Small Molecule Inhibitors of Protein Kinases in Cancer- How to Overcome Resistance

R. Griffith[1,3,*], M.N. Brown[1], A. McCluskey[1,3] and L.K. Ashman[2,3]

[1]*School of Environmental and Life Sciences, Faculty of Science and IT, University of Newcastle, Callaghan NSW 2308, Australia;* [2]*School of Biomedical Sciences, Faculty of Health, University of Newcastle, Callaghan NSW 2308, Australia;* [3]*Hunter Medical Research Institute, John Hunter Hospital, New Lambton, NSW 2310, Australia*

Abstract: Small molecule protein kinase inhibitors show great promise as anti-cancer agents, however, *de novo* and acquired resistance present problems. These are reviewed and illustrated using the receptor tyrosine kinase, KIT, as an example. Emerging solutions are presented, such as targeting active kinase conformations.

Key Words: Anti-cancer agents, protein kinase inhibitors, drug design, active kinase conformation, inactive kinase conformation, selectivity.

INTRODUCTION

Protein kinases represent one of the largest gene families, comprising 518 different gene products in the human organism alone [1]. Kinases operate by adding the gamma phosphate from ATP to another protein. Typically, this gamma phosphate is transferred to either a tyrosine, serine, or threonine residue. Protein kinases can thus be divided into tyrosine kinases and serine/threonine kinases. The transfer of the phosphate results in a conformational change in the protein, such that its activity is altered. Phosphorylation of tyrosine and serine residues also creates docking sites for interacting proteins. For example, phosphotyrosine containing peptides can recruit other proteins with SH2 or PTB domains, while phosphoserine containing motifs form binding sites for proteins such as 14-3-3 proteins. These interactions confer signalling specificity and influence subcellular localisation [2, 3]. Kinases play a key role in eukaryotic signal transduction cascades, and are structurally conserved in organisms as diverse as yeast and humans [4].

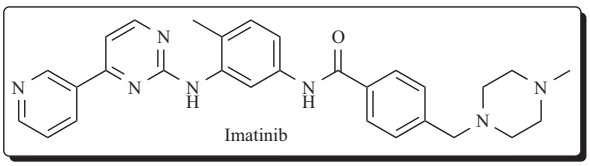
Cellular division or mitogenic signal pathways are amongst the many signalling pathways that rely, at some point, on protein kinases. As such, dysfunctional protein kinase activity has been associated with many forms of cancer (reviewed in [5]). Typically, enhanced kinase activity has been the result of kinase overexpression or mutations that lead to constitutive activity, i.e., kinases that are active even in the absence of an appropriate stimulus. Cancerous cells are known to display a greater than normal dependence on such aberrant signalling pathways; a phenomenon that has come to be known as 'oncogenic addiction' (reviewed in [6]).

Hence, kinases represent attractive drug targets for new therapeutics in the fight against cancer. Several small molecule inhibitors of various oncogenic protein kinases have been approved for clinical use or are currently in clinical trials, with many more promising compounds in pre-clinical development. Targeted small molecule kinase inhibitors have proven to be a major advance in the treatment of certain cancers (reviewed in [7]). However, this new class of anti-cancer drugs has not been without its share of problems. The initially encouraging results of Gleevec® (Novartis) [8-10], the first kinase inhibitor approved for clinical use, were soon followed by disappointing relapses and the development of resistance in many late stage tumours [11-14]. This compound has historically also been known as STI-571 and Imatinib, and will be referred to as Imatinib for the rest of this article.

Another problem arises from the high structural similarity between different kinases in their active conformations compared with the often divergent inactive conformations. This led early efforts in kinase inhibitor design to target the inactive conformation to improve drug selectivity. However, some oncogenic kinases have mutations in the activation loop of the kinase domain that render them constitutively active. These proteins are in many cases resistant to drugs that target the inactive conformation of the protein. Both major problems with protein kinase inhibitors as anti-cancer drugs thus arise, at least in part, from them targeting the inactive kinase conformation, and progress around the world in the development of inhibitors targeting active kinase conformations will be discussed here and illustrated by our own work on the receptor tyrosine kinase KIT.

IMATINIB- THE PROTOTYPE KINASE INHIBITORY DRUG

Imatinib has become famous as the first kinase inhibitor to be approved for clinical use. It is a phenyl-2-pyrimidine compound, as shown below.



Imatinib

*Address correspondence to this author at the School of Environmental and Life Sciences, Faculty of Science and IT, University of Newcastle, Callaghan NSW 2308, Australia; E-mail: renate.griffith@newcastle.edu.au

1389-5575/06 $50.00+.00

Imatinib was first developed by Novartis with the Platelet Derived Growth Factor Receptor tyrosine kinase (PDGFR) intended as its drug target. It has been shown to have activity against PDGFR, ABL, KIT, and more recently, LCK [15] protein kinases, and is also in clinical use against the PDGFRβ and KIT receptor tyrosine kinases. Imatinib has been used successfully in treatment of Chronic Myeloid Leukaemia (CML) and a sub-set of patients with Acute Lymphoblastic Leukaemia (ALL) where the target is the BCR/ABL fusion oncoprotein [16], and Gastrointestinal Stromal Tumours (GIST) where the target is mutant KIT or, less commonly, PDGFR [17]. Unfortunately, as an inactive conformation inhibitor, Imatinib was quickly shown to be inactive against certain kinase domain mutants of the target kinases, for example, the activating mutation D816V in the kinase domain renders KIT insensitive to the drug [18, 19]. The D816V KIT mutant, which was originally identified in a mast cell leukaemia [20], is common in adult systemic mastocytosis [21] and has also been found in acute myeloid leukaemia [22, 23], and testicular seminomas [24]. Cancers associated with this form of constitutively active KIT are expected to be intrinsically resistant to the drug and this has been demonstrated in systemic mastocytosis [25].

Moreover, secondary resistance frequently develops in patients who initially respond to Imatinib. This was first demonstrated in patients with CML and was shown to be most commonly due to secondary mutations in the ABL kinase domain. Such mutations can directly affect drug binding, or may act through conformational changes, particularly by favouring the active conformation [11, 14]. Similarly, secondary resistance to Imatinib has been reported in GISTs. The major oncogenic mutations in GISTs are not in the kinase domain, but in the intracellular juxtamembrane domain. These mutant forms of KIT are highly sensitive to Imatinib [19, 26], and GIST patients initially respond to Imatinib treatment, but subsequently relapse with refractory disease [27-31].

Imatinib's mode of action involves binding competitively in the ATP site of an inactive kinase conformation [32]. Several crystal structures of KIT have been published recently by Mol *et al.* [33, 34] (Fig. (**1**)). These clearly demonstrate that the kinase domain can exist in at least three different conformations: the autoinhibited inactive form, the Imatinib-inhibited inactive form, and the active form (reviewed in [35]).

To facilitate the discussion of Imatinib's binding mode, a global view of the kinase domain, highlighting the major structural features, is presented in Fig. (**1a**), using KIT as an example. Kinase domains typically have a bilobal structure, with the smaller, N-terminal part containing a β sheet and the αC helix. The larger C-terminal kinase lobe is dominated by 7 α-helices. The active site, where ATP binds, is located in the cleft between the two lobes. Important loop regions (highlighted in Fig. (**1a**)) include the G-rich nucleotide binding loop, and the activation loop (residues 810 – 839), which begins with the conserved DFG motif. The phenylalanine residue of the DFG motif (F811) is highlighted in Fig. (**1**). The activation loop also includes the peptide substrate-binding loop (P loop). Residues preceding the activation loop are termed hinge residues. Considerable rearrangement of the autoinhibited form is required to enable Imatinib binding, because in the absence of any inhibitors or substrates, the juxtamembrane region, which connects the kinase domain to the membrane spanning region, inserts partially between the two lobes of the kinase domain (Fig. (**1a**)). The juxtamembrane domain (JMD) thus has an autoinhibitory role, as was first shown by Wybenga-Groot *et al.* [36] for the Eph kinase. Biochemical data indicating an auto-inhibitory function of the JMD have also been published [37].

Imatinib binds in the cleft between the two kinase lobes with its two aromatic heterocycles mimicking the ATP purine system (Fig. (**1b**)). Hydrogen bonding with the "gatekeeper residue" (T670), and interaction with the hydrophobic pocket "guarded" by the gatekeeper residue *via* the phenyl ring in the centre of the molecule are the main reasons for the selectivity of Imatinib for kinases with a small gatekeeper residue capable of hydrogen bonding. Whilst these interactions stabilise the binding between Imatinib and the kinase, the phenyl ring at the piperazine end of the molecule inserts between the αC helix and the conserved DFG motif, thereby sterically preventing the two from coming together, and hence locking the kinase into an inactive conformation.

Upon activation of the kinase *via* ligand binding to the extracellular receptor regions, the mechanism of activation proceeds most likely through receptor dimerisation, leading to transphosphorylation, especially of the residues of the JMD. The autoinhibition is thus relieved, allowing ATP to bind to the enzyme and a conformational change to take place, so that the catalytic site is ready for the phosphorylation of substrate proteins. Fig. (**1c**) shows the active conformation of KIT [34], with ADP bound. The major difference between the inactive conformations (Fig. (**1a**, **1b**)) and the active conformation consists of a closing of the cleft between the two kinase lobes. This, as well as further changes highlighted in Fig. (**1c**), prevents Imatinib from binding to the active conformation. The role (if any) of phosphorylation of tyrosine residues in the activation loop in achieving an active conformation is not clear yet, and may also depend on the particular kinase studied. For example, KIT only contains one Y residue in the activation loop, and Mol *et al.* [33, 34] have shown that this is not phosphorylated in their active conformation crystal structure. Similarly, we have shown [38] that phosphorylation of this residue does not confer an energetic advantage to our model of the KIT kinase domain active conformation.

Molecular modelling work showed that the D816V substitution in KIT disrupts a hydrogen bond formed by the aspartate side chain that stabilised the inactive conformation of the kinase. Thus the active conformation of the kinase domain, to which Imatinib cannot bind, is strongly favoured in this Imatinib-insensitive mutant [38]. Inspection of the crystal structures of KIT also revealed that this hydrogen bond is present in the inactive conformation of the kinase domain, but not in the active conformation [33, 34].

Another inhibitor belonging to the same structural class, AMN107, (structure shown below) is currently undergoing pre-clinical testing and shows great promise, particularly in the area of overcoming resistance to Imatinib [39-42] including the resistance observed for the D816V mutation of KIT

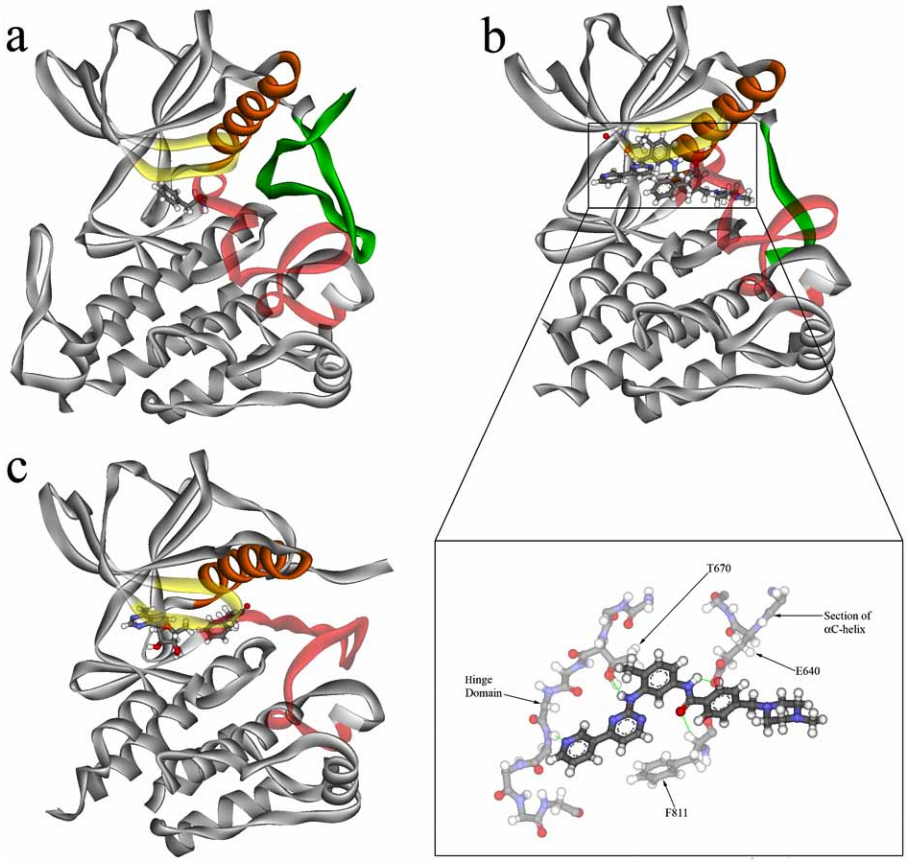


Fig. (1). Crystal structures of three different conformations of the kinase domain of the KIT receptor tyrosine kinase [33, 34]. The protein backbone is shown as a grey ribbon, with the αC helix coloured orange, a section of the nucleotide binding loop yellow, the activation loop red, and the juxtamembrane domain green. The phenylalanine sidechain of the conserved DFG motif (F811) at the beginning of the activation loop is shown and coloured according to element (grey:C; white:H; red:O; blue:N).

a: Autoinhibited inactive conformation. The juxtamembrane (green) is inserted between the two kinase lobes.

b: Imatinib-bound inactive conformation.

TOP: Global view, the gatekeeper residue (T670) sidechain and Imatinib are displayed and coloured by element. The juxtamembrane domain is not completely resolved, but has moved out of the cleft between the two kinase lobes. The F811 sidechain has swung down to accommodate Imatinib.

BOTTOM: Close-up view, showing sidechains of residues interacting with Imatinib coloured by element. Hydrogen bonds are shown as green lines.

c: Active conformation. The juxtamembrane domain is not resolved. ADP is shown bound into the cleft coloured by element. The two phosphate groups of ADP are not shown for clarity. The major changes in comparison to the two inactive conformations have occurred in the activation loop (red) and the αC helix (orange), but changes occur also in other structural elements, such as the nucleotide binding loop (yellow). Overall, the active conformation has a more closed cleft between the two kinase lobes, and this does not allow Imatinib to bind.

[41]. AMN107 has been shown to be ATP competitive for ABL [42]. It binds with higher affinity than Imatinib to the inactive conformation in a similar, but subtly different manner. It is, however, not able to overcome the resistance against the gatekeeper mutant, T315I, of ABL.

QUINAZOLINES

This group of compounds includes two compounds that have already been approved for clinical use, Iressa® (Gefitinib), and Tarceva® (Erlotinib), developed by AstraZeneca and OSI pharmaceuticals, respectively. Gefitinib and Erlotinib are both 4-anilinoquinazolines (see Table **1**), and both inhibit Epidermal Growth Factor Receptor (EGFR). Members of the EGFR kinase family are widely expressed in epithelial tumours. Other promising anilinoquinazolines that are currently undergoing clinical trials are GW-2016, CI-1033, ZD-6474. As with Gefitinib and Erlotinib, these inhibitors typically target an EGFR family kinase, such as Human Epider-

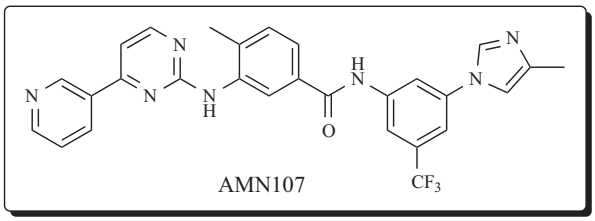
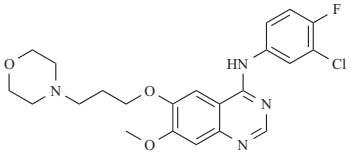
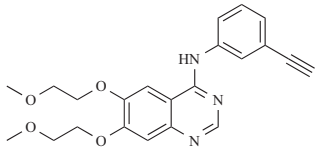
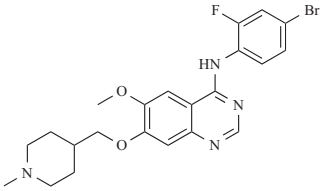
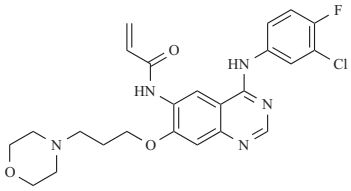
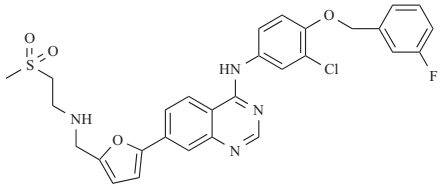
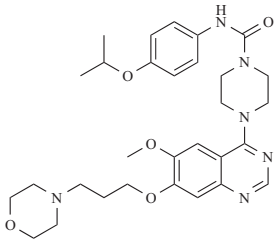
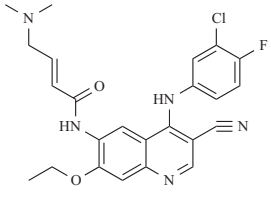


Table 1. Selected Quinazoline Inhibitors

Compound	Structure	Clinical Development	Kinase Target	Development
Gefitinib		Approved	EGFR	AstraZeneca
Erlotinib		Approved	EGFR	OSI Pharmaceuticals
ZD 6474		Phase II	EGFR VEGFR-2	AstraZeneca
CI 1033		Phase II	EGFR sub-family	Pfizer
GW 2016		Phase III	EGFR HER-2 HER-4	GlaxoSmithKline
MLN 518		Phase I	FLT-3	Millenium Pharma-ceuticals
EKB 569*		Phase II	EGFR HER-2	Wyeth Ayerst

* EKB 569 is not a quinazoline.

mal growth factor Receptor 2 (HER-2/Erb-B2), and/or HER-4; and they also target Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2). A structurally closely related inhibitor is the quinoline derivative EKB-569. MLN-518 is a piperazinylquinazoline in development by Millenium Pharmaceuticals, and is another molecule based on a quinazoline template that has progressed to clinical trials. In contrast to the anilinoquinazolines mentioned above, MLN-518 has a piperazinyl group at the 4-position of the core quinazoline scaffold. It was developed to inhibit the FLT-3 tyrosine kinase, an important target in AML [43] however, it has also been shown to have submicromolar activity against KIT, Neurotropic Tyrosine Receptor Kinase 1 (NTRK-1) and PDGFRβ tyrosine kinases [15]. A selection of quinazolines undergoing clinical testing is shown in Table **1** above.

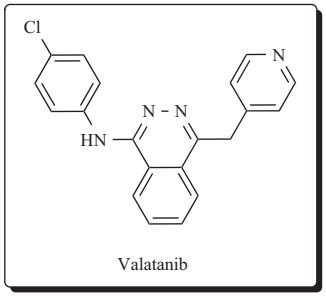
The crystal structures of several 4-anilinoquinazoline inhibitors bound to target kinases have been published [44, 45], and their mode of action has been elucidated. The fused ring system lines up along the hinge region of the kinase, with the nitrogen at position 1 of the ring system accepting a hydrogen bond with from a backbone NH group of the target kinase. The anilino nitrogen atom reaches around the gatekeeper residue such that the aromatic ring probes the hydrophobic pocket in a manner that is similar, but not identical, to interactions with the hydrophobic pocket made by Imatinib. Evidence that these compounds are capable of binding the active conformations of these kinases may be found in that MLN-518 is known to inhibit the constitutively active D816V KIT mutant that confers resistance to Imatinib [46], and that the crystal structure of Erlotinib bound to the EGFR kinase is consistent with the active conformation of that enzyme [44]. Erlotinib is much more compact than Imatinib and doesn't protrude out of the cleft between the C- and N-terminal kinase lobes as far as Imatinib. Therefore, Erlotinib can bind to the active conformation, where this cleft is closed.

OTHER STRUCTURAL CLASSES

The examples given above are merely two classes of the many and diverse chemical scaffolds known to inhibit tyrosine kinases, unique only in the sense that both classes have members that are currently approved for clinical use.

Phthalazines

A very promising phthalazine compound in clinical development targeting VEGFR-2 is the anilinophthalazine Valatanib [47]. A number of phthalazine derivatives are currently in pre-clinical development, for example, those discovered recently by Sridhar and colleagues [48]. Data regarding the potential binding mode of this class of compounds are not available presently.



Valatanib

Staurosporines

The inhibitor staurosporine was originally isolated in 1977 from the bacterium *Streptomyces staurosporeus*, and was shown to be an ATP competitive protein kinase inhibitor, the first such compound discovered (reviewed in [49]). This original compound is far too unselective to be considered a drug candidate, but it has spawned a whole family of variants that may eventually find use in the clinic. The ability of the staurosporine derivative PKC412 (Table **2**) to overcome the Imatinib resistant D842V PDGFR mutation indicates that this class of compounds is quite tolerant to active kinase conformations, as this mutation is analogous to the D816V mutation in KIT [30]. Staurosporines currently in clinical development include LY-333531, UCN-01, and PKC 412. A selection of known biologically active staurosporines is shown in Table **2**.

Indolinones

The anti-kinase activity of the indolinone class of kinase inhibitors was first reported in 1997 by Mohammadi and colleagues [50], in a publication that also included two co-crystal structures of two different indolinones bound to FGFR. The crystal structures indicate that the nitrogen on the five-membered ring forms an intramolecular hydrogen bond to the indolinone oxygen, such that a planar pseudo six-membered ring is formed [50]. Inhibitory activity against the KIT receptor tyrosine kinase was first reported in 2001 for this class of compounds [51]. Indolinone derivatives have also been shown to inhibit constitutively activated KIT mutants, including Imatinib-resistant D816 mutations in the kinase domain [52, 53], indicating that they are capable of binding active kinase conformations. Although most indolinones that advanced to clinical trials were discontinued due to harmful side effects, SU 11248 (Table **3**) is still in clinical development. It is noteworthy that this compound was one of the least specific of all the compounds tested by Fabian [15] (see below). A selection of indolinones that have undergone clinical testing is shown in Table **3**.

Pyrido-Pyrimidines

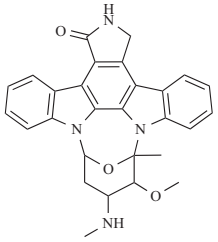
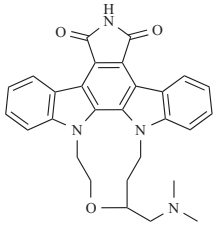
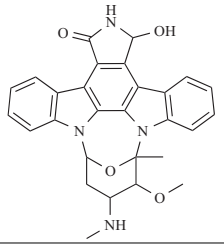
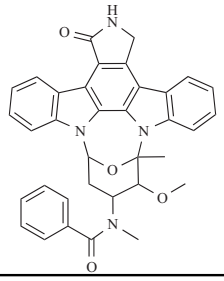
This group of compounds was first reported to have kinase inhibitory activity in 1996 [54], a discovery that soon led to an entire series of related compounds undergoing pre-clinical development. Elucidation of the crystal structure of PD 173955 in complex with ABL [55] revealed that this class of compound was capable of binding kinase active conformations, a finding that was supported by data reporting successful inhibition of constitutively active, Imatinib-resistant mutants of ABL [56].

The structure of PD 173955, a representative of this compound class, is shown below.

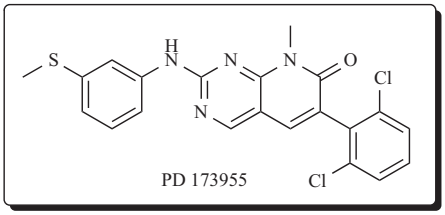
Tyrphostins

The discovery of the naturally occurring selective EGFR inhibitor erbstatin in 1986 demonstrated that a kinase inhibitor could show selectivity against a single protein kinase, when it was shown that this compound inhibited EGFR but

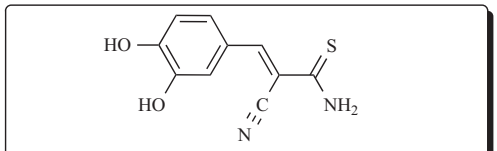
Table 2. Selected Staurosporine Based Inhibitors

Compound	Structure	Clinical Development	Kinase Target	Developement
Staurosporine		NA	NA	NA
LY333531		Phase III	PKC-β	Eli Lilly
UCN-01		Phase II	PKC-α	Kyowa Hakko Kogyo
PKC412		Phase II	PKC-β	Novartis

not cyclic AMP dependant protein kinase (cAMPDPK) [57]. Tyrphostins were eventually developed from the natural



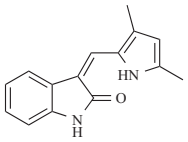
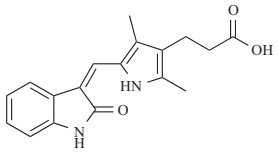
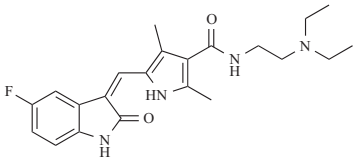
template provided by erbstatin and as such have great historical significance in this field. Their activity was first described in 1988 by Yaish and colleagues [58], and they were amongst the first kinase inhibitors to undergo preclinical testing after the staurosporines. A representative structure is shown below. Modification of the core tyrphostin skeleton by Levitski and colleagues eventually led to the proliferation of kinase inhibitor structural classes that characterises the modern state of the art, e.g., indoles, bicyclic quinoxalines, and tricyclic quinoxalines [59]. Structurally, tyrphostins may be characterised as benzylidene malononitrile compounds. An efficient way to create small (12 compounds) focussed



Compound 12 from Yaish *et al.,* (1988) [58], a tyrphostin.

libraries based on the tyrphostin skeleton has recently been published by McCluskey *et al*. [60]. Although no tyrphostin has so far undergone clinical testing, preclinical research with this class of compounds is ongoing, with some representatives being tested when administered along with other known drugs [61].

Table 3. Selected Indolinone Inhibitors

Compound	Structure	Clinical Development	Kinase Target	Development
SU 5416		Phase III Discontinued	VEGFR	Sugen/Pfizer
SU 6668		Phase I	VEGFR PDGFR FGFR	Sugen/Pfizer
SU 11248		Phase III	VEGFR-2 PDGFR FLT-3 KIT	Sugen/Pfizer

LIGAND-BASED DESIGN OF KINASE INHIBITORS

In light of the recent explosion of compounds reported in the literature as having anti-kinase activity, it is now possible to identify certain common chemical features shared by all ATP competitive kinase inhibitors. As the biologically appropriate ligand for kinases, ATP itself may serve as an obvious template for drug design. However, it must be taken into account that a variety of kinases that are structurally and functionally distinct from protein kinases also bind ATP; it is likely that any inhibitor based on this molecule alone will bind to a large number of non-target proteins. For this reason, some naturally occurring protein kinase inhibitors, such as staurosporine, have been taken into account as additional guides for drug development [62].

The discovery of other natural kinase inhibitors, such as flavopiridol, has contributed to the identification of universal features shared by all ATP competitive protein kinase inhibitors. These compounds are typically highly planar, featuring multiple heterocyclic aromatic ring systems or extended ring systems with multiple conjugated double bonds. The aromatic ring typically contains nitrogen heteroatoms. Imatinib is conspicuous in that it does not have an extended aromatic ring system, however, crystallographic analysis indicates that the two nitrogen containing aromatic rings of this molecule adopt a highly planar orientation upon binding to the protein. The aromatic nitrogen atoms typically make stabilising hydrogen bonds with the target enzyme. In the case of the indolinones, the highly planar aromatic ring system is extended to include a third ring in a novel fashion. Crystallographic data indicate that in binding to FGFR, an intramolecular hydrogen bond is formed between the pyrrole nitrogen and the indolinone oxygen such that the two aromatic ring systems adopt a planar arrangement, with the hydrogen bond forming a pseudo six membered ring [50]. SU 4948, which is substituted with a phenyl instead of the pyrrole ring and is therefore not capable of forming this hydrogen bond, does have activity against the FGFR kinase, however, the crystal structure indicates that a planar arrangement of the two aromatic ring systems is adopted even in the absence of the intramolecular hydrogen bond [50]. SU 4948 completely lacks activity against PDGFR family members, whereas similar compounds capable of forming the intramolecular hydrogen bond interact with these receptors [63]. The planar heterocyclic aromatic system generally forms the core of the kinase inhibitor, while substituents off the core typically dictate the specificity of the inhibitor for a particular kinase. In the case of Imatinib, specificity is determined by the side chain beginning with the amine group off the pyrimidine ring, with the amine hydrogen bonding with the threonine gatekeeper residue, and the adjacent phenyl ring inserting into the hydrophobic pocket behind the gatekeeper.

A computer-generated pharmacophore has been derived by Aronov and Murcko [64], and this allows rapid virtual screening of compound libraries for molecules with a potential for nonselective kinase inhibition. The authors suggest that such lead compounds can then be refined for the required selectivity.

BROAD SPECTRUM INHIBITORS- TARGETING ANGIOGENESIS

It has recently emerged that kinase inhibitors with limited selectivity can still be useful clinical agents. The indolinones, already discussed above, are one example of this. These compounds inhibit the active conformations of several related receptor tyrosine kinases, including KIT, VEGFR, EGFR, and PDGFR. All of these kinases play a role in tumour angiogenesis, so these broad spectrum inhibitors attack the cancer at a number of different levels [53, 65]. SU11248

(see Table **3**) is one of the most "unselective" inhibitors tested by Fabian *et al.* [15], but has progressed to phase III clinical trials, indicating that broad spectrum inhibitors of low toxicity may be feasible. Several anilinoquinazolines have also been shown to act this way [66]. These drugs not only inhibit the core growth factors responsible for oncogenic growth, but also inhibit angiogenesis, or the infiltration of the tumour with blood vessels to support such oncogenic growth.

The thienopyrimidine ureas, as another example, have been shown to inhibit the KIT and EGFR receptor tyrosine kinases [67]. Homology models suggest that this group of compounds only binds to the inactive conformation of their target kinases.

DISCRETE STRUCTURAL ELEMENTS LEND SPECIFICITY

Recent research indicates that the inhibitory profile of a given kinase inhibitor may in fact have very little to do with the sequence similarity of the kinases it binds do. Much more important are the common structural elements between the inhibited kinases. In a recent experiment [15], a set of 20 small molecule kinase inhibitors was tested against a panel of 113 different protein kinases, in what is the most comprehensive test of inhibitory activity to date. The results were surprising in that they showed that the inhibitory activity of a particular compound was not confined to or clustered in a series of closely related kinases. For example, it was shown that Imatinib, in addition to its known activity against PDGFR, KIT, and ABL, also inhibits the distantly related Src family kinase LCK, and has weak activity against 11 other kinases.

Further, Imatinib is known to inhibit both KIT and PDGFR-β with high affinity, but not the closely related kinase FLT-3. The reason for this is that Imatinib exploits the hydrophobic pocket at the back of the ATP binding site, which is guarded by a small threonine residue in KIT and PDGFR (the gatekeeper, see Fig. (**1b**)). Imatinib also hydrogen bonds directly with the gatekeeper residue. In FLT-3, the gatekeeper residue is the much larger phenylalanine, hence blocking access to the hydrophobic pocket and preventing Imatinib from binding. Moreover, the structural reason that Imatinib also binds to LCK, a very distant relative of KIT and PDGFR-β, is that LCK nevertheless has a small gatekeeper residue. In the particular case outlined here, the substitution of a single amino acid, threonine, for a more bulky amino acid, resulted in complete insensitivity to the drug.

Research has thus far turned to analysing the therapeutically validated kinase space, or the kinome, to explore the diverse structural motifs that may be utilised by different drug-like molecules [68-71]. In this approach, a particular kinase is viewed as a collection of structural elements that may be used as 'selectivity filters' for a particular kinase inhibitor to take advantage of. Vindication of this method of drug design may be found in the work of Cohen and colleagues [72]. This group identified two selectivity filters for the RSK group of protein kinases- the gatekeeper residue, and a cysteine in a conserved glycine rich loop common to protein kinases. It was found that only the RSK proteins had both of these selectivity filters. The group then designed a drug by adding substituents to a core ATP mimetic that would interact with both the gatekeeper residue *via* hydrogen bonding and a hydrophobic group that projected into the hydrophobic pocket, and present an electrophile to the cysteine such that a covalent bond would form between the cysteine and the inhibitor. The result was a highly potent, selective and irreversible inhibitor of the RSK kinase. The authors of this work then went on to perform an elegant validation of this approach. Site directed mutagenesis resulted in both of the two selectivity filters being engineered into kinases that previously only housed one. This immediately resulted in a reversal of the drug resistance previously observed for these enzymes.

CONCLUSIONS

Early efforts into kinase inhibition focused on the inactive conformation and ultimately culminated in the development of Imatinib. High selectivity for a specific kinase was thought to be of paramount importance and this was believed to be easier to achieve by targeting inactive conformations, which are more divergent in structure. However, it was found that cancer cells can develop resistance to Imatinib *via* mutations that favour the active conformation of the given kinase drug target. This led to research into a more complete characterisation of the clinically relevant kinase space [68], and ultimately resulted in the approach outlined by Cohen *et al.* [73], which could conceivably be applied to inhibitors that target the active conformation of a kinase.

It may be argued that inhibitors that depend on interaction with single, non-conserved amino acids in their target kinases may be as vulnerable to resistance causing mutations as inactive conformation inhibitors. In the approach outlined by Cohen [73], sensitivity to the drug was demonstrated in other kinases by the simple expediency of substituting the correct amino acids into those non-target kinases. It is just as reasonable to assume that resistance to the drug could develop by single amino acid substitution of these key interacting residues in a biological system. One of the particular selectivity filters employed by Cohen was the gatekeeper residue, and resistance to Imatinib, which also takes advantage of the gatekeeper residue, has already been demonstrated in the gatekeeper residue mutation T315I in ABL [74] and the corresponding mutant in KIT [28]. Hence, it is quite reasonable to assume that selective active conformation inhibitors may have limited advantages in terms of vulnerability to resistance-causing mutations over inactive conformation inhibitors.

However, research indicates that over half of the mutations that confer resistance to Imatinib in ABL do not involve residues that directly contact the inhibitor [11, 14], i.e., the mutations confer resistance by increasing the preference for the active conformation of the kinase, such that Imatinib is unable to bind. In this case, an active conformation inhibitor would have avoided over half the Imatinib-resistant mutations and would therefore represent a significant advantage over Imatinib.

The work by Fabian and colleagues [15] has shown that supposedly selective kinase inhibitors often have activity against wholly unexpected kinases, even in drugs that have been approved for clinical use or are in clinical trials. This indicates that kinase inhibitor selectivity is not as important

as was originally assumed; only enough selectivity to avoid particularly debilitating side effects is required. Inhibition of some kinases may be "safe"; Fabian *et al.* speak of "appropriate" specificity. Hence, nonspecific active conformation inhibitors may be much better tolerated than initially thought. That this should be so is understandable for two reasons. First, biological signalling pathways in multicellular organisms often display a large degree of operational redundancy; the loss of a single pathway is therefore quite tolerable. Secondly, cancerous cells often become very dependent on their malfunctioning growth pathways, a phenomenon known as oncogenic addiction [75]. For these cells, the loss of that particular pathway is not tolerable.

Moreover, work by various groups has indicated that in many cases, less selective kinase inhibitors may actually be more clinically effective than more selective kinase inhibitors, where the inhibitor is active against a range of kinases that are all known to be active in cancerous growth, such as angiogenesis inhibitors.

Two solutions to the problem present themselves. First, the production of active conformation inhibitors with just enough selectivity to not cause serious side effects could be advantageous. This approach can be summarised as designing a small number of inhibitors to inhibit a large number of kinases. Resistance of one kinase will be overcome in that the inhibitor will still be active against all the others. The second solution is to produce multiple kinase inhibitors for a single kinase target; the opposite approach as above; and to use these kinase inhibitors concurrently in the clinic. This approach would require very selective inhibitors as the side effects from each drug would accumulate. Ideally, the inhibitors should target a diversity of conformations and/or binding sites of the kinase. In this approach a particular target kinase would have to undergo multiple mutations in order to gain total resistance to the treatment regimen. Similar combination therapies are currently clinically used against HIV infections [76].

The receptor tyrosine kinase, KIT, is used in our laboratories as an excellent model in the design of new small molecule inhibitors of protein kinases. Mutant KIT is involved in cancers which can be treated with the clinically important inhibitor, Imatinib. Other mutant forms of KIT are involved in other, Imatinib-insensitive cancers (*de novo* resistance), and mutants responsible for acquired resistance to Imatinib upon clinical treatment are also known. Crystal structures are available for various conformations of KIT, including Imatinib-bound KIT, and any emerging mutants of clinical relevance can quickly be modelled and visualised, using the crystal structures as templates [38]. Apart from assays measuring the effect of potential inhibitors on kinase activity, cell-based screens are also available for wildtype and various mutant forms of KIT [19]. These can easily compare inhibitors against inactive and active kinase conformations, as well as taking into account general kinase inhibition (toxicity) and bioavailability. We have already tested the activities of a variety of tyrphostin analogues [60], and promising results have been obtained (data not published). The data generated in these medium throughput assays are amenable to computer-aided pharmacophore and docking approaches to elucidate the ideal structural features of an inhibitor targeting the active conformation of a small number of kinases involved in oncogenic signalling pathways.

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