Measuring and interpreting the selectivity of protein kinase inhibitors

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Abstract Protein kinase inhibitors are a well-established class of clinically useful drugs, particularly for the treatment of cancer. Achieving inhibitor selectivity for particular protein kinases often remains a significant challenge in the development of new small molecules as drugs or as tools for chemical biology research. This review summarises the methodologies available for measuring kinase inhibitor selectivity, both *in vitro* and *in cells*. The interpretation of kinase inhibitor selectivity data is discussed, particularly with reference to the structural biology of the protein targets. Measurement and prediction of kinase inhibitor selectivity will be important for the development of new multi-targeted kinase inhibitors.

Keywords Protein kinase · Selectivity · Specificity · Polypharmacology

Introduction

Protein kinases catalyse the transfer of the terminal phosphate group from adenosine triphosphate (ATP) to the hydroxyl group of a serine, threonine or tyrosine residue of the kinase itself or another protein substrate. The addition of phosphate groups can create new recognition sites for other proteins to bind to or may alter the conformation of the phosphorylated protein and change its activation state or function [35, 73, 124]. Protein kinase enzymes play pivotal roles in signal transduction, transfer of a signal from the cell membrane to the nucleus and cell-cycle control. Some

tyrosine kinase (TK) domains are part of receptor tyrosine kinases; proteins that span the cell membrane and are involved in transducing growth and survival signals into the cell in response to external growth factor stimuli. Because of their importance in cell-cycle progression, cell growth, apoptosis and metabolism amongst other processes, modulating protein kinase activity has the potential to treat diseases, particularly cancer and other signalopathies [89]. Kinases involved in inter- and intracellular signalling are frequently found to be mutated in cancer cells, leading to constitutive over or under activation. This abnormal signalling can result in uncontrolled cell growth and proliferation and so by specifically targeting the signal transduction pathways in which oncogenic kinases are involved it may be possible to prevent aberrant cell growth and other behaviours [10, 39, 40, 112].

Kinases were not necessarily considered good drug targets when the study of pharmacological inhibitors began [145]. As the cellular concentrations of ATP are high, typically 1–5 mM [64, 135], it was thought that a high concentration of drug would be needed for efficacy with ATP-competitive inhibitors, bringing potential toxicity problems. Another concern was that the large number of protein kinase enzymes, all sharing a common cofactor and similar three-dimensional structure of the catalytic site, might confound attempts at developing adequately selective drugs. As small-molecule inhibitors of protein kinases are now well established as clinically useful drugs, particularly for the treatment of cancer [37, 40], it can be seen that these problems have not proven to be insurmountable.

There are currently eight approved kinase inhibitors in use in the clinic which bind in the ATP binding site (Table 1). These target a range of protein kinases, although tyrosine kinase inhibitors dominate, and are used to treat a variety of cancer types [133]. Considerable medicinal

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Name	Structure	Targets	Disease
Sunitinib		VEGFR PDGFR c-Kit FLT3	RCC GIST
Erlotinib		EGFR	NSCLC Pancreatic cancer
Gefitinib		EGFR	NSCLC
Imatinib		Ab1	CML ALL GIST
Nilotinib		Ab1 PDGFR c-Kit	CML
Sorafenib		Raf pan kinase	RCC HCC
Dasatinib		Src Ab1 broad spectrum	CML ALL
Lapatinib		EGFR HER-2	Breast cancer

standing of kinase biology shows that compounds with a broader pattern of inhibitory activities can be effective [80, 84, 116]. Nevertheless, such compounds still need to be selective inhibitors of a well-defined set of kinase enzymes. This review summarises the methodologies available for measuring and interpreting kinase inhibitor selectivity, both in vitro and in cells, and describes how these data could be applied to the development of new multi-targeted kinase inhibitors.

Protein kinase structure and selective inhibition

There are more than 500 protein kinases coded for in the human genome. These have been grouped into families based on their similarity in amino acid sequence of the catalytic domain [100]. They comprise of two main domains, an N- and C-terminal domain. The cofactor ATP binds to the backbone of the linker region between the domains, also called the hinge region (Fig. 1) [35, 75, 82, 104].

Many protein kinases have a catalytically active and inactive state. In the inactive state, it has been noted that the structures of the catalytic domain are more diverse. When the kinase is activated, often by phosphorylation on the activation loop (Fig. 1), it adopts a shape which is able to bind ATP with the binding site open and the necessary residues correctly orientated to carry out the phosphate transfer [55, 75]. The DFG motif is a conserved sequence of three amino acids, aspartate (D), phenylalanine (F) and glycine (G) which forms part of the ATP binding site in the

active kinase. A further conserved aspartate residue in the catalytic domain may aid in the phosphate transfer by deprotonating the substrate hydroxyl group enabling it to more readily attack and remove the terminal phosphate from ATP [75]. The catalytic domain in the active conformation has been well characterised and an early pharmacophore model built to describe the ATP binding site has been successfully used for rational drug development [136] (Fig. 2). Although the ATP binding site is quite conserved, the adjacent areas not occupied by ATP itself are more variable, particularly the interior hydrophobic pocket and the solvent-exposed specificity surface. Thus, by making inhibitors that bind to these areas, more specificity may be obtained [35]. An example of a kinase inhibitor which targets these areas is dasatinib (Fig. 3) [95].

Kinase inhibitors can target the active or inactive conformations of the enzyme and may be directed towards the ATP-binding site, the substrate binding site or allosteric sites [89, 124, 143]. There are benefits and drawbacks associated with each of these strategies. One benefit of targeting the active state is that the ATP binding site is well characterised and many X-ray crystal structures are available which can aid in the design of new inhibitors. The availability of X-ray crystal structures and the rigidity and conserved structure of the ATP binding domain enable rational inhibitor design using docking and virtual screening methods [24, 113]. On the other hand, as the ATP binding site structure of active kinases is so conserved throughout the class, it may be more difficult to gain specificity for a particular kinase.

Fig. 1 X-ray crystal structure of CDK2 with ATP (black) bound with key structural motifs highlighted. Adapted from PDB: 1HCK

Fig. 2 Traxler model showing binding site for ATP in protein kinases [136]. Grey shading indicates non-conserved regions. GK gatekeeper residue

Fig. 3 X-ray crystal structure of dasatinib in Abl (Adapted from PDB: 2GQG)

For inactive kinases, although specificity is possibly easier to obtain as a greater diversity of protein conformations is possible, less is known about these conformations, so designing inhibitors becomes more of a challenge [55, 94]. Nevertheless, general pharmacophore models of inhibitor binding to inactive kinases have been developed (Fig. 4), and there are a number of kinase inhibitors which target this inactive state, such as sorafenib (Fig. 5). A key feature of the inactive kinase protein structure is the opening up of an extended hydrophobic pocket as the activation loop adopts the 'DFG-out' position and disrupts the arrangement of the catalytic residues. A version of this model has been applied to rationally design inhibitors that bind and stabilise the inactive conformation of protein kinases. Through *in silico* modelling of the catalytic site in the inactive conformation, it was proposed and subsequently confirmed that the incorporation of a large, lipophilic trifluoromethylbenzamide group at certain positions of known active conformation inhibitors

Fig. 5 X-ray crystal structure of sorafenib in b-Raf (Adapted from PDB: 1UWH)

could transform them into inhibitors binding the inactive kinase conformation [106].

One of the main problems that has emerged with targeting inactive kinases is the susceptibility to loss of inhibitor activity due to mutation. In an active kinase, a mutation preventing the binding of an inhibitor will often abolish kinase activity (by preventing ATP binding or catalysis) and, therefore, cannot be tolerated. In contrast, inhibitors bind to different residues in the inactive kinase, mutations of which are often less important for ATP binding and catalytic activity and are, therefore, better tolerated. The mutated form remains a functional enzyme, but one to which the inhibitor can no longer bind [104]. This has been demonstrated in the case of imatinib where mutations in the catalytic domain of the Abl kinase can lead to imatinib resistant chronic myeloid leukaemia (CML), a disease that is driven by the Bcr-Abl oncogene [62]. There are a number of possible mutations leading to imatinib resistance [132]. Many patients have only one single point mutation preventing drug binding and leading to relapse.

Fig. 4 Pharmacophore model of inactive kinase conformation with sorafenib bound. Adapted from [94] and PDB: 1UWH. GK gatekeeper residue

One of these mutations is T315I, which is a gatekeeper mutation preventing inhibitor access to the hydrophobic pocket and removing one of the crucial hydrogen bond donors used for imatinib binding [62, 143].

Targeting sites on protein kinases other than the ATP binding site is a means of circumventing the possible problem of competing with high cellular ATP levels. Substrate or protein partner binding sites can be targeted [22, 31], although these often involve protein–protein interactions over large surface areas which are difficult to inhibit with small molecules [80]. Some kinases also contain allosteric sites, pockets remote from the ATP binding site into which inhibitors can bind, altering the overall conformation of the catalytic domain and inhibiting the enzyme (Fig. 6) [41, 90, 134]. These may be more easily targeted by small molecules than substrate binding sites as they do not involve disrupting protein–protein interactions, but not all kinases have a known suitable allosteric site. Nonetheless, because allosteric inhibitors may avoid many of the problems associated with ATP-competitive kinase inhibition, there is a lot of interest in finding them [23]. In particular, allosteric sites offer the possibility of very highly selective or mono-specific inhibitor profiles. Proof of concept for this approach has been demonstrated by the discovery of potent and highly selective inhibitors of Akt that bind to an allosteric site located between the kinase domain and the N-terminal regulatory pleckstrin homology domain of the kinase [17, 28, 93]. The inhibitors are selective not only for Akt over other very closely homologous enzymes in the AGC sub-family but can also differentiate between the three isoforms of the kinase.

The importance of determining selectivity

Many kinase inhibitors already in clinical use to treat cancer are in fact not selective for one particular kinase but

target several [84]. This can not only bring efficacy benefits where the other kinases inhibited are also oncogenic or involved in driving cellular malignancy but it also potentially increases the risk of unwanted effects and toxicity. Of course, side effects can be due to interaction with other enzymes, ion channels or receptors as well as the inhibition of other kinases. It is important, therefore, to know what biomolecular targets drugs can interact with and what enzymes they can inhibit [59].

In recent years, it has been pointed out that there can be benefits to having selective but non-specific inhibitors as drugs [46, 71, 116]. One is that a spectrum of kinase inhibitory activities can lead to approved drugs being effective for treating diseases other than those initially targeted. Imatinib, developed and first used in the clinic as a Bcr-Abl inhibitor to treat CML, was subsequently shown to have clinical efficacy against gastrointestinal stromal tumours (GIST) due to its inhibition of c-Kit [47, 145]. Dasatinib is a dual Src/Abl kinase inhibitor which was shown to be effective against a range of tumour types. The compound also inhibits PDGFR and its activity has been attributed to its multi-targeting of tyrosine kinases [33, 95, 145].

Nilotinib was developed as a second generation agent for CML in response to the observation of drug resistance in imatinib-treated patients. The compound has activity against almost all known mutant forms of Bcr-Abl [105]. It also inhibits c-Kit and PDGFR which are oncogenic targets in a number of tumours such as GIST [43, 56]. Again, this is a multi-tyrosine kinase inhibitor and this multitargeting is a potential benefit in the clinic as the same drug can be used to treat a variety of cancers and overcome some resistance problems. The clinically approved agents sunitinib and sorafenib are also inhibitors of multiple tyrosine kinases [36, 141].

Lapatinib is a dual inhibitor of Erb-2 and EGFR [117, 123]. Overexpression of either Erb-2 or EGFR receptor tyrosine kinases is implicated in a range of cancer types,

Fig. 6 X-ray crystal structures; **a** Allosteric MEK inhibitor bound in a pocket adjacent to the ATP binding site (Adapted from PDB: 1S9J); **b** Allosteric Chk1 inhibitor bound at a site remote from ATP binding site (Adapted from PDB: 3F9N)

and overexpression of both in ovarian cancer leads to poorer prognosis. Thus, a dual inhibitor of these targets is a potentially desirable profile. Tumour growth arrest by lapatinib was shown in human tumour xenografts [142], and the compound was approved for the treatment of breast cancer in 2007. Other groups have also investigated dual Erb-2/EGFR inhibitors [119, 139].

The concept of targeting a defined range of protein kinases is now increasingly apparent in reports of preclinical drug discovery, and there are many examples of dual inhibitors in the recent literature. These sometimes target kinases in the same kinome sub-family [42] but can also be directed against two functionally related kinases from different kinome sub-families, as in the example of dual inhibitors of Chk1 and Wee1 which are both involved in the G2 cell cycle checkpoint [130]. Studies by several groups have shown that dual inhibitors of the protein kinase mTOR and the lipid kinase phosphoinositide 3-kinase (PI3K) may have better efficacy in certain cancer types compared to more specific inhibitors [5, 20, 32, 51, 67, 110, 118]. The design of dual mTOR/PI3K inhibitors addresses a potential limitation of the mTOR inhibitor rapamycin. Inhibition of mTOR by rapamycin and analogues causes a compensatory upregulation of the upstream signalling kinase Akt due to release of a negative feedback loop [108]. By simultaneously inhibiting the pathway at two points—upstream and downstream of Akt—this can be overcome. Although PI3K is a lipid kinase rather than a protein kinase, it is also involved in oncogenic pathways and contains an ATP binding site similar to the protein kinases, allowing it to be inhibited with similar compounds.

Thus, for drugs, inhibiting more than one kinase may be beneficial as long as these are therapeutically useful targets, for example involved in overactive pathways in cancer. However, knowledge and control of selectivity is still important as off-target kinase inhibition can also cause toxicity [68, 144]. For example, the inhibition of AMPK and some tyrosine kinases has been linked with cardiotoxicity through the sensitivity of cardiomyocytes to disruption of cellular energy production [57]. A number of TKs have been implicated including Abl, which has an identical kinase domain to the fusion protein Bcr-Abl that is the primary target of imatinib in CML. To overcome the cardiotoxicity of Abl inhibitors, a structure-based approach involved adding a methyl group to imatinib to prevent binding to Abl and Bcr-Abl while retaining other TK inhibitory activities [54]. As the anti-tumour activity of imatinib in some tumour types is due to inhibition of kinases other than Bcr-Abl, notably c-KIT in GIST, new compounds without the Abl activity still have the potential for efficacy in these diseases.

Measuring the selectivity and understanding the common features of the binding of unselective kinase inhibitors

may help in the design of less promiscuous inhibitors or at least allow hits from screening libraries to be prioritised to select scaffolds with greater potential for specificity [7, 8]. This approach may also be useful in the design of multi-targeted inhibitors with controlled selectivity profiles by defining chemical classes that have a consistent bias towards a clear selectivity pattern. Moreover, there is an argument that inhibitors that are to be used as pharmacological tools to understand biological systems need to be as selective as possible [3]. If a compound inhibits multiple kinases, its biological effects may not be easily attributable to specific targets, making their function more difficult to elucidate. At the least, knowledge of the kinome inhibitory profile of tool inhibitors is an important part of assessing their fitness for use as pharmacological probes [14].

Recently, it has become common practice that the inhibitory activity of new inhibitors will be measured against a panel of protein kinases [70, 109]. Many groups choose to concentrate on kinases in the same family and, therefore, with similar ATP binding site sequences [114, 148]. Another approach is to measure effects on functionally related kinases, as inhibiting more than one enzyme on the same signalling pathway may make a significant difference to the action of a drug [51]. Many others screen against a wide variety of kinases to get an overall view of selectivity [70, 109]. This wider kinome profiling was not always applied, and it is now being found that compounds which were thought to be mono-specific or highly selective inhibitors are not as selective as first believed [14].

Measuring the inhibition of substrate phosphorylation

There are many ways of measuring kinase activity, and these are reviewed regularly in the literature [97, 107]. They have varying advantages and disadvantages, and different techniques may be more suitable for a particular kinase or compound set (Fig. 7). One of the first and most widely used assays involves radiolabelled [^{32}P]- or [^{33}P]-ATP. This allows the direct detection of phosphorylation of a substrate peptide or protein by a kinase of interest [69]. Cohen et al. have pioneered screening for kinase inhibitor selectivity using panels of enzymes, often using incorporation of [^{33}P] into substrates in 96-well plate format [13, 14, 44]. Most recently they have screened 65 compounds against 70–80 protein kinases, showing that many inhibitors previously described as ‘selective’ have activity on a number of protein kinases. Some have activity on related family members and others have broader kinome-wide activity. However, many were also shown to be highly specific with respect to the panel of kinases used [14].

Fig. 7 A schematic to summarise the various methods for detecting inhibition of the phosphorylation activity of kinases relevant to selectivity profiling

While measuring incorporation of radioactive phosphate directly provides few sources of interference in the assay, there can be associated limits on the amount of radioactivity that can be used and the need for specialist disposal. As a result, non-radioactive methods are also widely used, such as fluorescent and luminescent endpoint assays, although these may have their own disadvantages [97, 121]. One is the fact that a significant number of organic compounds are coloured or fluorescent and can interfere with the results [25, 65]. Another is that many of the assay formats require specific phosphopeptide antibodies to detect the conversion of substrate. Although there is an available phosphotyrosine antibody which can be used for any phosphorylated tyrosine residue [121], it has proven much harder to find general antibodies recognising serine/threonine phosphorylation, and separate antibodies are needed for each peptide used. Thus, significant time and effort may be needed to develop antibody reagents for such assays [120].

Another more recently introduced way of directly measuring substrate phosphorylation is by using mobility shift assays. This method involves the electrophoretic separation of phosphorylated and non-phosphorylated short peptides based on their charge. The peptides are tagged with a fluorescent marker for detection [49, 76]. These assays can be carried out in 384-well plate format and have been used in high-throughput to screen 32,000 compounds [115] as well as for screening inhibitors against kinase panels. Electrophoretic separation assays have been compared with a radiometric assay format and found to be more reliable [29]. In our own research, we have used this technology to profile both potent kinase inhibitors and also to investigate the selectivity patterns of low molecular weight fragments (MW < 200) tested at high compound concentrations ([131]; Smyth et al. unpublished results).

Measuring the binding affinity of kinase inhibitors

As well as biochemical assays to measure phosphorylation, competitive binding assays can be used to measure the dissociation constant (K_d) of an inhibitor-kinase complex (Fig. 8). These generally involve an ATP mimic or competitive inhibitor which is used as a probe and is in competition with the inhibitor of interest. The K_d can then be calculated from the amount of probe bound to the kinase compared to a control (Fig. 8a). The probes are often attached to fluorescent markers in order to detect them [87]. In one case, staurosporine or close analogues have been used as probes without additional markers, since excitation of the compounds at 296 nm results in emission at 378 and 396 nm [74]. The fluorescence is enhanced on binding to the ATP binding site of the kinase, allowing a comparison between binding in wells with and without inhibitor. Although this study looked at screening compounds against a single kinase, Src, one could see the possibility of using the same probes to profile an inhibitor against a number of kinases. There was interference from some of the compounds tested, as they fluoresced at the same wavelength as the staurosporine analogues.

Ambit Biosciences have described a binding assay to determine the K_d of up to 38 kinase inhibitors against 317 protein kinases using an immobilised 'probe' ligand which binds to the ATP binding site [50]. Human kinases were expressed fused to a bacteriophage and were incubated with the immobilised probe and the inhibitor of interest (Fig. 8b). The kinases bound to either the probe ligand or the unbound test compound. After a wash step, the amount of kinase bound to the immobilised probe was determined by quantifying the immobilised bacteriophage using plaque assays or quantitative polymerase chain reaction. The binding constants obtained by this method agreed well

Fig. 8 Summary of various methods for detecting the binding of inhibitors to kinases.

a Displacement of labelled probe compounds; **b** phage display of kinase domains with competitive binding to inhibitors or immobilised probe ligands; **c** affinity chromatography using immobilised inhibitor

with both literature K_d values and the K_i or IC_{50} values from phosphorylation inhibition assays [50, 77].

Another way of assessing inhibitor binding to kinases is to use affinity chromatography (Fig. 8c). This technique has been applied to look for inhibitors of specific kinases by immobilising the enzyme onto beads and running a solution of small molecules through. By coupling this to mass spectrometry, the ligands which bind can be identified [129]. The same approach can also be used to look at inhibitor selectivity by immobilising the inhibitor and running proteins or cell lysates through the functionalised resin. Bound proteins are eluted from the affinity resin, purified by gel electrophoresis or Western blotting and identified by immunoblotting, protein staining or mass spectrometry. One limitation with immunoblot detection is

that the choice of antibodies determines which protein targets are detected. This approach was used to investigate the interactions of sunitinib, and a number of new targets were found [60]. Affinity chromatography of cell lysate, followed by electrophoretic separation of the captured proteins and their identification by mass spectrometry, was employed to determine the cellular targets of the EGFR inhibitor gefitinib [149]. Immobilised roscovitine was also studied in this way, and binding to pyridoxal kinase was discovered in addition to the expected targets [12]. Although effective for identifying targets and detecting the pattern of proteins an inhibitor can bind, one limitation with affinity chromatography of this type is the inability to immediately quantify the relative strength of binding when cell lysates are used. The relative abundance of each kinase

present may affect how much of each target is captured as well as the strength of the binding interaction. The heterogeneous affinity-based methods measure apparent K_d values and may not always reflect the K_d values in solution. However, binding affinities for different compounds can be compared; thus, the relative selectivities of multiple inhibitors can be evaluated.

A biophysical method for assessing inhibitor binding to kinases uses thermal shift data [52]. This compares the difference in the melting temperature (T_m), the temperature at which the protein unfolds, in the presence or absence of the inhibitor. The degree of denaturation is measured using a peptide-binding dye. Tight binding inhibitors stabilise the peptide in its folded conformation, raising the T_m . Using this technique, the selectivity of 156 kinase inhibitors against a panel of 60 serine/threonine kinases was assessed.

Microarrays, miniaturisation and proteomic methods

In order to improve efficiency, some of the assays described above have been miniaturised so they can be run on 1536- and 3456-well plates and used for high-throughput screening and kinase profiling [18, 120]. The main advantages to these techniques are the reduction in the amount of materials needed and the increased amount of information obtained. Even more screening can be carried out at once by using microarrays, of which there are numerous types involving the immobilisation of peptides, proteins or ligands (Fig. 9a, b). A number of types of surfaces are used, including glass, nanowell and 3D-surface structures. Immobilisation can be carried out by using high affinity interactions such as streptavidin–biotin, surface adsorption or covalent interactions. These and other considerations are covered in detailed reviews [48, 98, 146]. To determine the inhibitory profile of single compounds against multiple kinases, the most useful approach would be to use a protein array [96]. However, there are problems associated with immobilising enzymes on chips as they may denature easily and lose activity. The orientation of the bound proteins with respect to the surface is also important so that none of the binding sites are blocked or altered by the immobilisation. Different post-translational modifications may lead to proteins with varying activity so it may be necessary to make a number of forms of the protein in order to investigate the activity fully. These issues have meant that interactions are not always seen where expected, and it is advisable to use multiple techniques to confirm the activities observed [88].

Peptide substrates or test compounds have been simply arrayed on glass slides and other assay components sprayed onto the chips, avoiding some of the problems associated

with immobilised protein microarrays [99]. Potential inhibitors can be arrayed and the enzymes and fluorescence-tagged substrates subsequently sprayed onto the chip. Various detection methods are used, for example, in some cases the substrates fluoresce where they have been phosphorylated by the enzyme of interest. This method has been adapted for kinase inhibitor profiling using an immobilised tyrosine-containing peptide substrate on streptavidin slides [72]. The test inhibitors were arrayed on top of the peptide and the appropriate kinase was sprayed onto the slide. As the phosphorylated peptide was immobilised, wash steps could be included in the assay, and antibodies were used to recognise the phosphorylated tyrosine followed by a second fluorescent-labelled antibody. The technique was demonstrated using four kinases, but extension to a wider panel could be envisaged.

Patterns of kinase inhibitor selectivity can emerge as part of proteome-wide screening as well as from dedicated kinase profiling. For example, the yeast three-hybrid approach has generated new insight into the selectivity of cyclin-dependent kinase (CDK) inhibitors [19]. In this case, the compounds were covalently attached to the dihydrofolate reductase (DHFR) inhibitor methotrexate via a PEG spacer. A DNA-binding domain was fused to DHFR, and cDNA libraries were used to express large numbers of proteins fused to an activation domain (Fig. 9c). On binding to a protein, the CDK inhibitor-methotrexate conjugate brought the DNA binding and activation domains into close proximity, inducing detectable expression of the reporter gene. Interestingly, it was noted that the only targets identified for the CDK inhibitor purvalanol B were kinases, giving a wider context to the selectivity pattern. A detailed review of proteomic methods is available [83].

The development of tandem mass spectrometry (MS) techniques for the parallel quantification of the protein content of cell lysates has provided a set of analytical methods that can be adapted for kinase inhibitor selectivity profiling [147]. In particular, the isobaric tags for relative and absolute quantification (iTRAQ) reporter reagents for labelling of peptides prior to MS analysis [122] have been used in conjunction with affinity chromatography to assess the affinities of kinase inhibitors for endogenously expressed protein kinases [16]. Affinity chromatography was conducted with beads coated with a mixed population of seven, non-selective kinase inhibitors (Fig. 9d). This matrix was shown to effectively capture 183 distinct protein kinases from K562 tumour cell lysates. Three Bcr-Abl kinase inhibitors (dasatinib, imatinib, bosutinib) were added at different concentrations to the cell lysate before affinity chromatography. The bound proteins from the control and drug-treated affinity purifications were digested with trypsin and labelled with different iTRAQ reagents. The combined proteolytic digest was analysed by tandem MS to

Fig. 9 Schematic showing measurement of inhibitor selectivity using **a** a microarray of immobilised kinases; **b** a microarray of immobilised substrate peptides; **c** the yeast-3-hybrid proteomic method (*AD* activation domain, *BD* DNA binding domain); **d** tandem MS detection of altered binding of cellular kinase proteins to an affinity matrix following treatment with an inhibitor; **e** tandem MS detection of competitive binding to kinases using a reactive chemical label

detect the variation in pull-down of kinases between the drug-treated and control samples. Dose–response curves for inhibitor binding to approximately 150 kinases were generated from these data. An alternative approach used competition of inhibitors with specific chemical-labelling

probes for the kinase ATP binding site to derive modified peptides for MS analysis [111]. ADP- or ATP-bearing reactive terminal acyl groups, further substituted with a biotin group, were incubated with cell lysates (Fig. 9e). The positions of the reactive acyl group of the probes, close to

conserved lysines at least one of which is present in almost all protein kinases, led to specific transfer of the biotinylated tag to the kinase. Approximately 75–80% of the enzymes in the human, mouse and dog kinomes were effectively labelled by these acyl phosphates. After trypsin digestion, biotinylated fragment peptides were captured using streptavidin-coated beads. Identification and quantification of the labelled peptides was achieved by tandem MS. As with the iTRAQ method discussed above, comparison of the labelled peptides obtained with inhibitor treated and control lysates allowed the construction of dose–response curves to determine inhibitor-binding affinities. The selectivity profile obtained for staurosporine using this technique contained anomalies when compared to that produced by conventional biochemical or phage-display methods, with the affinities for some kinases being two orders of magnitude less than the values reported from phage-display. Differences in activation state between recombinant enzymes and endogenously expressed full-length kinases may account for some of this variation. It has been suggested that the kinases from lysates are likely to preserve important post-translational modifications and protein partner interactions that will modify the activity of the enzymes, although the significance and biological relevance of these differences remains unclear [111].

Understanding the limitations of in vitro kinase selectivity assays

Most of the assay formats described above are not conducted in cells, so how predictive are they of the selectivity of an inhibitor in a living organism? The difficulties encountered in translating in vitro biochemical selectivity data to the cellular context has been the subject of an excellent and detailed review by Knight and Shokat [81]. Encouragingly, these authors found that for several distinct chemotypes of kinase inhibitor, the biochemical affinities of a large set of compounds against three kinases did correlate with a target-specific cellular activity, for example phosphorylation of a downstream protein substrate or secretion of a protein. Nevertheless, there are numerous examples where observed cellular selectivity is not in agreement with the apparent biochemical selectivity profile of an inhibitor, some of which are described in the following section.

One factor that complicates the interpretation of in vitro kinase selectivity profiles is the co-dependence of the inhibitor potency on the intrinsic affinity of the inhibitor and the kinetics of the enzyme with respect to its cofactor, ATP. In a typical phosphorylation assay for reversible, ATP-competitive inhibitors, the inhibitor po-

tency is expressed as an IC_{50} value. This is a function of the intrinsic affinity of the inhibitor for the kinase (dissociation constant K_i) and the degree of competition from ATP. The latter varies with the concentration of ATP in the assay ($[ATP]$) and the affinity of the kinase for ATP (expressed as $K_{m,ATP}$), as described by the Cheng–Prusoff equation [34].

$$IC_{50} = K_i(1 + [ATP]/K_{m,ATP}) \quad (1)$$

When $[ATP]$ is at or below the $K_{m,ATP}$ then measured $IC_{50} \sim K_i$. Kinase selectivity profiling is often carried out with $[ATP]$ set to the approximate $K_{m,ATP}$ for each kinase tested so that the resulting selectivity profile directly reflects the intrinsic affinities of the inhibitors. The effect on the selectivity profile of testing compounds in phosphorylation assays at different fixed ATP concentrations rather than matching $[ATP]$ to $K_{m,ATP}$ has been investigated [63]. It was found that ATP concentrations of 10 and 100 μM made little difference to the profile.

In cells, the $[ATP]$ is generally much higher than $K_{m,ATP}$ and under these conditions, differences in $K_{m,ATP}$ between the enzymes can dominate over the intrinsic biochemical affinity in determining the inhibitor selectivity. Many protein kinases have $K_{m,ATP}$ in the range of 10–100 μM [81], and for this group, there is usually a correlation of changes in biochemical affinity with potency in cells, i.e. there is a reasonable expectation that selectivity patterns in vitro can manifest in cells. However, there are also significant outliers with very high or low values for $K_{m,ATP}$. An inhibitor with apparently equivalent intrinsic affinity for two kinases in a biochemical assay will inhibit one kinase more potently in cells if its $K_{m,ATP}$ is significantly higher. The interplay of inhibitor K_i , kinase $K_{m,ATP}$ and the concentration of ATP is illustrated in the hypothetical example in Table 2 for an inhibitor with an intrinsic affinity $K_i = 10$ nM tested against three kinases of increasing $K_{m,ATP}$ under conditions of increasing $[ATP]$. Such analysis can be used to estimate the approximate concentrations at which, for example, 50% occupancy of

Table 2 Idealised values for the potency (IC_{50}) of an inhibitor with intrinsic affinity $K_i=10$ nM in an ATP-competitive assay for three kinases A–C with varying $K_{m,ATP}$ at increasing concentrations of ATP

Kinase enzyme	$K_{m,ATP}$ (μM)	[ATP]=3 μM Inhibitor IC_{50} (nM) if inhibitor $K_i=10$ nM	[ATP]=30 μM	[ATP]=1 mM
A	3	20	110	3,340
B	30	11	20	340
C	300	10	11	43

Values calculated using the Cheng–Prusoff equation. $[ATP]$ in cells is typically estimated at 1–5 mM

the kinase binding site in cells should be achieved with a given inhibitor [6, 27]. However, it is important to note that this assumes no limits to cell permeability of the compound, which may further reduce the potency achieved in cellular assays. Moreover, although the occupancy of the active site may reflect engagement of the inhibitor with the target enzyme as measured, for example, in a binding assay or a direct measurement of an immediate downstream phosphorylation by the kinase, it does not guarantee the same response in a broader phenotypic assay, e.g. growth inhibition, which depends also on the sensitivity to perturbation of the signalling network in which the kinase participates [27].

Not all kinases can be used in biochemical assays in their full-length form and may require removal of transmembrane or regulatory domains. The partial protein constructs or isolated kinase domains available for in vitro experiments may have different activity from the native enzymes, i.e. different $K_{m,ATP}$ which will be reflected in the selectivity profile. The differences between protein constructs may be particularly apparent when assaying allosteric kinase inhibitors. For example, potent allosteric inhibitors of Akt require the presence of the pleckstrin homology (PH) domain of the kinase to show inhibitory activity [17] and will not inhibit phosphorylation by the isolated kinase domain of the protein. The inhibitor binding site has been shown to be located between the kinase and PH domains [28].

A further complication arises when the selectivity profiles of inhibitors that bind and stabilise inactive kinase conformations are considered. Phosphorylation assays depend on the presence of active kinase in the assay to generate a detectable phosphorylated substrate. If there is a dynamic equilibrium between active and inactive conformations of a kinase under the conditions of the assay, then inhibitors that only bind and stabilise the inactive conformation may still be detectable, since stabilisation of the inactive form depletes the amount of active kinase and inhibits the phosphorylation reaction. However, the interconversion of active and inactive conformations of kinases in vivo is more complex, being actively regulated by other enzymes, i.e. other kinases and phosphatases or binding partners. In these circumstances, competitive binding assays to unactivated forms of the kinase may be a more appropriate way to assess the inhibitor affinities.

The in vitro profiling methods have advantages of consistency and scale, allowing many compounds and many kinases to be screened in as similar conditions as possible. Decisions on whether to use a binding assay or measure phosphorylation, whether to use colorimetric, fluorescent, radiometric or luminescence endpoints and what format to use are all important. It has been noted that different assay formats can give different results, so one

approach is to carry out a number of assay types in parallel to look for consistent patterns [78, 107, 128].

Overall, in vitro selectivity assays can give insight into a fundamental property of the small molecule inhibitors: their intrinsic affinities for the different biological targets. The possibility of any targeted pharmacological effect is contingent on an interaction at the molecular level. The kinase selectivity patterns of compounds do give useful insight into the possible interactions an inhibitor can participate in and how that varies with structure. However, the biological significance of any selectivity pattern must be interpreted in the context of the cellular and tissue biology of the potential targets.

Kinase selectivity profiling in cells

There have been a number of cellular assays developed in order to address the limitations of in vitro kinase profiling. Fluorescence resonance energy transfer (FRET) assays give a fluorescent signal when a donor and acceptor fluorophore are in close proximity, as happens when a phosphorylated peptide attached to one fluorophore is recognised by a phosphospecific antibody attached to the other. Recent improvements in the sensitivity of the assay by introducing metal-chelating fluorophores, unnatural amino acids incorporating fluorophores and engineering the peptide substrate have not only allowed FRET to be used in cells but also enabled more than one protein to be monitored at once [4, 137]. This has been developed for the high-throughput screening of compounds against kinases in a 96-well plate format using HEK-293 (human) cells. Although different reporters are needed for different kinases, many have already been developed, and one could see this approach being used to look at kinase selectivity in cells [2].

A further approach describes the use of yeast cells with individually engineered kinases being used to map a transcriptional profile resulting from selective inhibition in the cell [85]. The kinases are expressed with a mutation in the ATP binding site that renders them sensitive to an ATP analogue that is usually too large to bind and, thus, has no effect on the wild-type enzymes. The effect of specific inhibition of each kinase in the cell can, therefore, be observed separately on gene expression microarrays. The cellular targets of a novel small molecule inhibitor in cells can be inferred from comparing the observed transcription profile with that due to inhibition of the single mutated kinases. It has been pointed out that although many targets can be identified in theory, the data may be difficult to interpret when many kinases are being inhibited, and the technique may be most useful for relatively selective compounds [11].

A cellular assay which can be used for high-throughput screening in 1,536-well format has been described [103]. To evaluate the assay in comparison to biochemical assays, 35 tyrosine kinases were screened against about 1,400 inhibitors. A library was generated containing an engineered panel of 35 Ba/F3 mouse cell lines each having a tyrosine kinase domain fused to a constitutively activating receptor/dimerisation domain. Compounds were cytotoxic if they inhibited the particular TK expressed in that cell line, enabling quantification of the inhibitory activity. Comparison to in vitro enzymatic assays showed very variable correlation. Many compounds which were active in cells showed activity in the biochemical assay. However, there were also instances where compounds which had nanomolar activity in the cellular assay only partially inhibited the relevant kinase at 10 μ M in the biochemical assay. Some of these discrepancies were attributed to inhibitors targeting inactive kinase conformations which would only be present in low abundance in the biochemical assay.

An example where kinase selectivity in vitro was shown to translate to the cellular context was seen with ATP-competitive inhibitors of Akt [92]. The inhibitor **1** (Fig. 10a) was 30-fold selective for PKA over Akt in a biochemical assay. The selectivity in cells was probed by monitoring the specific, and different, downstream effects of inhibiting these enzymes, where a similar selectivity for inhibition of PKA over Akt was observed. It is noteworthy that the phenotypic effects of inhibition of different kinases may require different assay formats for detection. Another example illustrates the case where in vitro selectivity does not translate to an equivalent cellular selectivity [126]. A differential in sensitivity was observed in cells with an inhibitor with no intrinsic difference in affinity for two kinases. The CDK1 inhibitor BMI-1026 (Fig. 10b) showed similar potency against CDK1 and CDK2 (8 and 2 nM IC_{50} s, respectively) in biochemical assays. However, on treating cells at 40 nM, only CDK1-dependent cell cycle arrest could be seen. Although at higher concentrations, CDK2-dependent cell cycle arrest was also observed, the

results suggested that this cell line was inherently more sensitive to CDK1 inhibition. Compound **2** (Fig. 10c) also illustrates a case where in vitro selectivity and cellular selectivity do not correlate [86]. Here, biochemical assays showed **2** to be 20-fold selective for inhibition of CDK1 over Pho85. However, in cells, only an effect due to inhibition of Pho85 was observed. This was attributed to a differential sensitivity between CDK1 and Pho85 to inhibition.

Although the intrinsic affinities of inhibitors may often be reflected in their phenotypes in cellular systems, it can also be seen that the selectivity of compounds may change between biochemical and cellular assays. This could result from differences in $K_{m,ATP}$ of the kinases as discussed above, differences in the abundance of the kinases in the cells or differences in the sensitivity of the cells to inhibition of particular pathways. This last factor may enhance the cellular selectivity of a small molecule if the intrinsic inhibitor affinities and the inherent pathway sensitivities align. In interpreting the in vitro data, it might be necessary to attain a large threshold, perhaps 1,000- or 10,000-fold selective, in order to be assured that the in vitro kinase selectivity will dominate in the cellular context. For this reason, it is important to test compounds in relevant cellular assays, although biochemical kinase profiling remains a useful and tractable first step in looking broadly at the potential activities and selectivity of compounds across the kinome.

There are additional factors to consider when moving from cells into living organisms that will affect the translation of a biochemical or cellular selectivity profile into physiological effects. Differential distribution of the drug to tissues, coupled with the possibility of differential expression of kinases between tissues, will modify the significance of the apparent selectivity profile. For example, although Sorafenib was rationally designed to inhibit Raf kinase, and has good potency on this target, it also inhibits a number of other kinases, including many of the growth factor receptor tyrosine kinases which are involved in angiogenesis. Some of the clinical efficacy of the drug can be ascribed to inhibition of these kinases in normal

Fig. 10 Examples of correlation (a) and non-correlation (b, c) of biochemical and cellular inhibitor selectivity between two kinases

	Compound 1		BMI-1026		Compound 2	
Target	AKT	PKA	CDK1	CDK2	CDK1	Pho85
Biochemical IC_{50}	3–23nM	0.1nM	8.3nM	2.3nM	20nM	400nM
Specific Cellular Response	330–1000nM	10–33nM	40nM	80–100nM	>50,000nM	400nM

blood vessel epithelia, preventing development of new blood supply from surrounding tissue to the tumour, in addition to the inhibition of enzymes in the tumour cells themselves [140].

Analysing and quantifying kinase selectivity

As has been described, there are a number of ways of measuring the inhibitory activity of a compound against a number of kinases, but how can this be analysed and quantified? This is a complex question which has been addressed in various ways. Tabulation of results allows all data to be seen. This is a great benefit but does not readily give a simple answer to the question, ‘which inhibitor is the most specific?’. Other ways of looking at the results include plotting them onto a kinase dendrogram [100], which is particularly useful for looking at the pattern of inhibition, but does not always differentiate between those kinases against which the inhibitor was shown to be inactive and those against which it was not tested. It is also possible that the kinome dendrogram, which delineates the similarity of the amino acid sequences, does not always show the kinases grouped in the most relevant way for chemical biology and drug discovery. It has been noted that although inhibitors often only inhibit kinases in the same family, other compounds may show some activity in many different families of kinases [77]. Although the total amino acid sequences may be similar in two kinases, they may fold to form different three-dimensional structures at the ATP binding site. Conversely, two kinases with quite different amino acid sequences from different families may be more similar at the ATP site and form a very similar shaped pocket. Thus in chemogenomics, the structure–activity relationships (SAR) of inhibitors and their relationship to the identity of important amino acid residues which form the ATP binding site are considered when defining the determinants of inhibitor specificity [66, 79]. In one such study, SAR were used to redesign the traditional Sugen kinome dendrogram [100]. It was noted that many of the kinases which are closely related in the phylogenetic dendrogram remain clustered in the SAR similarity dendrogram, but there were also some significant differences [15].

Researchers at Ambit Biosciences have suggested one way of quantifying the selectivity, the selectivity score (S) [77]. This is calculated by dividing the number of kinases for which the inhibitor binds with a K_d lower than a specific value (e.g. 3 μM) by the number of kinases tested (generating an $S_{(3\ \mu\text{M})}$, for example). A value of $S = 1$ indicates a completely unselective inhibitor and values closer to zero characterise more selective compounds. This of course can be used to calculate an S value at any concentration and using either IC_{50} or single point

percentage inhibition data. The same authors also looked at the number and distribution of kinases that should be screened for a good estimation of selectivity across the kinome. Their results suggested that where smaller panels were used, having a representative number from each family was better than selecting the kinases completely randomly. They also noted that the smaller the panel of kinases, the larger the variation in S .

Another measure of selectivity is the Gini co-efficient (G) [63]. This is a more complex calculation and involves ranking the inhibition potencies and plotting the Lorenz curve (cumulative fraction of total inhibition against cumulative fraction of kinases). The more this curve is displaced from the straight diagonal, which corresponds to all kinases being inhibited to the same degree, the more selective the compound is. The displacement is represented by the coefficient G , where a value of $G = 0$ indicates a completely unselective compound and inhibitor specificity increases as G approaches unity. The effect of variation of the size of the kinase panel on the Gini coefficient was studied and larger panels were preferred as the standard deviation in the coefficient was lower. A panel of at least 50 kinases was suggested as a minimum.

These calculations give an overall idea of specificity, the degree to which activity is concentrated against one defined target, but they do not take into account the pattern of selectivity of a compound for a family or subset of kinases. In the example shown in Fig. 11, three low molecular weight compounds based on the same chemical scaffold were tested against a limited panel of 24 kinases at a concentration of 30 μM ([131]; Smyth et al. unpublished results). Comparison of the Gini coefficients and S values for **3** and **4** indicates that both score **3** as having greater specificity than **4**, i.e. the total inhibitory activity observed is concentrated in fewer enzymes for **3** than for **4**. It can be seen from the dendrogram plots that **3** appears to be selective for a set of tyrosine kinases while **4** inhibits mainly AGC and CK1 sub-family members. The dendrogram of compound **5** shows an apparently higher specificity, with inhibition of one kinase in the CK1 subfamily dominating the pattern. The S value reflects this, being considerably lower than for either **3** or **4**. However, the Gini coefficient does not indicate a better specificity than compound **3**. This highlights the difference in the mathematical techniques between scoring inhibition relative to a single cut-off or using a cumulative approach. In this example, both coefficients are calculated using single concentration, percentage inhibition data. For the S value, $S_{(40\%)}$ was scored relative to 40% inhibition at 30 μM of the inhibitor, with activity below this threshold not counting as a hit. This approach is mirrored in the dendrogram plots presented here where threshold levels are used. In calculating the Gini coefficient the percentage inhibition at all the

Fig. 11 Selectivity profiles of three low molecular weight compounds (3, 4, 5) from one chemical series and their corresponding Gini coefficients (G) and $S_{(40\%)}$ values. Percentages shown are percentage inhibition at 30 μ M. The kinase dendrogram was adapted and

reproduced with permission from Cell Signaling Technology, Inc. (www.cellsignal.com). With sponsorship by Cell Signaling Technology and Sugen, the figure was originally presented as a poster in Science to accompany the first analysis of the complete human kinome [100]

kinases tested is incorporated, including the many for which low inhibition is measured. From the point of view of achieving controlled polypharmacology profiles in new kinase inhibitors, both considerations of the degree *and* the pattern of selectivity are important. Selectivity patterns such as those exhibited by 3 and 4, if repeated in a larger panel of kinases, and if constant within a series of structural analogues as potency was increased, might represent useful starting points for developing inhibitors with defined and qualitatively different polypharmacology.

Predicting specificity and selectivity

As kinase screening becomes more common, there have been many different approaches suggested for improving specificity and selectivity. The multiplexing of high-throughput screening so that compound libraries are screened against many kinases in parallel has been proposed as an alternative to single target-centric discovery of new starting points for drug discovery [61]. The availability of the miniaturised and high-throughput methods discussed earlier makes this a realistic proposition. Researchers at Vertex

proposed a pharmacophore for kinase ‘frequent hitters’ which involved comparing the structures of inhibitors shown to have activity on a set of five protein kinases [9]. The pharmacophore is suggested as a filter to remove promiscuous inhibitors from screening libraries or as the start-point for new medicinal chemistry projects. The ‘2–0 rule’ has also been introduced to help identify likely kinase inhibitors [8]. The ‘2–0 rule’ states that a compound is likely to have kinase activity if it contains: two or more heteroaromatic nitrogens, one or more heteroaromatic NH groups, one or more aniline and one or more nitriles. This can be used to avoid common motifs or to identify potential new kinase inhibitor scaffolds, with a 5-fold enrichment in the probability of identifying a kinase inhibitor motif found in the test set.

X-ray crystallography data are particularly valuable in examining in detail the differences between the ATP binding site in active and inactive conformations or between different kinases [91, 101]. Where co-crystal structures of kinases with various compounds from the same chemical class are solved, these can be used to improve the specificity by structure-based design [52, 53]. For example, a single amino acid difference was found to be the major determinant of selectivity for Akt versus PKA

in two series of Akt inhibitors [27, 45]. A library of fragments has been screened against 10 kinases to investigate if these very low molecular weight compounds show intrinsic specificity [1]. It was noted that even the fragments had some selectivity pattern, inhibiting only one or two kinases at test concentrations of 1–100 μ M. The fragments were elaborated using X-ray crystallography as one of the main tools to make decisions about where to add new functional groups. When the fragments were grown to these larger inhibitors, sub-micromolar inhibitory activity was seen on all kinases tested, but most compounds only hit one or two kinases very potently.

Other ways of predicting possible kinase targets for compounds are to compare the amino acid sequences of the catalytic sites or particular residues within them [127]. One very important residue in distinguishing between inhibitors is the gatekeeper residue. This is found at the entrance to the hydrophobic pocket. If the residue is small, the hydrophobic pocket is accessible, but larger gatekeepers block this pocket and stop inhibitors which occupy this part of the catalytic domain from binding. For example, compound **3** (Fig. 11) inhibited five out of seven tyrosine kinases tested. The five kinases, in which compound **3** was active, all had a serine gatekeeper. The two kinases which were not inhibited at the test concentration had larger methionine and leucine gatekeeper residues, suggesting a possible rationale for the observed selectivity.

In addition to looking at the kinase structure, the available structure–activity data can be used to look for kinases that are inhibited by structurally similar compounds [102, 125]. The distances between key features in the kinases and in possible inhibitors can be compared [138]. These and other similar *in silico* methods are part of what has become known as ‘chemogenomics’, ‘the discovery and description of all possible drugs for all possible drug targets’ [30]. Several groups have looked at using a selection of these methods in order to predict which kinases will be inhibited by a compound. Some good correlations between calculated and experimental data were found as long as the compounds used for training were sufficiently structurally similar to those subsequently tested [21, 26, 66]. An analysis of the binding modes of many kinase inhibitors, as determined by X-ray crystallography, has suggested simple rules-of-thumb for predicting the orientation of typical ATP-competitive inhibitor scaffolds within the binding site [58].

Conclusions

There have been great advances over the last decade in measuring and predicting kinase selectivity and obtaining more selective kinase inhibitors. Multiple methods are

available for profiling the activity of kinase inhibitors *in vitro* and in cells. *In vitro* methods give insight into the intrinsic pattern of affinities of small molecules for their enzymic targets. However, these intrinsic selectivity patterns may be modified when translated to cells. To obtain a good understanding of the selectivity profile, multiple screens should be run in both enzymatic and cellular formats. It is already apparent that the treatment of disease could benefit from developing drugs with a controlled kinase polypharmacology. Measuring, understanding and predicting the selectivity of small molecule kinase inhibitors will be essential in the development of such therapies.

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References

1. Akritopoulou-Zanze I, Hajduk PJ (2009) Kinase-targeted libraries: The design and synthesis of novel, potent, and selective kinase inhibitors. *Drug Discov Today* 14(5–6):291–297
2. Allen MD, DiPilato LM, Rahdar M, Ren YR, Chong C, Liu JO, Zhang J (2006) Reading dynamic kinase activity in living cells for high-throughput screening. *ACS Chem Biol* 1(6):371–376
3. Andersen CB, Wan Y, Chang JW, Riggs B, Lee C, Liu Y, Sessa F, Villa F, Kwiatkowski N, Suzuki M, Nallan L, Heald R, Musacchio A, Gray NS (2008) Discovery of selective aminothiazole Aurora kinase inhibitors. *ACS Chem Biol* 3(3):180–192
4. Aoki K, Kiyokawa E, Nakamura T, Matsuda M (2008) Visualization of growth signal transduction cascades in living cells with genetically encoded probes based on Förster resonance energy transfer. *Philos Trans R Soc B* 363(1500):2143–2151
5. Apsel B, Blair JA, Gonzalez B, Nazif TM, Feldman ME, Aizenstein B, Hoffman R, Williams RL, Shokat KM, Knight ZA (2008) Targeted polypharmacology: discovery of dual inhibitors of tyrosine and phosphoinositide kinases. *Nat Chem Biol* 4(11):691–699
6. Arienti KL, Brunmark A, Axe FU, McClure K, Lee A, Blevitt J, Neff DK, Huang L, Crawford S, Pandit CR, Karlsson L, Breitenbucher JG (2005) Checkpoint kinase inhibitors: SAR and radioprotective properties of a series of 2-arylbenzimidazoles. *J Med Chem* 48:1873–1885
7. Aronov AM, Bemis GW (2004) A minimalist approach to fragment-based ligand design using common rings and linkers: application to kinase inhibitors. *Proteins* 57(1):36–50

8. Aronov AM, McClain B, Stuver Moody C, Murcko MA (2008) Kinase-likeness and kinase-privileged fragments: toward virtual polypharmacology. *J Med Chem* 51(5):1214–1222
9. Aronov AM, Murcko MA (2004) Toward a pharmacophore for kinase frequent hitters. *J Med Chem* 47(23):5616–5619
10. Arslan MA, Kutuk O, Basaga H (2006) Protein kinases as drug targets in cancer. *Curr Cancer Drug Targets* 6(7):623–634
11. Bach S, Blondel M, Meijer L (2007) Evaluation of CDK inhibitor selectivity: from affinity chromatography to yeast genetics. In: Smith P, Yue E (eds) *Inhibitors of Cyclin-Dependent Kinases as Anti-Tumor Agents*. CRC, New York, pp 103–119
12. Bach S, Knockaert M, Reinhardt J, Lozach O, Schmitt S, Baratte B, Koken M, Coburn SP, Tang L, Jiang T, D-C L, Galons H, Dierick J-F, Pinna LA, Meggio F, Totzke F, Schaechtele C, Lerman AS, Carnero A, Wan Y, Gray N, Meijer L (2005) Roscovitine targets, protein kinases and pyridoxal kinase. *J Biol Chem* 280(35):31208–31219
13. Bain J, McLauchlan H, Elliott M, Cohen P (2003) The specificities of protein kinase inhibitors: an update. *Biochem J* 371(1):199–204
14. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevornic I, Arthur JSC, Alessi DR, Cohen P (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408(3):297–315
15. Bambrorough P, Drewry D, Harper G, Smith GK, Schneider K (2008) Assessment of chemical coverage of kinome space and its implications for kinase drug discovery. *J Med Chem* 51(24):7898–7914
16. Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, Mathieson T, Perrin J, Raida M, Rau C, Reader V, Sweetman G, Bauer A, Bouwmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol* 25(9):1035–1044
17. Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, Kahana JA, Kral AM, Leander K, Lee LL, Malinowski J, McAvoy EM, Nahas DD, Robinson RG, Huber HE (2005) Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem J* 385(2):399–408
18. Beasley JR, McCoy PM, Walker TL, Dunn DA (2004) Miniaturized, ultra-high throughput screening of tyrosine kinases using homogeneous, competitive fluorescence immunoassays. *Assay Drug Dev Technol* 2(2):141–151
19. Becker F, Murthi K, Smith C, Come J, Costa-Roldan N, Kaufmann C, Hanke U, Degenhart C, Baumann S, Wallner W, Huber A, Dedier S, Dill S, Kinsman D, Hediger M, Bockovich N, Meier-Ewert S, Kluge AF, Kley N (2004) A three-hybrid approach to scanning the proteome for targets of small molecule kinase inhibitors. *Chem Biol* 11(2):211–223
20. Bilanges B, Torbett N, Vanhaesebroeck B (2008) Killing two kinase families with one stone. *Nat Chem Biol* 4(11):648–649
21. Birault V, Harris CJ, Le J, Lipkin M, Nerella R, Stevens A (2006) Bringing kinases into focus: efficient drug design through the use of chemogenomic toolkits. *Curr Med Chem* 13(15):1735–1748
22. Blum G, Gazit A, Levitzki A (2000) Substrate competitive inhibitors of IGF-1 receptor kinase. *Biochemistry* 39(51):15705–15712
23. Bogoyevitch MA, Fairlie DP (2007) A new paradigm for protein kinase inhibition: blocking phosphorylation without directly targeting ATP binding. *Drug Discov Today* 12(15&16):622–633
24. Breda A, Basso LA, Santos DS, de Azevedo WF Jr (2008) Virtual screening of drugs: score functions, docking, and drug design. *Curr Comput-Aided Drug Des* 4(4):265–272
25. Burns S, Travers J, Collins I, Rowlands MG, Newbatt Y, Thompson N, Garrett MD, Workman P, Aherne W (2006) Identification of small-molecule inhibitors of protein kinase B (PKB/AKT) in an AlphaScreen high-throughput screen. *J Biomol Screen* 11(7):822–827
26. Caffrey DR, Lunney EA, Moshinsky DJ (2008) Prediction of specificity-determining residues for small-molecule kinase inhibitors. *BMC Bioinf* 9
27. Caldwell JJ, Davies TG, Donald A, McHardy T, Rowlands MG, Aherne GW, Hunter LK, Taylor K, Ruddie R, Raynaud FI, Verdonk M, Workman P, Garrett MD, Collins I (2008) Identification of 4-(4-aminopiperidin-1-yl)-7H-pyrrolo[2, 3-d] pyrimidines as selective inhibitors of protein kinase B through fragment elaboration. *J Med Chem* 51(7):2147–2157
28. Calleja V, Laguerre M, Parker Peter J, Larjani B (2009) Role of a novel PH-kinase domain interface in PKB/Akt regulation: structural mechanism for allosteric inhibition. *PLoS Biol* 7(1): e17
29. Card A, Caldwell C, Min H, Lokchander B, Hualin X, Sciabola S, Kamath Ajith V, Clugston Susan L, Tschantz William R, Leyu W, Moshinsky Deborah J (2009) High-throughput biochemical kinase selectivity assays: panel development and screening applications. *J Biomol Screen* 14(1):31–42
30. Caron PR, Mullican MD, Mashal RD, Wilson KP, Su MS, Murcko MA (2001) Chemogenomic approaches to drug discovery. *Curr Opin Chem Biol* 5(4):464–470
31. Castaneda G, Clark K, Wang S, Tsui V, Wong M, Nicholas J, Wickramasinghe D, Marsters JJC, Sutherlin D (2006) CDK2/cyclinA inhibitors: targeting the cyclinA recruitment site with small molecules derived from peptide leads. *Bioorg Med Chem Lett* 16(6):1716–1720
32. Chaisuparat R, Hu J, Jham BC, Knight ZA, Shokat KM, Montaner S (2008) Dual inhibition of PI3K alpha and mTOR as an alternative treatment for Kaposi's Sarcoma. *Cancer Res* 68(20):8361–8368
33. Chen Z, Lee FY, Bhalla KN, Wu J (2006) Potent inhibition of platelet-derived growth factor-induced responses in vascular smooth muscle cells by BMS-354825 (dasatinib). *Mol Pharmacol* 69(5):1527–1533
34. Cheng Y-C, Prusoff WH (1973) Relation between the inhibition constant (K_i) and the concentration of inhibitor which causes fifty per cent inhibition (I₅₀) of an enzymic reaction. *Biochem Pharmacol* 22(23):3099–3108
35. Cherry M, Williams DH (2004) Recent kinase and kinase inhibitor x-ray structures: mechanisms of inhibition and selectivity insights. *Curr Med Chem* 11(6):663–673
36. Christensen JG (2007) A preclinical review of sunitinib, a multitargeted receptor tyrosine kinase inhibitor with anti-angiogenic and antitumour activities. *Ann Oncol* 18(Suppl 10): x3–10
37. Cohen P (2002) Timeline: protein kinases - the major drug targets of the twenty-first century? *Nat Rev Drug Discov* 1(4):309–315
38. Cohen P (2009) Targeting protein kinases for the development of anti-inflammatory drugs. *Curr Opin Cell Biol* 21(2):317–324
39. Collins I, Garrett MD (2005) Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. *Curr Opin Pharmacol* 5(4):366–373
40. Collins I, Workman P (2006) Design and development of signal transduction inhibitors for cancer treatment: experience and challenges with kinase targets. *Curr Signal Transduction Ther* 1(1):13–23

41. Converso A, Hartingh T, Garbaccio RM, Tasber E, Rickert K, Fraley ME, Yan Y, Kreatsoulas C, Stirdivant S, Drakas B, Walsh ES, Hamilton K, Buser CA, Mao X, Abrams MT, Beck SC, Tao W, Lobell R, Sepp-Lorenzino L, Zugay-Murphy J, Sardana V, Munshi SK, Jezequel-Sur SM, Zuck PD, Hartman GD (2009) Development of thioquinazolinones, allosteric Chk1 kinase inhibitors. *Bioorg Med Chem Lett* 19(4):1240–1244
42. Dandu R, Zulli AL, Bacon ER, Underiner T, Robinson C, Chang H, Miknyoczki S, Grobelny J, Ruggeri BA, Yang S, Albom MS, Angeles TS, Aimone LD, Hudkins RL (2008) Design and synthesis of dihydroindazolo[5, 4-a]pyrrolo[3, 4-c]carbazole oximes as potent dual inhibitors of TIE-2 and VEGF-R2 receptor tyrosine kinases. *Bioorg Med Chem Lett* 18(6):1916–1921
43. Davies SL, Bolos J, Serradell N, Bayes M (2007) Nilotinib. *Drugs Future* 32(1):17–25
44. Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351(1):95–105
45. Davies TG, Verdonk ML, Graham B, Saalau-Bethell S, Hamlett CCF, McHardy T, Collins I, Garrett MD, Workman P, Woodhead SJ, Jhoti H, Barford D (2007) A structural comparison of inhibitor binding to PKB, PKA and PKA-PKB Chimera. *J Mol Biol* 367(3):882–894
46. de Jonge MJA, Verweij J (2006) Multiple targeted tyrosine kinase inhibition in the clinic: all for one or one for all? *Eur J Cancer* 42(10):1351–1356
47. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CDM, Joensuu H (2002) Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 347(7):472–480
48. Diaz-Mochon JJ, Tourniaire G, Bradley M (2007) Microarray platforms for enzymatic and cell-based assays. *Chem Soc Rev* 36(3):449–457
49. Dunne J, Reardon H, Trinh V, Li E, Farinas J (2004) Comparison of on-chip and off-chip microfluidic kinase assay formats. *Assay Drug Dev Technol* 2(2):121–129
50. Fabian MA, Biggs WH, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA, Ciceri P, Edeen PT, Floyd M, Ford JM, Galvin M, Gerlach JL, Grotzfeld RM, Herrgard S, Insko DE, Insko MA, Lai AG, Lelias J-M, Mehta SA, Milanov ZV, Velasco AM, Wodicka LM, Patel HK, Zarrinkar PP, Lockhart DJ (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol* 23(3):329–336
51. Fan Q-W, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D, Shokat KM, Weiss WA (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 9(5):341–349
52. Fedorov O, Marsden B, Pogacic V, Rellos P, Mueller S, Bullock AN, Schwaller J, Sundstroem M, Knapp S (2007) A systematic interaction map of validated kinase inhibitors with Ser/Thr kinases. *Proc Natl Acad Sci U S A* 104(51):20523–20528
53. Fedorov O, Sundstroem M, Marsden B, Knapp S (2007) Insights for the development of specific kinase inhibitors by targeted structural genomics. *Drug Discov Today* 12(9&10):365–372
54. Fernandez A, Sanguino A, Peng Z, Ozturk E, Chen J, Crespo A, Wulf S, Shavrin A, Qin C, Ma J, Trent J, Lin Y, Han H-D, Mangala LS, Bankson JA, Gelovani J, Samarel A, Bornmann W, Sood AK, Lopez-Berestein G (2007) An anticancer C-Kit kinase inhibitor is reengineered to make it more active and less cardiotoxic. *J Clin Invest* 117(12):4044–4054
55. Fischer PM (2004) The design of drug candidate molecules as selective inhibitors of therapeutically relevant protein kinases. *Curr Med Chem* 11(12):1563–1583
56. Fletcher JA, Rubin BP (2007) KIT Mutations in GIST. *Curr Opin Genet Dev* 17(1):3–7
57. Force T, Krause DS, Van Etten RA (2007) Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition. *Nat Rev Cancer* 7(5):332–344
58. Ghose AK, Herbertz T, Pippin DA, Salvino JM, Mallamo JP (2008) Knowledge based prediction of ligand binding modes and rational inhibitor design for kinase drug discovery. *J Med Chem* 51(17):5149–5171
59. Gleeson MP (2008) Generation of a set of simple, interpretable ADMET rules of thumb. *J Med Chem* 51(4):817–834
60. Godl K, Gruss OJ, Eickhoff J, Wissing J, Blencke S, Weber M, Degen H, Brehmer D, Orfi L, Horvath Z, Keri G, Mueller S, Cotten M, Ullrich A, Daub H (2005) Proteomic characterization of the angiogenesis inhibitor SU6668 reveals multiple impacts on cellular kinase signaling. *Cancer Res* 65(15):6919–6926
61. Goldstein DM, Gray NS, Zarrinkar PP (2008) High-throughput kinase profiling as a platform for drug discovery. *Nat Rev Drug Discov* 7(5):391–397
62. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 293(5531):876–880
63. Graczyk PP (2007) Gini Coefficient: a new way to express selectivity of kinase inhibitors against a family of kinases. *J Med Chem* 50(23):5773–5779
64. Gribble FM, Loussouarn G, Tucker SJ, Zhao C, Nichols CG, Ashcroft FM (2000) A novel method for measurement of submembrane ATP concentration. *J Biol Chem* 275(39):30046–30049
65. Gribbon P, Sewing A (2003) Fluorescence readouts in HTS: no gain without pain? *Drug Discov Today* 8(22):1035–1043
66. Harris CJ, Stevens AP (2006) Chemogenomics: structuring the drug discovery process to gene families. *Drug Discov Today* 11(19–20):880–888
67. Guillard S, Clarke PA, Te Poele R, Mohri Z, Bjerke L, Valenti M, Raynaud F, Eccles SA, Workman P (2009) Molecular pharmacology of phosphatidylinositol 3-kinase inhibition in human glioma. *Cell Cycle* 8(3):443–453
68. Hasinoff BB, Patel D, O'Hara KA (2008) Mechanisms of myocyte cytotoxicity induced by the multiple receptor tyrosine kinase inhibitor sunitinib. *Mol Pharmacol* 74(6):1722–1728
69. Hastie CJ, McLauchlan Hilary J, Cohen P (2006) Assay of protein kinases using radiolabeled ATP: a protocol. *Nat Protoc* 1(2):968–971
70. Hill RJ, Dabbagh K, Phippard D, Li C, Suttman RT, Welch M, Papp E, Song KW, K-C C, Leaffer D, Kim Y-N, Roberts RT, Zabka TS, Aud D, Dal Porto J, Manning AM, Peng SL, Goldstein DM, Wong BR (2008) Pamapimod, a novel p38 mitogen-activated protein kinase inhibitor: preclinical analysis of efficacy and selectivity. *J Pharmacol Exp Ther* 327(3):610–619
71. Hopkins AL, Mason JS, Overington JP (2006) Can we rationally design promiscuous drugs? *Curr Opin Struct Biol* 16(1):127–136
72. Horiuchi KY, Wang Y, Diamond SL, Ma H (2006) Microarrays for the functional analysis of the chemical-kinase interactome. *J Biomol Screen* 11(1):48–56
73. Hubbard SR, Till JH (2000) Protein tyrosine kinase structure and function. *Annu Rev Biochem* 69:373–398

74. Iyer GH, Taslimi P, Pazhanisamy S (2008) Staurosporine-based binding assay for testing the affinity of compounds to protein kinases. *Anal Biochem* 373(2):197–206
75. Johnson LN, Noble MEM, Owen DJ (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* 85(2):149–158
76. Johnson M, Li C, Rasnow B, Grandsard P, Xing H, Fields A (2002) Converting a protease assay to a Caliper® format LabChip system. *JALA* 7(4):62–68
77. Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM, Zarrinkar PP (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* 26(1):127–132
78. Kashem MA, Nelson RM, Yingling JD, Pullen SS, Prokopowicz AS III, Jones JW, Wolak JP, Rogers GR, Morelock MM, Snow RJ, Homon CA, Jakes S (2007) Three mechanistically distinct kinase assays compared: measurement of intrinsic ATPase activity identified the most comprehensive set of ITK inhibitors. *J Biomol Screen* 12(1):70–83
79. Kinnings SL, Jackson RM (2009) Binding site similarity analysis for the functional classification of the protein kinase family. *J Chem Inf Model* 49(2):318–329
80. Klebl BM, Mueller G (2005) Second-generation kinase inhibitors. *Expert Opin Ther Targets* 9(5):975–993
81. Knight ZA, Shokat KM (2005) Features of selective kinase inhibitors. *Chem Biol* 12(6):621–637
82. Knighton DR, Zheng J, Ten Eyck LF, Ashford VA, Nguyen Huu X, Taylor SS, Sowadski JM (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* 253(5018):407–414
83. Krishnamurthy R, Maly DJ (2007) Chemical genomic and proteomic methods for determining kinase inhibitor selectivity. *Comb Chem High Throughput Screen* 10(8):652–666
84. Krug M, Hilgeroth A (2008) Recent advances in the development of multi-kinase inhibitors. *Mini-Rev Med Chem* 8(13):1312–1327
85. Kung C, Kenski DM, Dickerson SH, Howson RW, Kuyper LF, Madhani HD, Shokat KM (2005) Chemical genomic profiling to identify intracellular targets of a multiplex kinase inhibitor. *Proc Natl Acad Sci U S A* 102(10):3587–3592
86. Kung C, Kenski DM, Krukenberg K, Madhani HD, Shokat KM (2006) Selective kinase inhibition by exploiting differential pathway sensitivity. *Chem Biol* 13(4):399–407
87. Lebakken CS, Kang HC, Vogel KW (2007) A fluorescence lifetime-based binding assay to characterize kinase inhibitors. *J Biomol Screen* 12(6):828–841
88. Lee Y-S, Mrksich M (2002) Protein chips, from concept to practice. *Trends Biotechnol* 20(Suppl 12):S14–S18
89. Li B, Liu Y, Uno T, Gray N (2004) Creating chemical diversity to target protein kinases. *Comb Chem High Throughput Screen* 7(5):453–472
90. Li Y, Liang J, Siu T, Hu E, Rossi MA, Barnett SF, Defeo-Jones D, Jones RE, Robinson RG, Leander K, Huber HE, Mittal S, Cosford N, Prasit P (2009) Allosteric inhibitors of Akt1 and Akt2: discovery of [1, 2, 4]triazolo[3, 4-f][1, 6]naphthyridines with potent and balanced activity. *Bioorg Med Chem Lett* 19(3):834–836
91. Liao JJ-L (2007) Molecular recognition of protein kinase binding pockets for design of potent and selective kinase inhibitors. *J Med Chem* 50(3):409–424
92. Lin X, Murray JM, Rico AC, Wang MX, Chu DT, Zhou Y, Rosario MD, Kaufman S, Ma S, Fang E, Crawford K, Jefferson AB (2006) Discovery of 2-pyrimidyl-5-amidothiophenes as potent inhibitors for AKT: synthesis and SAR studies. *Bioorg Med Chem Lett* 16(16):4163–4168
93. Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, Hartman GD, Huff JR, Huber HE, Duggan ME (2005) Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. *Bioorg Med Chem Lett* 15(3):761–764
94. Liu Y, Gray NS (2006) Rational design of inhibitors that bind to inactive kinase conformations. *Nat Chem Biol* 2(7):358–364
95. Lombardo LJ, Lee FY, Chen P, Norris D, Barrish JC, Behnia K, Castaneda S, Cornelius LAM, Das J, Dowsyko AM, Fairchild C, Hunt JT, Inigo I, Johnston K, Kamath A, Kan D, Klei H, Marathe P, Pang S, Peterson R, Pitt S, Schieven GL, Schmidt RJ, Tokarski J, Wen M-L, Wityak J, Borzilleri RM (2004) Discovery of N-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino) thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem* 47(27):6658–6661
96. Lueking A, Cahill DJ, Müller S (2005) Protein biochips: a new and versatile platform technology for molecular medicine. *Drug Discov Today* 10(11):789–794
97. Ma H, Deacon S, Horiuchi K (2008) The challenge of selecting protein kinase assays for lead discovery optimization. *Expert Opin Drug Discov* 3(6):607–621
98. Ma H, Horiuchi KY (2006) Chemical microarray: a new tool for drug screening and discovery. *Drug Discov Today* 11(13–14):661–668
99. Ma H, Horiuchi KY, Wang Y, Kucharewicz SA, Diamond SL (2005) Nanoliter homogeneous ultra-high throughput screening microarray for lead discoveries and IC₅₀ profiling. *Assay Drug Dev Technol* 3(2):177–187
100. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* 298(5600):1912–1916, 1933–1934
101. Marsden BD, Knapp S (2008) Doing more than just the structure—structural genomics in kinase drug discovery. *Curr Opin Chem Biol* 12(1):40–45
102. McGregor MJ (2007) A pharmacophore map of small molecule protein kinase inhibitors. *J Chem Inf Model* 47(6):2374–2382
103. Melnick JS, Janes J, Kim S, Chang JY, Sipes DG, Gunderson D, James L, Matzen JT, Garcia ME, Hood TL, Beigi R, Xia G, Harg RA, Asatryan H, Yan SF, Zhou Y, Gu X-J, Saadat A, Zhou V, King FJ, Shaw CM, Su AI, Downs R, Gray NS, Schultz PG, Warmuth M, Caldwell JS (2006) An efficient rapid system for profiling the cellular activities of molecular libraries. *Proc Natl Acad Sci U S A* 103(9):3153–3158
104. Noble MEM, Endicott JA, Johnson LN (2004) Protein kinase inhibitors: insights into drug design from structure. *Science* 303(5665):1800–1805
105. O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, Cowan-Jacob SW, Lee FY, Heinrich MC, Deininger MW, Druker BJ (2005) In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. *Cancer Res* 65(11):4500–4505
106. Okram B, Nagle A, Adrian FJ, Lee C, Ren P, Wang X, Sim T, Xie Y, Wang X, Xia G, Spraggon G, Warmuth M, Liu Y, Gray NS (2006) A general strategy for creating "inactive-conformation" Abl inhibitors. *Chem Biol* 13(7):779–786
107. Olive DM (2004) Quantitative methods for the analysis of protein phosphorylation in drug development. *Expert Rev Proteomics* 1(3):327–341

108. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J, Rosen N (2006) mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 66(3):1500–1508
109. Pagano MA, Bain J, Kazimierczuk Z, Sarno S, Ruzzene M, Di Maira G, Elliott M, Orzeszko A, Cozza G, Meggio F, Pinna LA (2008) The selectivity of inhibitors of protein kinase CK2: an update. *Biochem J* 415(3):353–365
110. Park S, Chapuis N, Bardet V, Tamburini J, Gallay N, Willems L, Knight ZA, Shokat KM, Azar N, Viguie F, Ifrah N, Dreyfus F, Mayeux P, Lacombe C, Bouscary D (2008) PI-103, a dual inhibitor of Class IA phosphatidylinositol 3-kinase and mTOR, has antileukemic activity in AML. *Leukemia* 22(9):1698–1706
111. Patricelli MP, Szardenings AK, Liyanage M, Nomanbhoy TK, Wu M, Weissig H, Aban A, Chun D, Tanner S, Kozarich JW (2007) Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* 46(2):350–358
112. Paul MK, Mukhopadhyay AK (2004) Tyrosine kinase - role and significance in cancer. *Int J Med Sci* 1(2):101–115
113. Peach ML, Tan N, Choyke SJ, Giubellino A, Athauda G, Burke TR Jr, Nicklaus MC, Bottaro DP (2009) Directed discovery of agents targeting the Met tyrosine kinase domain by virtual screening. *J Med Chem* 52(4):943–951
114. Peifer C, Krasowski A, Haemmerle N, Kohlbacher O, Dannhardt G, Totzke F, Schaechtele C, Laufer S (2006) Profile and molecular modeling of 3-(indole-3-yl)-4-(3, 4, 5-trimethoxyphenyl)-1 H-pyrrole-2, 5-dione (I) as a highly selective VEGF-R2/3 inhibitor. *J Med Chem* 49(25):7549–7553
115. Perrin D, Fremaux C, Scheer A (2006) Assay development and screening of a serine/threonine kinase in an on-chip mode using caliper nanofluidics technology. *J Biomol Screen* 11(4):359–368
116. Petrelli A, Giordano S (2008) From single- to multi-target drugs in cancer therapy: when aspecificity becomes an advantage. *Curr Med Chem* 15(5):422–432
117. Petrov KG, Zhang Y-M, Carter M, Cockerill GS, Dickerson S, Gauthier CA, Guo Y, Mook RA, Rusnak DW, Walker AL, Wood ER, Lackey KE (2006) Optimization and SAR for dual ErbB-1/ ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. *Bioorg Med Chem Lett* 16(17):4686–4691
118. Raynaud FI, Eccles S, Clarke PA, Hayes A, Nutley B, Alix S, Henley A, Di-Stefano F, Ahmad Z, Guillard S, Bjerke LM, Kelland L, Valenti M, Patterson L, Gowan S, de Haven Brandon A, Hayakawa M, Kaizawa H, Koizumi T, Ohishi T, Patel S, Saghir N, Parker P, Waterfield M, Workman P (2007) Pharmacologic characterization of a potent inhibitor of class I phosphatidylinositol 3-kinases. *Cancer Res* 67:5840–5850
119. Rheault TR, Caferro TR, Dickerson SH, Donaldson KH, Gaul MD, Goetz AS, Mullin RJ, McDonald OB, Petrov KG, Rusnak DW, Shewchuk LM, Spehar GM, Truesdale AT, Vanderwall DE, Wood ER, Uehling DE (2009) Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. *Bioorg Med Chem Lett* 19(3):817–820
120. Rodems SM, Hamman BD, Lin C, Zhao J, Shah S, Heidary D, Makings L, Stack JH, Pollok BA (2002) A FRET-based assay platform for ultra-high density drug screening of protein kinases and phosphatases. *Assay Drug Dev Technol* 1(1 Pt 1):9–19
121. Ross H, Armstrong CG, Cohen P (2002) A non-radioactive method for the assay of many serine/threonine-specific protein kinases. *Biochem J* 366(3):977–981
122. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlett-Jones M, He F, Jacobson A, Pappin DJ (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 3(12):1154–1169
123. Rusnak DW, Affleck K, Cockerill SG, Stubberfield C, Harris R, Page M, Smith KJ, Guntrip SB, Carter MC, Shaw RJ, Jowett A, Stables J, Topley P, Wood ER, Brignola PS, Kadwell SH, Reep BR, Mullin RJ, Alligood KJ, Keith BR, Crosby RM, Murray DM, Knight WB, Gilmer TM, Lackey K (2001) The characterization of novel, dual ErbB-2/EGFR, tyrosine kinase inhibitors: potential therapy for cancer. *Cancer Res* 61(19):7196–7203
124. Scapin G (2006) Protein kinase inhibition: different approaches to selective inhibitor design. *Curr Drug Targets* 7(11):1443–1454
125. Sciabola S, Stanton RV, Wittkopp S, Wildman S, Moshinsky D, Potluri S, Xi H (2008) Predicting kinase selectivity profiles using Free-Wilson QSAR analysis. *J Chem Inf Model* 48(9):1851–1867
126. Seong Y-S, Min C, Li L, Yang JY, Kim S-Y, Cao X, Kim K, Yuspa SH, Chung H-H, Lee KS (2003) Characterization of a novel cyclin-dependent kinase 1 inhibitor, BMI-1026. *Cancer Res* 63:7384–7391
127. Sheinerman FB, Giraud E, Laoui A (2005) High affinity targets of protein kinase inhibitors have similar residues at the positions energetically important for binding. *J Mol Biol* 352(5):1134–1156
128. Sills MA, Weiss D, Pham Q, Schweitzer R, Wu X, Wu JJ (2002) Comparison of assay technologies for a tyrosine kinase assay generates different results in high throughput screening. *J Biomol Screen* 7(3):191–214
129. Slon-Usakiewicz JJ, Dai J-R, Ng W, Foster JE, Deretey E, Toledo-Sherman L, Redden PR, Pasternak A, Reid N (2005) Global kinase screening. Applications of frontal affinity chromatography coupled to mass spectrometry in drug discovery. *Anal Chem* 77(5):1268–1274
130. Smaill JB, Baker EN, Booth RJ, Bridges AJ, Dickson JM, Dobrusin EM, Ivanovic I, Kraker AJ, Lee HH, Lunney EA, Ortwin DF, Palmer BD, Quin J III, Squire CJ, Thompson AM, Denny WA (2008) Synthesis and structure-activity relationships of N-6 substituted analogues of 9-hydroxy-4-phenylpyrrolo[3, 4-c]carbazole-1, 3(2H, 6H)-diones as inhibitors of Wee1 and Chk1 checkpoint kinases. *Eur J Med Chem* 43(6):1276–1296
131. Smyth LA, Matthews TP, Collins I (2009) Kinase-directed libraries inspired by the natural product Indirubin. Abstracts of Papers, 237th ACS National Meeting, Salt Lake City, UT, United States, Mar. 22–26, 2009: ORGN - 352
132. Soverini S, Colarossi S, Gnani A, Rosti G, Castagnetti F, Poerio A, Iacobucci I, Amabile M, Abruzzese E, Orlandi E, Radaelli F, Ciccone F, Tiribelli M, di Lorenzo R, Caracciolo C, Izzo B, Pane F, Saglio G, Baccarani M, Martinelli G (2006) Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive Patients: by the GIMEMA working party on chronic myeloid leukemia. *Clin Cancer Res* 12(24):7374–7379
133. Taylor RD, Bailey S. "Small molecule oral kinase inhibitor in oncology – UCB datasheet." from www.ucb-group.com
134. Tecle H, Shao J, Li Y, Kothe M, Kazmirski S, Penzotti J, Ding Y-H, Ohren J, Moshinsky D, Coli R, Jhawar N, Bora E, Jacques-O'Hagan S, Wu J (2009) Beyond the MEK-pocket: can current MEK kinase inhibitors be utilized to synthesize novel type III NCKIs? Does the MEK-pocket exist in kinases other than MEK? *Bioorg Med Chem Lett* 19(1):226–229
135. Traut TW (1994) Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem* 140(1):1–22

136. Traxler P, Furet P (1999) Strategies toward the design of novel and selective protein tyrosine kinase inhibitors. *Pharmacol Ther* 82(2–3):195–206
137. Turk BE (2005) Measuring kinase activity: finding needles in a haystack. *Nat Methods* 2(4):251–252
138. Vieth M, Sutherland JJ, Robertson DH, Campbell RM (2005) Kinomics: characterizing the therapeutically validated kinase space. *Drug Discov Today* 10(12):839–846
139. Waterson AG, Petrov KG, Hornberger KR, Hubbard RD, Sammond DM, Smith SC, Dickson HD, Caferro TR, Hinkle KW, Stevens KL, Dickerson SH, Rusnak DW, Spehar GM, Wood ER, Griffin RJ, Uehling DE (2009) Synthesis and evaluation of aniline headgroups for alkynyl thienopyrimidine dual EGFR/ErbB-2 kinase inhibitors. *Bioorg Med Chem Lett* 19(5):1332–1336
140. Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R, Kelley S (2006) Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* 5(10):835–844
141. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M (2008) Preclinical overview of sorafenib, a multi-kinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* 7(10):3129–3140
142. Xia W, Mullin RJ, Keith BR, Liu L-H, Ma H, Rusnak DW, Owens G, Alligood KJ, Spector NL (2002) Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 21(41):6255–6263
143. Zhang J, Yang PL, Gray NS (2009) Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* 9(1):28–39
144. Zhang X, Crespo A, Fernandez A (2008) Turning promiscuous kinase inhibitors into safer drugs. *Trends Biotechnol* 26(6):295–301
145. Zhang Z, Meier KE (2006) New assignments for multitasking signal transduction inhibitors. *Mol Pharmacol* 69(5):1510–1512
146. Zhu H, Snyder M (2003) Protein chip technology. *Curr Opin Chem Biol* 7(1):55–63
147. Zieske LR (2006) A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. *J Exp Bot* 57(7):1501–1508
148. Peifer C, Urich R, Schattel V, Abadleh M, Roettig M, Kohlbacher O, Laufer S (2008) Implications for selectivity of 3,4- diarylquinolinones as p38alpha MAP kinase inhibitors. *Bioorg Med Chem Lett* 18(4):1431–1435
149. Brehmer D, Greff Z, Godl K, Blencke S, Kurtenbach A, Weber M, Mueller S, Klebl B, Cotten M, Keri G, Wissing J, Daub H (2005) Cellular targets of gefitinib. *Cancer Res* 65(2):379–382