inhibition in the context of a living organism. This will allow more accurate evaluation of on- and off-target effects of the inhibitor that can be evaluated in relation to efficacy and toxicity.

Kinase inhibitor resistance mechanisms

As many kinase inhibitors exert their cytotoxic effects primarily by inhibiting a specific kinase, there is a strong selective pressure for cells to acquire resistance through mutations in the kinase gene that abrogate drug binding. Additional non-mutation kinase inhibitor resistance mechanisms have been documented, including target amplification in the case of BCR–ABL1 in CML patients[80] and upregulation of alternative kinase pathways such as hepatocyte growth factor receptor in the acquisition of resistance to EGFR kinase inhibitors that has been observed in lung cancer[81]. Owing to the rapid proliferation of cancer cells, the acquisition of mutations conferring drug resistance has become a recurring theme in the clinic. Indeed, resistance as a result of kinase mutations has been documented for inhibitors of BCR–ABL1 (Supplementary Information S4 (figure)), EGFR, FLT3, KIT and PDGFR[50,82–86]. To date the most extensive clinical and laboratory characterization of resistance-causing mutations has been performed for BCR–ABL1 in the context of imatinib and second-generation inhibitors. Additionally, it has been shown in several haematological tumours that quiescent stem cells are refractory to tyrosine kinase inhibitors, and these cell populations are probably also involved in resistance mechanisms[87,88].

Inhibitor resistance conferred by mutation at the gatekeeper residue — so called because the size of the amino acid side chain at this position determines the relative accessibility of a hydrophobic pocket located adjacent to the ATP binding site (hydrophobic pocket II, FIG. 1) — appears to be a common theme for a variety of kinases. Access to this pocket is important to many kinase inhibitors because hydrophobic interactions in this site are crucial for the binding affinity of the inhibitor. For example, the most recalcitrant of the BCR–ABL1 mutants is T315I, which harbours a mutation in the gatekeeper residue. Although the gatekeeper residue often comes in close contact with type 1 and type 2 ATP binding site inhibitors, it typically does not interact with ATP[33]. Consequently, mutation of the gatekeeper residue generally causes little or no change in kinase activity but has the potential to confer inhibitor resistance through a variety of biochemical mechanisms. The T315I gatekeeper mutation in BCR–ABL1 impedes drug binding through loss of a crucial hydrogen bonding interaction to the inhibitor and introduction of a steric clash. Mutation of the EGFR T790 residue to methionine induces resistance to the quinazoline-based inhibitors gefitinib and erlotinib[50,89] by increasing the affinity for ATP, which effectively weakens the affinity of ATP-competitive inhibitors.

Gatekeeper mutations have been found to confer inhibitor resistance in a number of additional cases. The gatekeeper mutation G697R in FLT3 induces resistance to the type 1 staurosporine derivative PKC412 (REF. 86). A systematic investigation of engineered gatekeeper mutations in ABL1, PDGFRβ, SRC and FGFR1 demonstrated that resistance can be achieved against a variety of selective and non-selective inhibitors of these kinases[90]. For example, the T341M mutation of SRC results in resistance to the type I pyrido[2,3-*d*]pyridinone PP58, mutation of T681I of PDGFRβ confers resistance to both PP58 and imatinib, and the V561M mutation of FGFR1 confers resistance to PP58 (REF. 90). There is currently debate as to whether the gatekeeper mutation is pre-existing or acquired following inhibitor treatment. The gatekeeper mutants of BCR–ABL1 (T315I), PDGFRα (T674I), EGFR (T790M) and KIT (T670I) are the most frequently reported mutants for each of these kinases. This suggests that mutation of the gatekeeper amino acid is likely to be a common occurrence in clinical kinase inhibitor resistance and that general methods to design inhibitors that can overcome this mutation would be useful.

Several strategies are being investigated to overcome kinase inhibitor resistance mutants. A first approach is to develop inhibitors that can tolerate diverse amino acids at the gatekeeper position as discussed for T315I BCR–ABL1 inhibitors in BOX 3. A second approach is to target the kinase with inhibitors that bind at alternative binding sites. For example, the ABL1 substrate binding site has been targeted by ON012380, a vinyl sulphone-containing inhibitor[91,92], and the myristate binding site has been targeted by the GNF2 class of inhibitors[39]. A third approach involves targeting other pathways that may be required for BCR–ABL1-mediated transformation, such as the chaperone function of HSP90 (REF. 93) or farnesyltransferase activity[94] These approaches have been demonstrated to work in cell culture and efforts are currently underway to apply them clinically.

Perspectives

Kinase inhibitor drug discovery has progressed dramatically in the past decade. In addition to the clinical approval of a number of new drugs, efficient approaches for the development of potent and selective inhibitors with desirable properties have become established. Kinase inhibitor design has benefited immensely from crystallography in the elucidation of binding modes and unexpected inhibitor-induced conformational rearrangements. Indeed, much of current kinase inhibitor discovery takes place through rational drug design rather than through high-throughput screening and empirical optimization on the basis of structure–activity relationships. Sophisticated

Steric clash
Atoms contain an electron shell with a defined radius that prevents atoms not engaged in a covalent bond from occupying the same volume. A steric clash occurs when two atoms are placed closer than the sum of their atomic radii.

Farnesyltransferase activity
Post-translational modification of proteins that consists of attaching an isoprenyl group to a cysteine residue.

Box 3 | **Development of mutant-targeting BCR–ABL1 inhibitors**

The discovery of imatinib resistance mutations has encouraged the successful development of new ATP-competitive inhibitors such as nilotinib (AMN107)[97] and dasatinib (BMS-354825)[98]. Nilotinib is a phenylaminopyrimidine that has approximately 20-fold higher cellular activity than imatinib and inhibits the majority of BCR (breakpoint cluster region)–ABL1 mutants, with the exception of T315I. Dasatinib is a thiazole-derived compound that has an approximately 50-fold lower cellular EC_{50} than imatinib and inhibits virtually all known BCR–ABL1 mutants, but again with the exception of the T315I mutant. Whereas nilotinib has a selectivity profile similar to imatinib (BCR–ABL1, PDGFR and KIT), dasatinib has a pan-tyrosine kinase inhibitor profile including the Src family (SRC, YES, FYN and LYN), BCR–ABL1, KIT, platelet-derived growth factor receptor (PDGFR), BMX, Eph family, vascular endothelial growth factor receptor 2 and TIE2 (REF. 99). Nilotinib binds to the inactive conformation (type 2, DFG out) whereas dasatinib binds to the active conformation of the ABL1 kinase domain (type 1, DFG in)[100]. Both inhibitors make a hydrogen-bonding interaction to the side-chain hydroxyl group of T315. The development of these inhibitors demonstrates that by increasing affinity to the ATP binding site through more extensive and complementary hydrogen-bonding and hydrophobic interactions, it is possible to overcome both a subset of the active site and distal mutations in BCR–ABL1. Owing to the direct steric intrusion of the isobutyl side chain and loss of a hydrogen-bonding interaction in the middle of the ATP cleft, however, the T315I mutation renders complete resistance to both compounds[101]

Two new inhibitors that are capable of inhibiting T315I BCR–ABL1 have recently been reported in conjunction with co-crystal structures with T315I BCR-ABL1: PPY-A[101] and PHA-739358 (REF. 102). Both inhibitors recognize the active conformation of the kinase (type 1, DFG in). The selectivity profile of PPY-A has not been reported but PHA-739358 is reported to have a broad selectivity profile (IC_{50} < 1 μM against the kinases ABL1, Aurora A, Aurora B, TRK (high affinity nerve growth factor receptor), RET, FGFRs, Src family, KIT, cyclin-dependent kinase 2 and endothelial growth factor receptor 2)[103]. The chemical structures of imatinib, nilotinib, dasatinib, PPY-A and PHA-739358 may be found in Supplementary Information S5 (figure).

proteomic approaches have been developed in conjunction with panels of enzyme assays to allow for a more thorough annotation of kinase inhibitor selectivity. It is fair to say that the chemical challenge of making potent and selective inhibitors with good pharmacological properties can be overcome for most kinases of interest using currently available technologies. Significant advances have been made to discover and validate the kinase targets that are key oncogenic drivers and therefore most appropriate to target with inhibitors. However, despite these advances a number of significant challenges remain. First, mutant inhibitor resistant kinases seem to evolve quickly, and appropriate multi-targeted inhibitors or combinations need to be planned in advance of clinical application.

Second, the mechanistic basis of unexpected toxicities observed during the preclinical and clinical stages of inhibitor development need to be studied more rigorously. Improved documentation of kinase inhibitor specificities, metabolites and observed toxicities would provide a valuable database for understanding whether there are particular kinases of which inhibition should be avoided or specific substructures that result in problematic metabolites. Given the prohibitively high costs of developing new drugs, a collective effort in this arena from all organizations engaging in human clinical trials with kinase inhibitors would be justified to avoid repeating mistakes. Such a database would be a tremendous asset to the research community owing to the unprecedented size of the protein kinase family in drug discovery and the fact that less than a quarter of the kinome has been pharmacologically interrogated to date.

Third, there is a need for more predictive tumour models and better ways to monitor target inhibition in humans in a minimally invasive fashion. As only a small fraction of the tumorigenesis that occurs in humans can be recapitulated in cell culture and animal models, we need a better ability to monitor the development of cancer in humans. This would include minimally invasive methods to discover and monitor biomarkers that may serve as landmarks for the evolution of a tumour in humans. One can imagine the implementation of new imaging modalities in conjunction with proteomic technologies to monitor changes in signalling proteins and metabolites. The development of early detection technologies may allow us to discover and eradicate tumours before they have acquired the full range of survival capabilities that make established tumours and metastases so resistant to therapy.

Fourth, as kinase signalling cascades involve an intricate array of interconnected circuits we need to develop more sophisticated modelling of how they are reprogrammed in response to oncogenic events and in the presence of inhibitors. This information will be vital to more rational application of combinations of inhibitors. Successfully meeting these challenges will require a highly interdisciplinary collaboration between chemistry, biology, computation, protein crystallography, pharmacology and clinical investigation. Thus, the future promises incremental advances against a disease that inevitably will affect us all.

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Competing interests statement.
The authors declare competing financial interests: see web version for details.

DATABASES

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PI3KCA
National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary/
AT7519 | dasatinib | erlotinib | gefitinib | imatinib | nilotinib | PKC412 | rapamycin | sorafenib | VX-680
Protein Data Bank: http://www.rcsb.org/pdb/home/home.do
1IEP | 1O6L | 1OPK | 1S9I | 1SM2
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
essential thrombocythemia | myelofibrosis | neuroblastoma | polycythaemia vera
UniProtKB: http://www.uniprot.org
ABL1 | ALK | BCR | BRAF | BTK | DDR1 | EGFR | FGFR1 | FKBP1A | FRAP1 | hepatocyte growth factor receptor | IGF1R | IL-3 | insulin receptor | JAK2 | KIT | M2 splice isoform of pyruvate kinase | NPM | NTRK2 | PDGFRα | PDGFRβ | PI3Kα | PI3Kβ | PI3Kγ | PTEN | RET | RSK1 | RSK2 | RSK4 | VEGFR2

FURTHER INFORMATION

N. S. Gray's homepage: http://research4.dfci.harvard.edu/gray_lab/home.htm
Catalogue of somatic mutations in cancer: http://www.sanger.ac.uk/genetics/CGP/cosmic/
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