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Through the "Gatekeeper Door": Exploiting the Active Kinase Conformation

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Introduction

The human genome comprises more than 500 protein kinases including serine/threonine, tyrosine and dual specificity kinases.[1,2] Because of the important physiological and pathological role of kinases, the human kinome represents one of the most important drug discovery opportunities in oncology and other therapeutic areas such as inflammation, autoimmune diseases, and metabolic disorders.[3−7]

Protein kinases phosphorylate their substrate proteins by catalyzing the transfer of the terminal γ-phosphate of the cosubstrate ATP[a] to the hydroxyl acceptor group on the side chains of serine, threonine, or tyrosine residues of the substrate proteins. Kinase structures present a highly conserved catalytic domain that consists of a bilobed structure with the ATP molecule and a Mg ion binding in a deep cleft located between the N- and C-terminal lobes.[8,9]

Since protein phosphorylation represents a key step in many crucial cellular processes like proliferation, differentiation, and apoptosis, the discovery of small molecule kinase inhibitors has attracted growing interest for novel drugs research and development as well as the identification of experimental tools for the understanding of the biological roles of this class of proteins.

The possibility of controlling cell proliferation and death by inhibition of a specific target kinase offers the opportunity to introduce the concept of targeted therapies in the treatment of cancer, providing a valid alternative to conventional chemotherapy. In the past, the main effort in kinase inhibitors development was aimed at the identification of highly potent and specific compounds inhibiting a single kinase. The initial strategy was to develop compounds that could mimic ATP binding to the kinase. This led to a first generation of small molecules kinase inhibitors (defined as type I inhibitors) normally targeting the ATP binding site of the enzyme in the active form, which is characterized by an open conformation of the activation loop. This open conformation is normally referred to as DFG "in" based on the position of the conserved triad aspartate−phenylalanine−glycine (DFG) at the beginning of the activation loop. The common feature of the type I compounds is their ability to bind to the ATP site mimicking the adenine ring in its interactions with the "hinge" residues of the protein.

However, the first kinase small molecule inhibitor to reach the market was imatinib **1** (Gleevec, Novartis) (Figure 1), a compound that recognizes a different form of the kinase.[10] Structural studies have demonstrated that imatinib **1**, a cKIT, Abl, and PDGFR inhibitor approved for chronic myeloid leukemia treatment, binds an inactive form of the target kinases characterized by a closed conformation of the activation loop (DFG "out") which prevents binding of both nucleotide and protein substrates. The flip of the DFG motif in the inactive form considerably changes the morphology of the ATP site and exposes an additional hydrophobic site. The discovery of the novel mode of binding of imatinib **1** was serendipitous, but following its success a new generation of kinase inhibitors, specifically targeting the DFG "out" form, emerged. This class of compounds, defined as type II inhibitors, binds to the same area occupied by the type I compounds but also extends to the additional hydrophobic site available in the inactive form.

Despite numerous drug discovery projects targeting kinases and considerable investments during the past few decades, only a few compounds have reached the market, leaving many potential cancer targets still undrugged. To date, eight kinase small molecules inhibitors have been approved by the FDA for cancer treatment. All these compounds are ATP competitors and follow either a type I (sunitinib **2**, erlotinib **3**, getifinib **4**, dasatinib **5**, and lapatinib **6**) or a type II design (imatinib **1**, sorafenib **7**, nilotinib **8**) (Figure 1). Compounds in the late phase of development as kinase inhibitors also fall within the same design paradigms with the exception of a few compounds, defined as allosteric inhibitors, which bind outside the ATP binding site.

The development of new kinase inhibitors is still a slow and problematic process. Generally, type I compounds suffer from widespread cross-reactivity among other members of the kinase target family, and intense medicinal chemistry optimization programs are required to modulate their activity. Also, fierce competition in the development of scaffolds that could mimic ATP has led to a crowded IP space.

The new opportunities opened by the type II compounds created great expectations, as targeting the allosteric site of the ATP pocket offered additional opportunities to control selectivity and introduce IP novelty. However, clinical evaluation of these compounds has highlighted how the acquisition of resistance-causing mutations of the kinase target limits their efficacy in cancer treatment.[11]

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[a]Abbreviations: ATP, adenosine 5′-triphosphate; FDA, Food and Drug Administration; IP, intellectual property; PDB, Protein Data Bank; SAR, structure−activity relationship; DFG, aspartate−phenylalanine−glycine.

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1; imatinib

2; sunitinib

3; erlotinib

4; gefitinib

5; dasatinib

6; lapatinib

7; sorafenib

8; nilotinib

Figure 1. Kinase inhibitors currently on the market.

Of late, the targeted therapies paradigm is also changing.[12] There is now a general agreement that given the complexity of cancer, in the absence of an "oncogene addiction" mechanism, a multitarget approach might be more effective than the one based on the inhibition of a single target.[13−15] In the discovery of novel kinase inhibitors, however, an uncontrolled promiscuous activity might pose considerable risks due to unforeseeable side effects and toxicity due to broad inhibition of cellular processes. To reduce these risks, a controlled multitarget therapy, based on the selective inhibition on a specific set of kinase targets, is required.[16] To achieve this, the rational design of kinase inhibitors with a controlled specificity may be one of the major challenges.

The wealth of structural information available on kinases has greatly promoted the rational design of kinase inhibitors and at the same time has provided a clear picture of the level of complexity of the kinase catalytic regulation and the several structural elements that are playing part in the process.[17−20]As a part of a study aimed at understanding how it is possible to take advantage of kinase structural elements to rationally guide compounds selectivity and improve type I and type II designs, an analysis of some recently published kinase inhibitors highlighted an additional kinase inhibitor design strategy. These compounds, which we classified as type I$^1/_2$ inhibitors, recognize the target kinases in the DFG "in" form. Type I$^1/_2$ compounds bind to the ATP site like type I compounds and extend to target the back cavity, establishing a defined set of conserved interactions with those residues characteristic of the type II design which is based on the DFG "out" form. Type I$^1/_2$ design could provide an additional tool in the rational design of compounds with the desired activity profile.

Type I Inhibitors

Many of the small molecule kinase inhibitors developed so far are compounds that adhere to the type I design. This may be the result of an initial bias introduced by the use of active phosphorylated kinases in the biochemical assays used for hit identification screening and lead optimization.

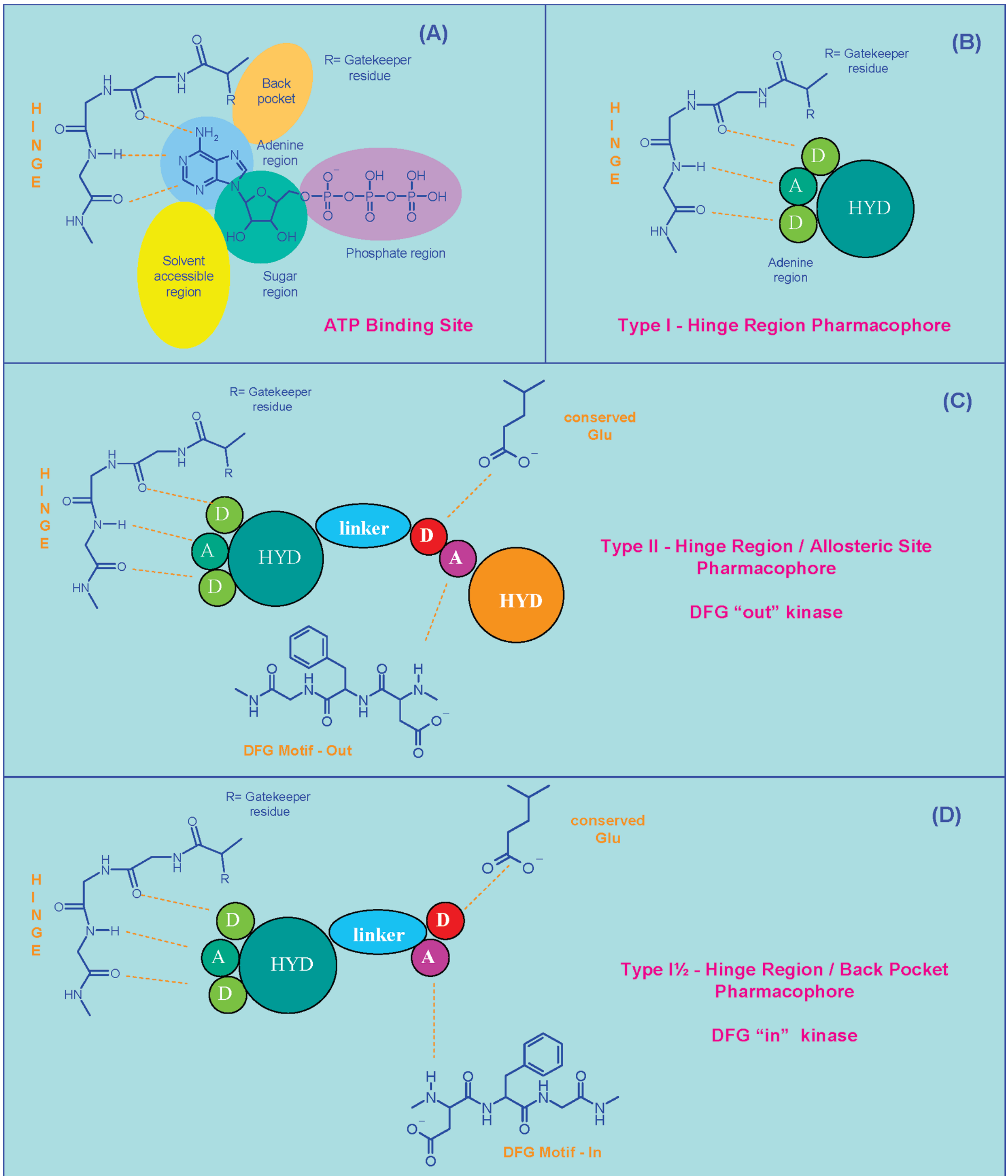


Figure 2. (A) ATP binding site regions and ATP interactions with the hinge residues. Hydrogen bonds are represented by orange dashed lines. (B) Type I kinase inhibitor pharmacophore representing the potential hydrogen bonds with the hinge region. (C) Type II kinase inhibitor pharmacophore representing interactions with the hinge region and the allosteric site present in the DFG "out" conformation. (D) Type $I^1/_2$ pharmacophore representing the potential interactions at the hinge and in the back cavity. Hydrogen bond donors are represented by circles labeled D, hydrogen bond acceptors by circles labeled A. The larger circles labeled HYD indicate the moieties, hydrophobic in nature, that occupy the adenine ring region (green) and the allosteric site (orange).

Type I inhibitors bind to the ATP site and are ATP-competitive. They typically recognize the enzyme in a catalytically active DFG "in" conformation. These molecules mimic the interactions of the adenine moiety (Figure 2A) and bind to the kinase through the formation of one to three hydrogen bonds with the backbone residues of the hinge region connecting the kinase N- and C-terminal lobes and establishing hydrophobic interactions in the adenine ring region. These interactions are described by the commonly accepted type I kinase pharmacophore (Figure 2B) which comprises a hydrogen bond acceptor, two hydrogen bond donors, and a hydrophobic moiety.[4,21,22]

A representative example of compounds belonging to the type I class of kinase inhibitors is sunitinib **2** (Sutent, SU11248, Pfizer, Figure 1), an oral multikinase inhibitor approved by the FDA in 2006 for the treatment of

gastrointestinal tumors and advanced renal-cell carcinoma.[23] This indolinone derivative is a promiscuous inhibitor having a dissociation constant of less than 3 μM for 165 out of a 290 kinases screening panel. Sunitinib **2** inhibits cellular signaling by targeting with remarkable affinity several kinases like PDGFR, VEGFR, c-KIT, RET, CSF-1R, IRK, FLT3, FGFR, CHK1, JAK1, and MPS1.[24,25]

Because of the high degree of sequence and structural similarity in the ATP binding site, cross-activity within the kinase target family is a common feature of type I compounds. However, such promiscuous activity is often related to undesirable side effects and toxicity.[26] To improve potency and modulate selectivity of the initial lead compounds, differences in ATP binding site residues among various kinases are usually targeted. This is normally carried out by introducing several modifications on the chemical scaffolds and substituents, often leading to extensive medicinal chemistry optimization programs.

Although reducing off-target activity in type I inhibitors may be challenging and resource intensive, clean selectivity profiles for type I inhibitors have been achieved. An example is the marketed drug erlotinib **3** (Tarceva , Roche-Genentech-Osi, Figure 2), a potent inhibitor of the tyrosine kinase EGFR effective in a subpopulation of non-small-cell lung cancer (NSCLC) patients harboring a somatic mutation in the catalytic domain of EGFR.[27−29]

Given the relative simplicity of the pharmacophore and the intense activity in the development of kinase inhibitors, it is becoming difficult to identify novel type I inhibitors, restricting the possibility for new intellectual property claims.

Type II Inhibitors

Different structural elements of protein kinases could be exploited to introduce potency and selectivity enhancing features in the early phases of design and development of novel chemical classes. An excellent example of the extent that structural elements can be used to guide compound design and contribute to the development of inhibitors with a specific activity/selectivity profile is represented by the DFG motif and the recent development of a second class of kinase inhibitors named type II.[21,30,31]

Type II inhibitors bind to and stabilize an inactive kinase form that is characterized by the DFG motif being in an "out" conformation. The different position of the DFG residues in the "out" form results in the opening of an additional cavity, the allosteric site, which is hydrophobic in nature and is specifically targeted by type II compounds. These inhibitors not only bind to the ATP region through a set of hydrogen bonds to the hinge, like type I compounds, but also exploit hydrogen bonding and hydrophobic interactions within the allosteric site.

An analysis of the way type II compounds bind to the protein stabilizing the DFG "out" inactive conformation recently led to the formulation of a type II kinase pharmacophore (Figure 2C). The type II pharmacophore can be viewed as an extension of the type I pharmacophore where the elements describing the interactions in the allosteric site are connected through a linker to the hinge-binding type I pharmacophore. In particular, the interactions taking place in the allosteric site of the DFG "out" inactive kinase involve a pair of hydrogen bonds between the ligand and the protein, one with the conserved glutamic acid present on αC-helix and the other with the backbone nitrogen of the DFG aspartic acid. The first interaction requires a hydrogen bond donor on the ligand; the latter requires a hydrogen bond acceptor. Immediately adjacent to the hydrogen bond donor−acceptor pair a hydrophobic moiety is required to form van der Waals interactions with residues of the allosteric site (Figure 2C).[21]

Imatinib **1** is a successful example of type II small molecules with global sales of 3.6 billion dollars in 2008. This molecule, however, was initially developed as type I inhibitor within a SAR-guided medicinal chemistry optimization program. Only subsequently, structural studies did highlight its novel mode of binding targeting the DFG "out" form of the kinase. Most of the other known type II inhibitors have also been developed by starting from their type I equivalent compounds following a chemistry-based approach rather than from de novo identified hits. This is probably due to the fact that, for type II compounds, the hit identification phase is particularly challenging. The lack of suitable biochemical assays and the limited availability of relevant structural information, in fact, limit both high-throughput screening (HTS) and structure-based in silico screening methods.[32]

The relation between the type I and type II inhibitors is clear, as the type I pharmacophore is a subset of the type II pharmacophore and they both display the same interactions in the adenine region of the ATP binding site. However, by specifically targeting the allosteric site, the type II design might offer some advantages compared to the type I design. The conformational variability of the DFG motif in the DFG "out" inactive state and the lower level of sequence conservation in the allosteric sites provide interesting opportunities for the development of selective kinase inhibitors.[19,20] Type II inhibitors very often are much more selective compounds than type I inhibitors.[26] Also, from an IP standpoint, the possibility of targeting the allosteric site can be used to introduce novelty in the design of kinase inhibitors.

Types I and II compounds normally show differences in their kinetics of binding and pharmacokinetics profiles. As a result of their more extensive interactions and generally higher potency, type II compounds typically display a lower dissociation rate (K_{off}) with extended residence time, which has a favorable impact on kinase inhibition.[33,34] Targeting the DFG "out" state of the kinase, which is normally characterized by a $K_{M,ATP}$ value higher than the corresponding value for the DFG "in" active state, also means that type II inhibitors face weaker competition from cellular ATP resulting in enhanced in vivo activity.[26] On the other hand type II compounds are characterized by higher molecular weight, which normally has a negative impact on cellular penetration and results in a lower ligand efficiency.[35,43]

However, the main problem in targeting the DFG "out" form of the kinases is that type II compounds are more vulnerable to the insurgence of resistance due to mutations in the kinase domain that result in the loss of activity of the inhibitor. A mutation preventing the binding of a type I inhibitor most likely would also result in the disruption of ATP binding leading to a dysfunctional kinase which would not be tolerated by the cell. On the contrary, mutations of residues not involved in ATP recognition or catalytic regulation (e.g., the gatekeeper residue) might still result in a functional enzyme but would prevent inhibitor binding. This is, for instance, the case of the imatinib **1** resistance inducing T315I mutation of the gatekeeper in Abl.[36]

Interestingly, the DFG "out" state required for the development of the type II inhibitors does not seem to be accessible to every kinase. It was first reported for the inactive conformation of the insulin receptor kinase (IRK) and was later

Table 1. Comparison of the General Properties of Type I, Type II, and Type I$^{1}/_{2}$ Kinase Inhibitors

	Type I	**Type II**	**Type I½**
Activation state	Active/Inactive	Inactive	Active/Inactive
DFG conformation	In/Out	Out	In
Apply to every kinase	Yes	Small/Medium gatekeeper	Small/Medium gatekeeper
Binding region	ATP site	ATP and allosteric sites	ATP site and back pocket
Hinge Hb	Yes	Yes	Yes
ATP competitive	Yes	Yes	Yes
Selectivity	Usually low but very selective inhibitors identified	The allosteric site offers additional opportunities to modulate selectivity	The back pocket offers additional opportunities to modulate selectivity
Inhibitor resistance	Yes. Mutations in ATP site	Yes. Mutations in ATP and allosteric sites	Yes. Mutations in ATP site
IP position	Crowded	Allosteric site offers opportunity for novelty	Back pocket site offers opportunity for novelty

experimentally observed also for Abl, p38α MAPK, bRAF, FLT3, KIT, LCK, HCK, KDR, TIE2, AUR-A, FMS/CSFR, MET, Src, Pyk2, and CDK6. The vast majority of the kinases for which the DFG "out" state was determined are part of the tyrosine kinase (TK) subfamily, but CMGC (p38α MAPK and CDK6), TKL (bRAF), and AGC (AUR-A) families are also represented. Hence, to date, only a very small portion of the structural data related to the kinome ($>$1700 structures for 162 different kinase domains) refers to the inactive DFG "out" state (15 different kinase domains, $<$10%).

As previously discussed, type II inhibitors (hence, the type II pharmacophore) are clearly related to the catalytically inactive DFG "out" form of the protein. This unique relation between pharmacophore, DFG state, and active/inactive form of the protein observed for the type II compounds does not hold true for type I compounds. This class of inhibitors normally binds to a catalytically active DFG "in" protein, but they can also bind to catalytically inactive DFG "in"/αC-helix "out" kinases (e.g., lapatinib **6** in EGFR kinase, PDB 1xkk). A type I inhibitor could even bind to an inactive DFG "out" kinase if its scaffold could satisfy the type I pharmacophore at the hinge without occupying the sugar/phosphate region where the DFG "out" Phe side chain is located. We hence suggest classification of kinase inhibitors as type I or type II based on the kinase pharmacophore that they recognize rather than DFG "in/out" or active/inactive state of the protein they bind to (e.g., sunitinib **2** binds to a DFG "out" cKIT but matches a type I pharmacophore, PDB code 3g0e). A detailed comparison of the binding features of the different kinase inhibitors classes can be found in Table 1.

Gatekeeper Residue and Type I$^{1}/_{2}$ Kinase Inhibitors

Type II design offers the opportunity to address some of the problems intrinsically related to a type I design (i.e., promiscuous activity and IP novelty). However, it is becoming apparent that targeting the inactive conformation poses a new range of problems. In light of this, it was of interest to reassess the opportunities offered by the DFG "in" form of the kinase in the design of inhibitors with a controlled selectivity profile and a favorable IP position.

From the structural analysis of recently published kinase inhibitors a class of novel kinase inhibitors that follow a new design paradigm emerged. These compounds specifically target the back cavity of the ATP in either catalytically active (DFG "in" and αC-helix "in") or inactive (DFG "in" and αC-helix "out") kinases and conform to a common pharmacophore. Because it can be viewed as a hybrid of the existing types I and II pharmacophores, we defined this novel pharmacophore as type I$^{1}/_{2}$ (Figure 2D). Type I$^{1}/_{2}$ inhibitors bind to the adenine ring region like type I compounds establishing hydrogen bonds with the hinge region and then extend into the back cavity of the ATP site to give specific interactions with those residues that are involved in the type II pharmacophore. Thus, following this design, it is possible to target a DFG "in" kinase with type II interactions that are typical of a DFG "out" form.

The back cavity is mainly hydrophobic and is not occupied by the natural cosubstrate ATP. Its shape and size are primarily controlled by the nature of the gatekeeper residue which is the first residue of the hinge connecting the C- and N-terminal lobes. When the gatekeeper residue has a bulky side chain (like Phe in CDK2, for example; Figure 3A), the back cavity is small and delimited by β-sheets 4 and 6 and by the gatekeeper side chain itself. When the gatekeeper is small (like Thr in bRAF, Figure 3B), its side chain no longer limits the size of the cavity which expands toward β sheet 5 and the αC-helix.

It is clear that targeting a kinase with a small gatekeeper by placing a substituent in the large back cavity may have the dual effect of increasing potency and increasing selectivity over those kinases that have a large gatekeeper and hence a small back cavity. In particular, it is interesting to note that in

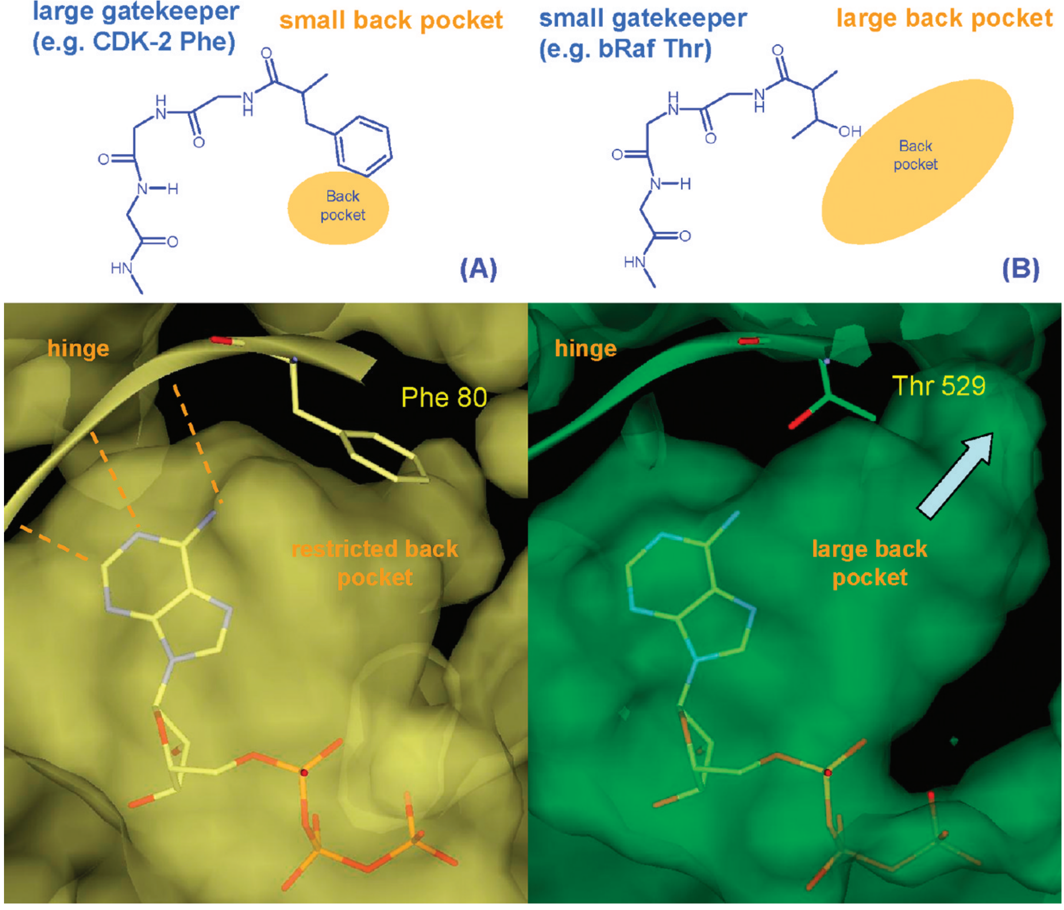


Figure 3. Comparison of the hydrophobic back pockets of a kinase proteins with a large (CDK2) and a small (bRAF) gatekeeper residue. (A) Molecular surface of ATP binding cavity in the ATP-CDK2 complex (PDB 1b39). The large gatekeeper residue Phe 80 limits the dimensions of the back pocket. (B) Molecular surface of the ATP binding cavity in the SB590885-bRAF complex (PDB 2fb8). The small gatekeeper residue Thr 529 allows a large back pocket. To facilitate comparison, the SB590885 ligand was removed and replaced by the ATP molecule after superimposition of the two structures. For clarity, only the gatekeeper residue and the hinge region of the protein are shown.

a DFG "in" kinase, not only does the presence of a small gatekeeper residue result in a larger cavity but the backbone atoms of the DFG triad and the conserved αC-helix glutamic acid also become exposed to the binding cavity and could interact with a putative ligand. These are the same conserved residues related to the type II pharmacophore observed in the DFG "out" inactive conformation.

The novel type $I^1/_2$ pharmacophore we propose contains all the elements of the type I pharmacophore describing the interactions at the hinge and the adenine ring region plus three elements of the type II pharmacophore: (i) the linker moiety, (ii) the hydrogen bond donor interacting with the Glu carboxylate, and (iii) the hydrogen bond acceptor interacting with the Asp backbone NH of the back cavity (Figure 2D).

The back cavity was first recognized as a druggable ATP subsite by Tong and co-workers who solved the structure of the mitogen-activated protein kinase 14 (MAPK14, also known as p38) in complex with an analogue of the highly specific *p*-fluorophenylpyridinylimidazole SB203580 inhibitor **9** (IC_{50}(MAPK14) = 48nM; Figure 4) showing that the compound occupies the back cavity.[37,38] Other kinase inhibitors developed in the past, such as dasatinib **5** or lapatinib **6**, target this area obtaining different levels of specificity.[39]

The back cavity is a poorly conserved area of the ATP binding site and, as a result of the different relative orientations of the C- and N-terminus lobes and the conformational flexibility of the DFG motif which can adopt several "in" like conformations, is characterized by a high level of plasticity among kinases.

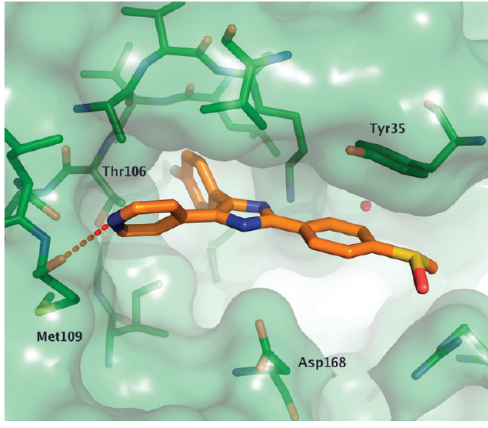
The possibility of targeting the back cavity following the specific type $I^1/_2$ pharmacophore, coupled with the intrinsic high level of plasticity and the lower sequence conservation, might offer the opportunity to rationally develop a new class of inhibitors either de novo or by conversion of type I scaffolds and provide an additional tool in the highly needed modulation of kinase inhibitors specificity.

Type $I^1/_2$ Kinase Inhibitors

Src Tyrosine Kinase. Gatekeeper Thr. An interesting example of type $I^1/_2$ compounds is the purine-based compound **10** (AP23464) developed by ARIAD (Figure 5) which targets Src tyrosine kinase with picomolar affinity.[40]

In the Src−**10** complex (PDB code 2bdj, Figure 5) it is possible to observe how the compound sits in the ATP pocket of the kinase in a DFG "in" state. The presence of the small gatekeeper residue Thr338 results in the opening of a large back cavity that exposes the conserved Glu310 and the backbone atoms of the DFG motif (Asp404, Phe405, and Gly406) offering the possibility of a type $I^1/_2$ ligand design.

The structural data clearly show how the binding mode of compound **10** satisfies the type $I^1/_2$ pharmacophore. Like a



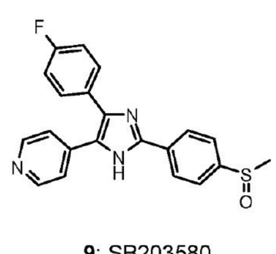


Figure 4. Chemical structure of the p38 inhibitor **9** and its mode of binding in the ATP binding site.

type I inhibitor, the compound interacts with the protein through two hydrogen bonds at the hinge: one between the acceptor N7 of the purine and the donor backbone NH of Met341, the other one between the aniline donor NH in position 6 of the purine and the carbonyl oxygen of Met341 that acts as acceptor. In addition to interactions at the hinge, the (3-hydroxyphenethyl)-N9 purine substituent penetrates deeply in the back cavity where the pharmacophore elements are present. The aromatic ring of the phenethyl moiety acts as linker unit and is surrounded by the hydrophobic side chains of Met314, Ile336, Thr338, and the hydrophobic part of Lys295. The hydroxyl group in meta position on the phenyl ring matches both the hydrogen bond acceptor and hydrogen bond donor type $\text{I}^{1}/_{2}$ pharmacophore elements of the back cavity, as it establishes hydrogen bonds with both Asp404 backbone NH (as acceptor) and Glu310 carboxylate (as donor). It is interesting to note that compound **10** was initially developed from purvalanol A, a 2,6,9-trisubstituted purine derivative optimized for CDK2 inhibition (IC_{50} = 70 nM for CDK2 and IC_{50} = 240 nM for Src), carrying on N9 an isopropyl group instead of the hydroxyphenethyl moiety. The kinase CDK2 is characterized by a phenylalanine as gatekeeper residue (Phe80). The bulky side chain of the gatekeeper severely limits the size of the back pocket in CDK2 which is just large enough to accommodate the isopropyl group. When the isopropyl group in purvalanol A is replaced by the hydroxyphenethyl moiety in derivative **10**, the activity on CDK2 drastically decreases (IC_{50} = 20.9 μM), as the phenethyl group can no longer be accommodated in the back cavity because of steric hindrance with the gatekeeper Phe80 side chain. When assessed against a panel of kinases, compound **10** showed very potent activity against Src (0.45 nM) and notable activity against Abl, FYN, YES, Lck, LYN, EGFR, HER2, PDGFR, FGFR, cKIT, and bRAF, all kinases characterized by a small gatekeeper residue.

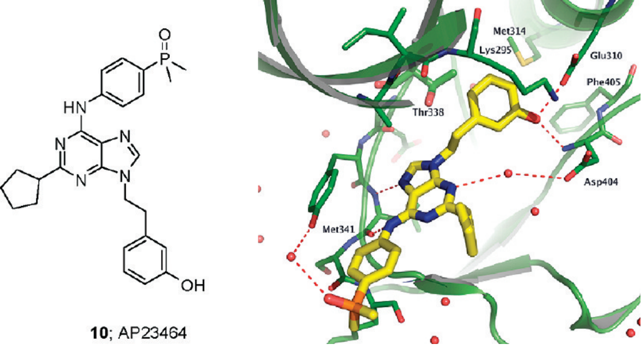


Figure 5. Chemical structure of the Src inhibitor **10** and its mode of binding in the ATP binding site.

Compound **10** is a good example of how a type $\text{I}^{1}/_{2}$ design may completely alter the kinase selectivity profile of a known scaffold, opening new opportunities for scaffold rescue, scaffold hopping, and the creation of intellectual property.[41] Moreover, the reduced incidence of resistant mutations observed for the compound **10** highlights the importance of targeting the kinase active conformation by design of new small molecule inhibitors aiming to have more efficient cancer treatments.[42]

Vascular Endothelial Growth Factor Receptor 2 (KDR). Gatekeeper Val. Recent work carried out by Amgen researchers on the KDR inhibitors *N*-alkyl(aryl)naphthamides provides another example of how a type $\text{I}^{1}/_{2}$ design could result in an improved selectivity profile.[35] Initial work on this scaffold provided access to low nanomolar KDR inhibitors and led to the identification of compound **11** (PDB code 3b8q, Figure 6) which demonstrated robust KDR activity (IC_{50} = 0.48 nM) and in vivo efficacy but also retained an undesired inhibitory activity against Aurora B, a serine–threonine kinase strongly involved in mitotic cellular process, and also against the nonreceptor tyrosine kinase Lck, involved in T cell receptor signal transduction. During medicinal chemistry activity the close analogue compound **12** (PDB code 3b8r, Figure 6) emerged displaying again a favorable activity against KDR (IC_{50} = 0.60 nM) but also a more restrictive selectivity profile in a small panel of kinases including Aurora A, Aurora B, Lck, KDR, TIE2, and MET.

Structural data provided insight into the origin of the different selectivity profile. From an analysis of the crystallographic structure it emerged that compound **11** is a type II inhibitor that binds to the KDR catalytic domain in a DFG "out" inactive state. The sp^2 nitrogen (acceptor) of the dimethoxyquinoline ring is involved in a hydrogen bond with the backbone amide NH of Cys919 of the hinge region (donor). The naphthyl moiety occupies the hydrophobic pocket facing the small gatekeeper (Val916 mutated to Thr in this structure) and projects the amide deeply into the back cavity. The carbonyl oxygen (acceptor) and the NH group (donor) of the amide are engaged in two hydrogen bonding interactions with the type II pharmacophore elements of the back cavity: the backbone NH of Asp1046 of the conserved DFG (donor) and the carboxylate on the side chain of the

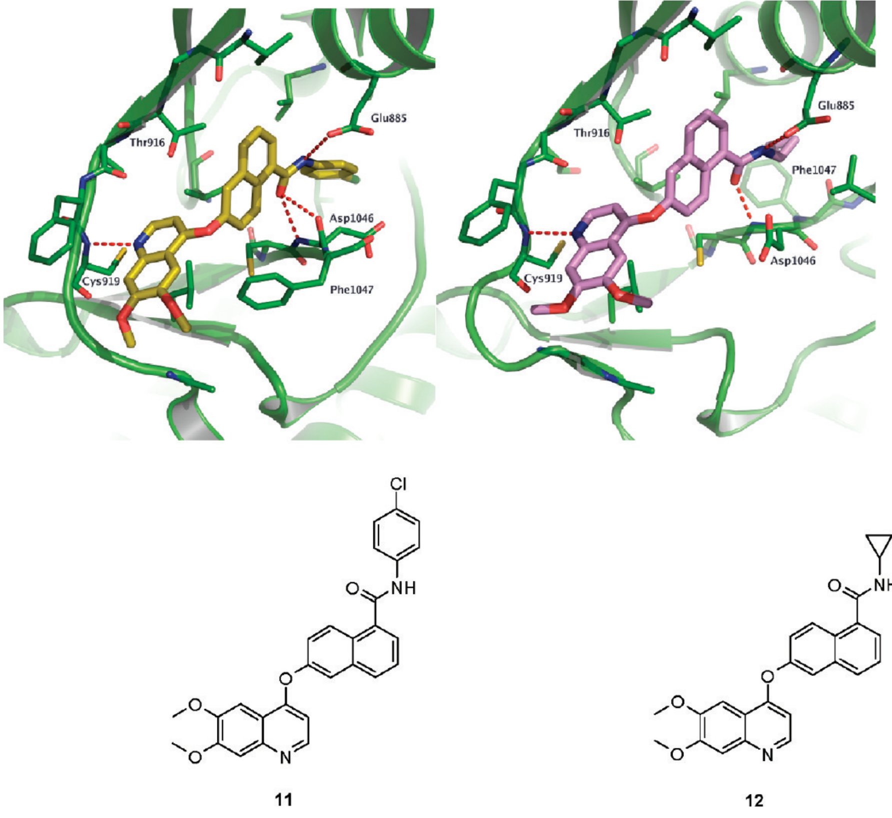


Figure 6. Chemical structure of the KDR inhibitors **11** and **12** and their mode of binding in the ATP binding site.

conserved Glu885 (acceptor). The *p*-chlorophenyl ring penetrates into the allosteric site of the DFG "out" state matching the terminal hydrophobic pharmacophore element of the type II model. On the contrary, compound **12**, in which the *p*-chlorophenyl group on the amide is replaced by a smaller and less hydrophobic cyclopropyl group, albeit adopting a very similar mode of binding, binds to the DFG "in" state of the protein following a typical type $\mathrm{I}^{1}/_{2}$ design. The quinoline system establishes the same interaction with the hinge, and the naphthyl moiety accesses the back cavity occupying the same area as in compound **11**. The small gatekeeper provides full access to the back cavity, allowing access to the type II pharmacophore elements even from the DFG "in" state, and the amide establishes the same interactions with Asp1046 and Glu885. It is very interesting to note that the *N*-alkyl(aryl)naphthamides scaffold occupies the adenine ring region of the ATP site and then develops into the back cavity without occupying the sugar and phosphate region. This allows this scaffold to bind to both DFG states of the kinase even when, in the presence of a DFG out, the ATP site is partially occupied by the DFG Phe side chain. The equilibrium between the DFG "in" and DFG "out" forms seems to be controlled by the nature of the alkyl(aryl) substituent.

These KDR inhibitors also provide evidence that the presence of a large hydrophobic moiety (like the *p*-chlorophenyl group in compound **11**) plays a crucial role in the type II inhibitors stabilization of the DFG "out" state in which the hydrophobic spine of the kinase is dismantled. In the DFG "in" form, the DFG Phe side chain is part of the hydrophobic spine, a structural element that stabilizes the active form of the kinase despite the unfavorable DFG Asp torsional angles adopted in this conformation. When the DFG flips in a "out" conformation, the Phe side chain moves away from its position and occupies the ATP pocket, introducing an element of instability by disrupting the hydrophobic spine. In the presence of compound **11**, a type II ligand, the terminal hydrophobic moiety *p*-chlorophenyl in the allosteric site partially re-establishes the hydrophobic spine, thus stabilizing the inactive kinase conformation.[19] On the contrary, in compound **12**, the smaller cyclopropyl ring does not provide enough stabilization to the hydrophobic spine and the DFG flips into the "in" state so that the Phe side chain can provide the required hydrophobic interaction.

When tested on a larger panel of 43 kinases, compound **12** displayed a nanomolar range potency for structurally related proteins like PDGFR, KIT, and FMS but not FLT3, which displays a large gatekeeper (Phe), and good selectivity versus EGFR, Src, p38, Abl, FGR, SYK, ZAP70, JAK2, IRK, and TIE2. Interestingly, in this case, the type II inhibitor compound **11** is less selective than the type $\mathrm{I}^{1}/_{2}$ analogue compound **12**, showing that conformational variability associated with the DFG motif in the "in" state and the variability in the back cavity sequence coupled with a type $\mathrm{I}^{1}/_{2}$ design can be exploited to produce selective and still potent compounds targeting the DFG "in" state of the kinase. A similar example, where the equilibrium between the DFG states of the protein can be modulated by the presence or absence of a large hydrophobic moiety in the back cavity, can be found in work carried out by GSK on biphenylamides targeting the p38α MAP kinase and in Amgen's work on Lck inhibitors.[43,44] Thus, by selection of the proper back cavity substitution, it is possible to induce or trap a preferred DFG state.

Polo-like Kinase 1. Gatekeeper Leu. An additional example of type $\mathrm{I}^{1}/_{2}$ pharmacophore matching molecule is represented by the compound **13** disclosed by Sunesis (Figure 7),

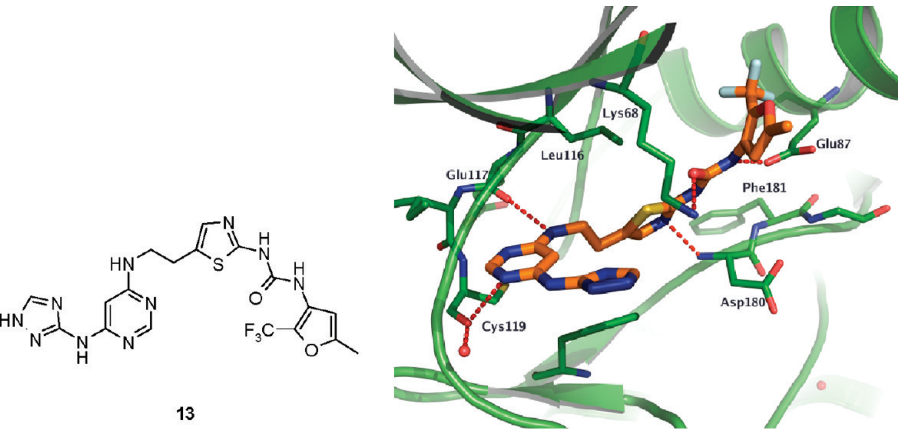


Figure 7. Chemical structure of the Sunesis PLK1 inhibitor **13** and its mode of binding in the ATP binding site.

identified as a polo-like kinase 1 inhibitor (IC_{50} = 720 nM) during the screening of an Aurora A targeted library.[45] Structural data (PDB code 3db6, Figure 7) show that the compound binds to the hinge of PLK1 through two hydrogen bonds: a strong one between the N1 pyrimidine nitrogen (acceptor) and the Cys119 backbone NH (donor), and a weaker one between the anilino NH on the pyrimidine and the carbonyl oxygen of Glu117.

The triazole substituent is placed in the sugar region, while the rest of the molecule extends from the 4-NH_2 group of the pyrimidine ring to occupy the large back cavity made available by the presence of the midsized gatekeeper residue Leu116. In this case the linker element of the pharmacophore is the aminoethyl group connecting the pyrimidine ring with the thiazole ring. The sp^2 nitrogen atom of the thiazole matches the type $I^1/_2$ acceptor pharmacophore feature in the back cavity, establishing a hydrogen bond with the backbone NH of the DFG Asp180. The type $I^1/_2$ donor pharmacophore feature in the same area is satisfied by one of the urea nitrogens which is hydrogen-bonded to the conserved Glu87. Interestingly, by comparison of the PLK1−**13** complex with the structure of PLK1 complexed with compounds that do not occupy the back cavity (such as the complex with BI6727, PDB 3fc2, for example), it is possible to observe that β-sheet 4 and the glycine rich loop move slightly upward to accommodate the trifluoromethylfuran part of the ligand. In particular the Lys68 side chain extends underneath the glycine rich loop and establishes a hydrogen bond to the urea carbonyl oxygen of the ligand. The trifluoromethylfuran moiety forces its way into the binding site, disrupting the Lys68−Glu87 salt bridge, and occupies the area made available by side chain rearrangement that follows the breaking up of the charged interaction. This ligand-induced effect seems to be made possible by the fact that PLK1 is characterized by a weaker Lys−Glu interaction (>4.0 Å). Other kinases, like cSrc, BTK, JNK3, or MPS1, present a similar feature that could be targeted, within a type $I^1/_2$ design.

In the three examples just described the functional group of the ligand matching the hydrogen bond acceptor feature of the type $I^1/_2$ pharmacophore in the back cavity area (i.e., the 3-hydroxyl group on the phenethyl moiety in the case of compound **10** bound to Src, the amide carbonyl in the case of compound **12**, and the sp^2 nitrogen of the thiazole in the case of compound **13** bound to PLK1) interacts with the backbone NH of DFG Asp (Asp180 in PLK1 and Asp404 in Src). In most cases the DFG Phe NH is engaged in an interaction with the conserved Glu residue (i.e., Glu310 in Src and Glu87 in PLK1) either directly or through a water molecule. There are instances however, where the same hydrogen bonding acceptor feature of the ligand interacts with the DFG Phe backbone NH instead, either replacing the water molecule that mediates the interaction or interposing itself between the interacting residues.

Checkpoint Kinase 1. Gatekeeper Leu. A specific example where the ligand interacts with the DFG Phe backbone NH is the novel checkpoint kinase 1 (CHK1) inhibitor compound **14** (Figure 8, IC_{50} = 6.2 nM), developed by Abbott from a class of tricyclic pyrazoles initially identified during an HTS campaign.[46]

The crystal structure of the complex between CHK1 and compound **14** (PDB code 2e9n, Figure 8) showed that the pyrazole nitrogen atoms of the scaffold make two hydrogen bonding interactions with the hinge residues: one between the sp^2 N1 nitrogen (acting as hydrogen bond acceptor) and the backbone NH of Cys87 (donor), and one between the N2 NH which donates to the carbonyl oxygen of Glu85. The midsized side chain of the gatekeeper residue Leu84 results in a large back cavity that is occupied by the biphenylhydroxyl moiety. Similar to compound **10** in Src, the biphenyl system provides the linker of the type $I^1/_2$ pharmacophore, which delivers the hydroxyl group matching both the hydrogen bonding donor and acceptor pharmacophore features present in the back cavity. The hydroxyl group replaces the water molecule that normally mediates the interaction between the conserved Glu55 and DFG Phe149 backbone NH. A new hydrogen bond network is then established where the hydroxyl group of the ligand interacts as hydrogen bond acceptor with DFG Phe149 backbone NH and as hydrogen bond donor with Glu55. An additional hydrogen bonding interaction is also established with Asn59.

When tested on a panel of 13 serine/threonine kinases, compound **14** showed a good selectivity profile (at least 200-fold more active against CHK1 than the other kinases with only three kinases hit in the micromolar range). Interestingly, compound **14** showed a limited activity against only one of the nine kinases with a large/long gatekeeper residue (AKT, IC_{50} = 4.43 μM, Met as gatekeeper), whereas of the four kinases with a small/medium sized gatekeeper, two were inhibited at low micromolar levels (AUR1, IC_50 = 8.09 μM; SGK, IC_{50} = 1.32 μM both with Leu as gatekeeper) and two were not inhibited (CHK2 and Src with Leu and Thr as gatekeeper residue). In these cases, differences in binding residues in the ATP region (e.g., Asn59, Tyr86, and Ser147

Figure 8. Chemical structure of the CHK1 inhibitor **14** and its mode of binding in the ATP binding site.

become Leu227, Leu303, and Thr367 in CHK2) rather than the interactions in the back cavity may be considered responsible for the selectivity.

The last two examples clearly show how a type $I^{1}/_{2}$ design can be applied not just when the gatekeeper residue is small, but with an appropriate chemistry strategy, it is possible to extend it to kinases with medium sized gatekeeper residues such as Leu, Ile, and Met. This could provide an interesting opportunity to develop more effective kinase inhibitors and a successful strategy for dealing with gatekeeper mutations responsible for many resistance mechanisms in oncology. Additional examples of type $I^{1}/_{2}$ inhibitors identified in the literature include inhibitor **15** developed by Ariad,[47] inhibitors **16**, **17**, **18**, **19** by GSK,[48−50] inhibitor **20** by BMS,[51] inhibitor **21** by Amgen,[52] inhibitors **22** and **23** by Bayer,[53,54] inhibitor **24** by Pfizer,[55] inhibitor **25** by Vertex,[56] inhibitor **26** by Wyeth,[57] and inhibitor **27** by Zurich University[58] targeting kinases like Src, Abl, Lck, p38, Ret, EphB4, VEGFR-2, Jak2, and bRAF (Figure 9).

Type $I^{1}/_{2}$ Pharmacophore: Relevance and Selectivity

The possibility of developing kinase inhibitors following a type $I^{1}/_{2}$ design should be evaluated target by target. Because a large and accessible back cavity is required, kinases with a small or medium sized gatekeeper will probably offer better opportunities for a successful type $I^{1}/_{2}$ inhibitors development whereas kinases characterized by a large gatekeeper might be more problematic. Despite this limitation, the relevance of the type $I^{1}/_{2}$ design can be extended to a considerable portion of the human kinome. An in-house analysis of 420 closely related kinase sequences highlighted that about 23% have a small gatekeeper with threonine being the most and alanine the least common residues (Table 2).[59] Very bulky gatekeepers like Phe and Tyr account for about 16% of the analyzed kinase sequences, whereas the majority, about 61% of the total, is characterized by midsized gatekeepers that are branched, like Leu and Ile, or characterized by a long but yet flexible side chain like Met (the most frequent gatekeeper residue of the analyzed set with 40% of the occurrences).[60] Clearly, small-sized gatekeeper residues (Gly, Ala, Ser, Cys, Val, and Thr) result in the larger back cavities. In this case several different chemical scaffolds are well suited to place substituent groups inside the back cavity providing easy access to the type $I^{1}/_{2}$ pharmacophore. On the contrary, gatekeeper residues with a large side chain (Phe, Tyr) considerably limit the size of the back cavity, virtually blocking the access to the type $I^{1}/_{2}$ pharmacophore. A different scenario occurs when a medium-size or flexible side chain gatekeeper is present. In this case, the possibility of placing a substituent in the back cavity and interacting with the type $I^{1}/_{2}$ pharmacophore is a fine balance between the type of gatekeeper residue and the core chemical scaffold of the ATP mimicking ligand. In particular, the chemical nature and the directional vector of the substituent projected toward the back pocket play a crucial role in a successful design aimed at occupying the back cavity and interacting with the type $I^{1}/_{2}$ pharmacophore. In the previously discussed PLK1−**13** complex, for instance, starting from an aminopyrimidine moiety binding at the hinge, access to the back cavity and interaction with the type $I^{1}/_{2}$ pharmacophore were achieved by introducing the flexible ethyl spacer which avoided clashes with the Leu gatekeeper. Methionine, which is characterized by a fairly bulky but yet flexible side chain, is another example. Even in the presence of a Met as gatekeeper, compounds can sometimes still access and occupy the back pocket by shifting the Met side chain with an induced-fit type of mechanism. One reported example is the 3 Å movement observed for the gatekeeper Met146 side chain in JNK3, when an imidazolopyrimidine-based inhibitor is bound.The gatekeeper movement allows the opening of the back cavity and the positioning of the dichlorophenyl moiety of the ligand.[61] Another recent example has been presented by Peifer and co-workers, where diarylisoxazoles or -imidazoles revealed potent and dual inhibitors of p38 (Thr as gatekeeper) and CK1 (Met as gatekeeper).[62]

The gatekeeper residues in protein kinases regulate the different sizes and the different levels of accessibility to the back cavity and type $I^{1}/_{2}$ interactions in the ATP pocket in a DFG "in" conformation kinase. This, together with the lower level of sequence conservation and a considerable plasticity of the back cavity, offers interesting opportunities for selective drug design aimed at achieving the desired activity profile for a given chemical class. In addition, a further control on the activity profile within the type $I^{1}/_{2}$ ligand design can be achieved considering the different distances between the pharmacophore elements, in particular, the distances between the ones shared with the hinge (type I pharmacophore) and

15; Ariad (Src-Abl)

16; GSK (Lck)

17; GSK (Lck)

18; GSK (p38)

19; GSK (RET)

20; BMS (p38)

21; Amgen (LCK)

22; Bayer (EphB4)

23; Bayer (VEGFR2)

24; Pfizer (b-Raf)

25; Vertex (Jak2)

26; Wyeth (b-Raf)

27; Zurich University (EphB4)

Figure 9. Additional structures of type $I^1/_2$ kinase inhibitors. All compounds target kinases with a small/medium gatekeeper residue. The region interacting with back cavity portion of the type $I^1/_2$ pharmacophore is indicated in blue. In parentheses is the kinase target for which the compound has been developed.

Table 2. Gatekeeper Residues Distribution in Kinome (420 Closely Related Kinase Sequences)[a]

gatekeeper	no. of kinases	%	size
Gly	1	0.24	S
Ala	1	0.24	S
Ser	3	0.71	S
Cys	2	0.48	S
Thr	76	18.10	S
Val	13	3.10	S
Ile	9	2.14	M
Leu	72	17.14	M
Met	169	40.24	M
Gln	8	1.90	M
Phe	63	15.00	L
Tyr	3	0.71	L

[a] Residues have been grouped in three sets according to size. Met has a long but flexible side chain and was classified as medium-sized.

the pharmacophore elements in the back cavity specific to type $I^1/_2$. For instance, the distance between the backbone NH of the hinge (the one responsible for the hydrogen bond acceptor feature in the adenine area of the type I) and the donor NH of the DFG Phe is 14.0 Å in CHK1, similar to 13.5 Å in bRAF, but it is 17.2 Å in JNK3. These different distances imply that starting from the same scaffold that interacts at the hinge level, a different linker, hence a different chemistry design, will be required to access the type $I^1/_2$ pharmacophore in CHK1 or bRAF and JNK3.

The gatekeeper residue might also play a relevant role in type II inhibition, as it might control which kinases can access the DFG "out" conformation. In fact most of the kinases for which type II inhibitors have been so far discovered display a small gatekeeper residue and in particular the threonine residue. As such, the nature of the gatekeeper residue can deeply affect the strategy underlying a kinase inhibitor development program, offering several opportunities to achieve potency and modulate the selectivity profile of the compounds.

Future Directions

As discussed, a totally clean selectivity profile is not necessarily the main goal in kinase inhibitors development. At the same time, a general promiscuity is highly undesirable because of unexpected off-target effects that might result in severe toxicity. It is now emerging that developing compounds that are characterized by a selective but not specific activity profile could provide a successful strategy in providing an effective answer to cancer therapeutic needs. The possibility of controlling compound selectivity is still a pressing need in the development of new generations of kinase inhibitors with the desired activity profile.

The growing number of type II kinase inhibitors that have been successfully marketed for the therapeutic treatment of several cancer forms is a step in the right direction, as they are often characterized by a cleaner selectivity profile. However, the future success of this kinase inhibitor design is hindered by a number of issues that might limit its availability. One of the major drawbacks is that type II compounds, targeting the DFG "out" form, may suffer from mutations of the molecular target that confer resistance often by stabilizing the kinase active conformation. Another problem associated with type II inhibitors is that, at present, it is not possible to assess how many and especially which kinases might adopt the DFG "out" conformation. Hence, the relevance of the type II inhibitors design could be limited to an uncertain portion of the kinome. Not knowing whether the DFG "out" might be available as a drug target and the limitations imposed by the lack of a type II specific screening assay technology platform could limit the further development of other type II inhibitors.[32] As the initially perceived advantages offered by targeting the DFG "out" binding mode are becoming increasingly questionable, the possibility of developing inhibitors with a controlled selectivity by targeting the active DFG "in" form of the kinase might offer considerable advantages.

The type $I^1/_2$ pharmacophore model described in this work targets the hinge, the adenine ring area, and the back cavity of the ATP binding site and acts on the DFG "in" active state of the kinase. In principle, the same design could be applied to DFG "in" inactive kinases (i.e., DFG "in" but αC-helix "out"). Targeting the back cavity by establishing hydrophobic and specific hydrogen bonding interactions (with the conserved Glu and DFG Asp NH) offers interesting opportunities to achieve potency and selectivity maintaining a good level of ligand efficiency. The intrinsic plasticity of the DFG motif and the conformational flexibility of its side chains could represent an additional opportunity to achieve the desired modulation. In the future, type $I^1/_2$ design could provide an additional tool and a different strategy in the successful design of a new generation of potent kinase inhibitors with a controlled activity profile, also targeting the growing number of resistant mutations emerging in patients. The type $I^1/_2$ design is also relevant for kinases with medium-sized gatekeepers that are often involved in such mutations (e.g., T315I in Abl).

It is also becoming apparent that in targeting kinases, it is not possible to identify a universal strategy that can be applied to achieve a successful and efficacious treatment. For each type of tumor, with its particular genetic makeup, and for each kinase target the most appropriate inhibition strategy, controlled selectivity vs multitarget, and the most appropriate inhibitor design (type I, type $I^1/_2$, or type II) should be specifically evaluated.

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Biographies

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Elena Ardini received her Laurea degree in Biology from Università degli Studi, Milano, Italy, in 1993. From 1993 to 2000 she was a postdoctoral fellow working with Dr. Sylvie Ménard at the Department of Experimental Oncology of the National Cancer Institute of Milan where she was involved in oncology projects mainly focused on investigating the role of tyrosine kinase receptors in breast cancer progression. In 1997, she spent 1 year as a Visiting Research Scientist in Prof. Joseph Schlessinger's laboratory at the Department of Pharmacology of New York University. In 2000 she joined Pharmacia & Upjohn in Nerviano, Italy (later Nerviano Medical Sciences), where she is currently Project Leader working in the Department of Biology.

Elena Casale received her Laurea degree in Pharmaceutical Chemistry from Università degli Studi, Milano, Italy, in 1989. She was a postdoctoral fellow at the Marshall Space Flight Center, NASA, Huntsville, AL, working in the Crystallography group under the supervision of Dr. D. Carter. She did further postdoctoral training with Prof. M. Bolognesi at the Department of Genetic Microbiology of the University of Pavia, Italy. In 1992 she joined Farmitalia Carlo Erba (later Pharmacia & Upjohn and currently Nerviano Medical Sciences), working in the Structural Chemistry Group. She is currently a Principal Research Scientist contributing to several research projects in the oncology area.

Mauro Angiolini received in 1995 a Laurea degree in Chemistry and a Ph.D. in Medicinal Chemistry in 1998, working under the supervision of Prof. Carlo Scolastico at the Università degli Studi, Milano, Italy. He joined Pharmacia & Upjohn in Nerviano (later Nerviano Medical Sciences) where he has been involved in many projects related to the discovery of new drugs in the oncology therapeutic area, with considerable focus on kinases inhibitors. In 2000 he spent 1 year as postdoctoral fellow in Prof. S. Hanessian group at Université de Montréal, Canada, focusing on application of the ring closing metathesis reaction. He is currently a Principal Research Scientist working in the Department of Medicinal Chemistry of the Oncology Division.

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