Protein kinases as targets for drug design

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REVIEW

ABSTRACT

Protein kinases have become the second most important group of drug targets after G protein coupled receptors. Deregulated kinase activity is the frequent cause of many diseases, especially cancer, where kinases regulate many aspects that control cell growth and death. Drugs inhibiting specific kinases are being developed to treat this disease, and some are already in clinical use. This article reviews the current strategies to inhibit protein kinase activity in cancer therapy and illustrates the application of bioinformatics to design novel protein kinase inhibitors.

Keywords: Protein kinases, cancer, receptor, virtual screening, docking, molecular modelling, drug design

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RESUMEN

Las proteínas quinasas como blancos en el diseño de fármacos. Las proteínas quinasas se han convertido en el segundo grupo más importante de proteínas utilizadas como blanco para el diseño de fármacos luego de los receptores asociados a proteínas G. La desregulación de la actividad de estas proteínas constituye la causa frecuente de varias enfermedades, especialmente del cáncer, donde estas quinasas regulan un conjunto de aspectos tan importantes como el control del crecimiento y la muerte celular. Se han desarrollado varias drogas para el tratamiento del cáncer que inhiben la actividad de quinasas específicas, algunas de los cuales ya están siendo utilizadas en la clínica. Este artículo resume las estrategias actuales para la inhibición de la actividad quinasa y su uso en la terapia del cáncer e ilustra la aplicación de la bioinformática en el diseño de nuevos inhibidores de proteínas quinasas.

Palabras clave: Proteínas quinasas, cáncer, receptor, pesquisa virtual, acoplamiento, modelación molecular, diseño de fármacos

*P*rotein kinases

Protein kinases regulate the majority of cellular pathways, especially those involved in signal transduction [1, 2] by catalyzing phosphorylation reactions. Phosphorylation consists on the delivering of a single phosphoryl group from the ATP to protein substrates; it may occur on hydroxyl side chains of serine, threonine and tyrosine amino acids, on imidazole nitrogen of histidine and the carboxyl oxygen at the side chain of aspartic acid residues. Phosphorylation usually results in a functional change of the substrates by shifting enzyme activity, cellular location, or association with other proteins.

More than 500 protein kinases are predicted to exist, based on the human genome sequencing, corresponding about 2% of all eukaryotic genes. Protein kinases are grouped in to three main classes, based upon substrate preferences: the most common are serine-threonine kinases (S/TKs), followed by tyrosine kinases (TKs), with the rarest being both S/TKs and TKs, the so-called dual-function kinases (DFKs) [3, 4]. TKs are classified in receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinase (NRTKs). Histidine kinases have also emerged as signalling enzymes [5] but because their novelty they will not be covered in this review.

Illustrative of how protein kinases can mediate signal transduction, is the Ras/Raf mitogen-activated protein kinase (MAPK) being one of the major signalling routes for the epidermal growth factor receptor (EGFR) tyrosine kinase [6]. EGFR is a 170 kDa RTK protein comprising three major functional domains: an extracellular ligand-binding domain, a hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase domain. EGFR exist on the cell surface as an inactive monomer and is activated upon the binding of a specific set of ligands (*e.g*, EGF, TGF-α, and others) to its extracellular domain, triggering the receptor dimerization and autophosphorylation [7, 8] on tyrosine residues within the C-terminal tail. After autophosphorylation, several signal transduction pathways downstream of EGFR become active [9]. The specific components of the MAPK cascade vary among different stimuli, but the architecture of the pathway usually includes a set of adaptors such as Grb2 that link the EGFR to a guanine nucleotide exchange factor (GEF) like the son of sevenless (Sos). GEF stimulates Ras protein, which in turn activates the core unit of the cascade composed of a MAPKKK (Raf), a MAPKK (MEK1/2) and MAPK (ERK). The activated ERK dimer regulates several targets in the cytosol (p90RSK) and also translocate to the nucleus where it phosphorylates a variety of transcription factors (*e.g*, c-Myc, STAT-3, ELK-1) culminating in DNA transcription (Figure 1). The tyrosine kinase receptor signal can be terminated by endocytosis of the phosphorylated receptor-ligand complex [7].

Normally, protein kinase activity is strictly regulated, however, under pathological conditions protein kinases can be deregulated, leading to alterations in the phosphorylation and resulting in uncontrolled cell division, inhibition of apoptosis, and other disease causing abnormalities [3]. Kinases and cancer have been

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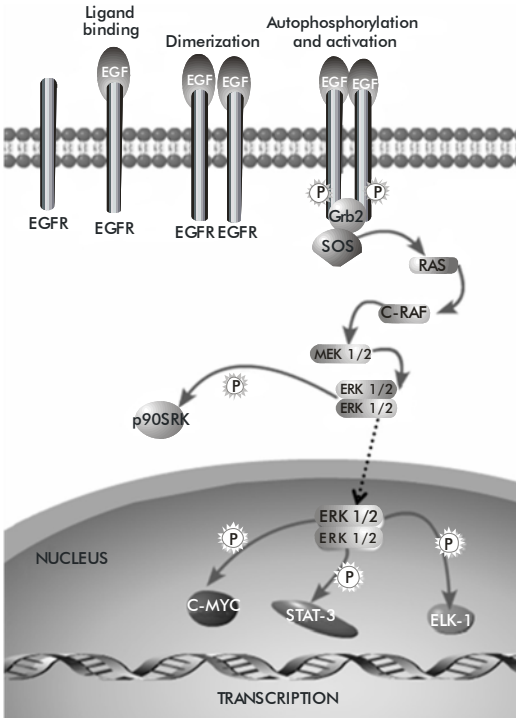

Figure 1. Schematic representation of the Ras/Raf mitogen-activated protein kinase signaling pathway. Epidermal growth factor receptor (EGFR) is activated by extracellular ligands, such as EGF (epidermal growth factor). Binding of EGF activates the tyrosine kinase activity of the cytoplasmic domain of the receptor. The EGFR becomes autophosphorylated on tyrosines. Docking proteins such as GRB2 contain SH2 domains bind to the phosphotyrosines of the activated receptor. GRB2 binds to the guanine nucleotide exchange factor SOS by way of an SH3 domain of GRB2. When the GRB2-SOS complex docks to phosphorylated EGFR, SOS becomes activated. Activated SOS promotes the removal of GDP from Ras. Ras can then bind GTP and become active. Activated Ras activates the protein kinase activity of RAF kinase, a serine/threonine protein kinase. RAF kinase phosphorylates and activates MEK, another serine/threonine kinase. MEK phosphorylates and activates mitogen-activated protein kinase (ERK). MAPK was originally called "extracelular signal-regulated kinase" (ERK) and latter was renamed "mitogen-activated protein kinase" (MAPK). This signal transduction cascades causes cell survival and proliferation providing also opportunities for feedback regulation and signal amplification.

Table 1. Kinase inhibitors approved for cancer treatment up to the end of 2006

Company	Inhibitor	Kinase targeted	Strategy	Indication	Entry
Bristol-Myers Squibb	Sprycel (dasatinib)	BCR-ABL, SRC family (Src, Lck, Yes, Fyn), c-kit, Epha2, and PDGFR-β	multiple TK ATP small inhibitor	CML	June, 2006
Pfizer	Sutent (sunitinib)	PDGFR-α, PDGFR-β,VEGFR-1, VEGFR-2 and VEGFR-3, kit, FLT3, CSF-1R and RET	multiple TK ATP small inhibitor	Kidney Cancer, GSIT	January, 2006
Amgen	Vectibix (panitumumab)	EGFR	recombinant, human mAb	Colorectal Cancer	September, 2006
Bayer/Onyx	Nexavar (sorafenib)	C-RAF, B-RAF, kit, FLT-3, VEGFR-2, VEGFR-3 and PDGFR-β	multiple S/TK and TK ATP small inhibitor	RCC	December, 2005
Imclone, Bristol-Myers Squibb	Erbitux (cetuximab)	EGFR, HER1, c-ErbB-1	recombinant, human/mouse chimeric mAb	Colorectal Cancer	February, 2004
Genentech and OSI Pharmaceuticals	Tarceva (erlotinib)	HER1, EGFR	TK ATP small inhibitor	NSCLC	November, 2004
AstraZeneca	Iressa (gefitinib)	EGFR	TK ATP small inhibitor	NSCLC	May, 2003
Novartis	Gleevec (imatinib)	PDGFR, SCF, c-kit	TK ATP small inhibitor	GIST	February, 2002
Novartis	Gleevec (imatinib)	BCR-ABL	TK ATP small inhibitor	CML	May, 2001
Genentech	Herceptin (Trastuzumab)	EGFR	recombinant, human mAb	Breast cancer	September, 1998

A reviewed on February 2007 [12].
MAb, monoclonal antibody; STK, serine threonine kinase; TK, tyrosine kinase; CML, Chronic Myeloid Leukemia; GIST, gastrointestinal stromal tumors; RCC, renal cell carcinoma; NSCLC, Non-Small-Cell Lung Cancer; PDGFR-α and PDGFR-β, platelet-derived growth factor receptors; VEGFR, vascular endothelial growth factor receptors; c-kit, stem cell factor receptor; FLT3, Fms-like tyrosine kinase-3; CSF-1R, colony stimulating factor receptor Type 1; RET, glial cell-line derived neurotrophic factor receptor; EGFR, Epidermal Growth Factor Receptor; HER1, Epidermal Growth Factor Receptor type 1; SCF, stem cell factor.

intimately linked for 30 years, since the first cellular oncogene, src, was discovered by Harold Varmus and Michael Bishop in 1975, and later found to encode a tyrosine kinase [4, 10]. Much effort is directed toward the development of protein kinase inhibitors and also, to understand the biology of the kinase systems and their role in cancer, allowing for the successful development of therapies targeting such mechanisms. About six protein kinase inhibitors have been approved for treating cancer in the last ten years (Table 1) and at least 40 are known to be undergoing clinical trials. However, a general drawback of the current small molecule inhibitors is the relative ease by which kinases can develop resistance to the drugs. New methods and treatments to increase response and to limit resistance are in development [11]. Some examples being: (a) to hit multiple tumour-promoting signalling pathways, either with smart drug combinations or with a single multitarget compound, (b) to administer the patients those drugs matching the genetic profile of their biopsed tumour, (c) to design combinations with ATP and substrate competitors and also, combinations of inhibitors that do not have an overlap in the specific mutation that confers the resistance and (d) to use inhibitors in combination with traditional chemotherapy.

Catalytic domain features

The key feature that distinguishes protein kinase family members from other proteins is the sequence of a contiguous stretch of approximately 250 amino acids that constitutes the catalytic domain, which is responsible for the nucleotide binding and phosphate transferring [13]. The solution of the crystallographic structure of several protein kinases has clarified somehow the functional role of particularly conserved residues in the binding of ATP and the protein substrate, and for the regulation of binding events [14, 15]. The first protein kinase structure was elucidated in 1991, and corresponded to cyclic AMP-dependent kinase (PKA) in complex with a natural peptide inhibitor [16]. Owing to the highly conserved nature of the core structure, a description of PKA catalytic core encompasses the main features of all members of the family and it is worth noting here.

The inactive PKA holoenzyme is a tetramer, composed of two regulatory and two catalytic subunits that become dissociated after the second messenger

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cAMP (cyclic-adenosine monophosphate) binds to the regulatory subunits. As a result, the catalytic component form of PKA (C-subunit) is composed of only one domain containing about 350 amino acids.

The C-subunit of PKA comprises two domains: a small ATP binding domain composed primarily of α helixes and a large substrate binding domain mostly composed of β strands. The active site is located at the interface between the small and large lobes forming a deep cleft. The nucleoside moiety of ATP is completely buried into this cleft, whereas the triphosphate arm and the peptide substrate are located at the mouth of the opening, where the phosphoryltransfer reaction takes place [17] (Figure 2).

ATP is anchored to several residues of the enzyme located at the start of the C-terminal domain and the linker region via hydrogen bonds to its adenine moiety and the ribose ring respectively. The triphosphate group is coordinated by two Mg^{2+} ions: Mg (1) chelating the β, γ phosphates of ATP and Asp-184, a strictly conserved residue in this protein family, and Mg (2) chelating the α, γ phosphates of ATP and two conserved residues located in the DFG motif and the catalytic loop respectively, named Asp-184 and Asn-171. DFG motif is composed of three highly conserved residues (Asp, Phe and Gly) located in the N-terminal region of the activation loop that adopts an appropriated conformation for the metal ion binding in the active kinase conformation. Additional stabilization of the phosphates may arise from the interaction of Lys-72 with α and β phosphates of ATP. Glu-91 positions Lys-72 by electrostatic interactions. The glycinerich loop fixes β phosphates of ATP by hydrogen bonding to backbone nitrogen of Phe-54 and Gly-55. Another crucial residue is Lys-168, conserved in all S/TKs and replaced by an Arg in members of the TKs [18], being perhaps the most critical residue at the active site because it has the potential to interact with components of both the small and large lobes. Lys-168 in PKA makes an electrostatic contact with the γ phosphate and also helps to anchor the peptide to the surface of the large lobe (Figure 3).

Two essential structural elements of the large lobe are the activation and catalytic loops. The activation loop regulates catalytic activity in most kinases by switching between different states in a phosphorylation-dependent manner [19]. In fully active kinases, the loop is stabilized in an open conformation by phosphorylation on serine, threonine or tyrosine residues within the loop, and in this conformation a β-strand in the loop provides a platform for substrate binding. In the case of PKA, the phosphorylation of the activation loop in Thr-197 residue enhances its catalytic activity by approximately three orders of magnitude [20].

The catalytic loop includes a conserved aspartic acid (Asp-166) which is the nearest ionisable residue to the hydroxyl group of the substrate. In the crystal structure of PKA with a peptide inhibitor, a direct hydrogen bond can be assigned between the carboxyl group of the Asp-166 and the hydroxyl group of the phosphoacceptor residue in the substrate [14] (Figure 3). The observation of this hydrogen bond in other protein kinase structures supports a general role for the carboxyl group [21]. While the essential presence of this residue has been established by mutational studies [21, 22], its exact function is still being debated. The current hypothesis is that it might position

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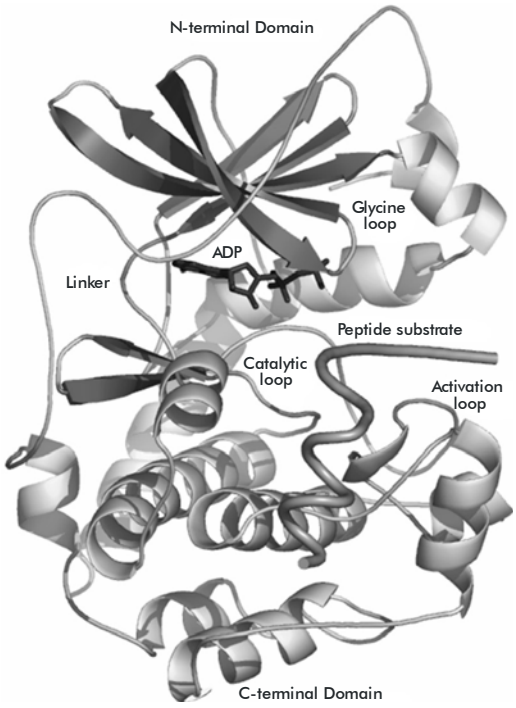

Figure 2. Ribbon representation of the catalytic subunit of PKA in complex with ADP and a substrate peptide, 1JBP [14].

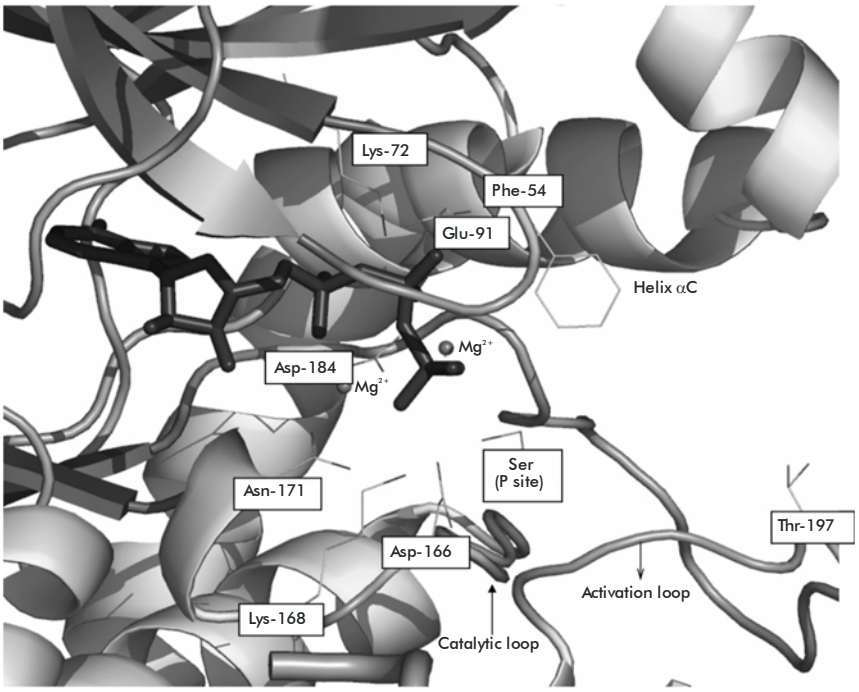

Figure 3. Schematic ribbon representation of the catalytic domain of PKA in complex with ATP (black sticks) mapping some of the key residues involve in ATP recognition (based on the PKA ternary complex with ATP and a peptide inhibitor, 1ATP [17] and the hydrogen bonding interaction between the catalytic conserved aspartate (Asp-166) in the catalytic loop and the Ser phosphoacceptor residue in the peptide substrate (based on the binary complex of PKA with a substrate peptide, 1JBP [14]).

the hydroxyl group, allowing the appropriate attack geometry on the ATP γ phosphate, with or without participation of a metal ion (Mg^{2+}) to stabilize the developing negative charge on the bridging oxygen, thereby easing the detachment of the leaving ADP molecule [23].

Substrate recognition

While the catalytic domain of these kinases is highly conserved, the sequence variation that is observed in the kinome provides for recognition of distinct substrates. A protein kinase is usually not specific to a single substrate, but instead can phosphorylate a whole "substrate family" having in common a consensus sequence. Consensus sequences refers to those sequence elements immediately surrounding the phosphoacceptor group that are essential for kinase recognition and phosphorylation [24] (Table 2). However, in many cases it has become apparent that consensus sequences do not exploit or reflect the complete binding capacity offered by the protein kinases. The natural substrate is, thus like to utilize additional binding determinants not present in the immediately environment of the active site [25] and for some kinases, other factors such as secondary/tertiary structure are also likely to be important for efficient phosphorylation [26].

*K*inase inhibition

Protein kinases were initially though to be unsuitable drug targets, largely because the unfavourably high degree of structural conservation within the catalytic domains of all protein kinases, particularly the ATP binding site. However, the degree of conservation of the ATP binding sites in the distinct protein kinases is not absolute, so it is possible to developed ATP mimicry with relatively high selectivity. Currently, the development of ATP mimics has been the most extensive exploited strategy for kinase inhibition, and most of protein kinase inhibitors currently approved by the Food and Drug Administration (FDA) or in different stages of clinical trials for cancer therapy, are directed toward the ATP binding pocket [27-29]. About four inhibition mechanisms of protein kinase activity using small organic molecules (a, b, c) or antibodies (d) have been developed: (a) an ATP-mimetic mechanism, (b) a substrate mimetic mechanism, (c) a mechanism locking the kinase into inactive conformation and (d) the recognition of ligand binding site in the receptor tyrosine kinase preventing receptor's activation [27, 30-32].

Targeting the kinase catalytic domain at the ATP binding site

Two small molecule inhibitors that target ATP binding to EGFR, Gefitinib (Iressa; AstraZeneca) and Erlotinib (Tarceva, OSI 774; Genentech, OSI Pharmaceuticals) (Figure 4) have been approved in 2003 and 2004 respectively, for the treatment of patients with advanced non-small cell lung cancer (NSCLC) [12]. Mutations that lead to EGFR overexpression have been associated with a number of cancers, including non-small cell lung cancer (NSCLC) [33]. Other inhibitors, like Flavopiridol (Aventis) (Figure 4), which targets cyclin dependent protein kinase (CDK), are undergoing clinical trials [29]. CDKs are involved in the regulation of cell cycle and its deregulation has been observed in many cancer types [43], which make them potential targets in anticancer drug research.

Table 2. Some protein kinases and their proposed substrate specificities. Phosphorylation sites are shown in boldface.

	Substrate name	Consensus sequence	Reference
PKA	cAMP-dependent protein kinase	R-R-X-**S/T**	34
Phk	phosphorylase kinase	-R-X-X-**S/T**-F-F-	35
CDK-2	cyclin-dependent kinase-2	-**S/T**-P-X-R/K-	36
CK-II	casein kinase 2	-**S/T**-X-X-E/D	37, 38
ERK2	extracellular-regulated kinase-2	-P-X-**S/T**-P-	39
c-Src	cellular form of the transforming agent of Rous sarcoma	E-E-I-**Y**-E/G-X-F	40
v-Fps	transforming agent of the Fujinami sarcoma virus		41
Csk	C-terminal Src kinase	I-**Y**-M-F-F-F-	42
InRK	insulin receptor kinase	-**Y**-M-M-M-	40
EGFR	epidermal growth factor receptor	-E-E-E-**Y**-F-	40

In contrast with the majority of ATP kinase inhibitors which occupy the whole ATP binding site, some others, like Imatinib (ST1571, Gleevec/Glivec; Novartis) occupy just part of the pocket, and most of its inhibitory effect comes from tight binding to other regions in the inactive form of its target kinase.

Imatinib is an inhibitor of the BCR-ABL abnormal tyrosine kinase implicated in the pathogenesis of chronic myeloid leukemia (CML) and acute lymphoblastic leukemia [44]. The three dimensional structure of ABL-Imatinib complex showed that Imatinib re-

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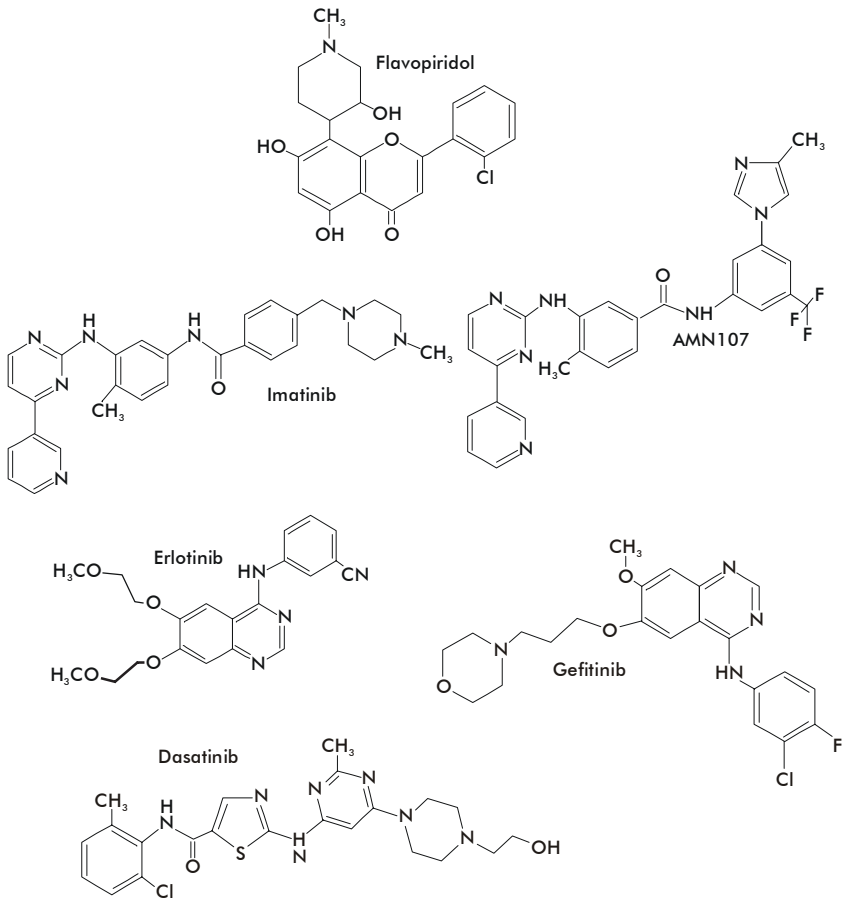

Figure 4. Chemical structures of some protein kinase ATP competitive inhibitors.

mains bound to the ATP binding cleft of the inactive conformation (close state) of ABL tyrosine kinase, establishing extensive contacts with important structural features of the kinase, such as the 'gatekeeper' residue, the p-loop and the activation loop (Figure 5). The binding of Imatinib stabilizes BCR-ABL protein in its closed form avoiding ATP to access the ATP site and thus, the inhibition of autophosphorylation and substrate phosphorylation [45]. Imatinib was approved for the treatment of CML in 2001. Despite the fact that the majority of CML positive patients receiving Imatinib responded to the treatment, a significant portion of patients chronically treated with it, developed resistance because of the development of mutations in the kinase domain of BCR-ABL, which interfere with the ability of the enzyme to adopt the inactive conformation required for Imatinib binding [46]. A variety of strategies derived from structural studies of the ABL-Imatinib complex have been developed, resulting in the design of novel ABL inhibitors, including AMN107 (Novartis), Dasatinib (BMS-354 825, Bristol-Myers Squibb), ON012380 (Onconova Therapeutics) and others [47]. Due to the structural similarity between AMN107 and Imatinib (Figure 4), AMN107 also requires the ABL protein to be in the inactive conformation for optimal binding. Using numerous BCR-ABL transformed hematopoietic cell lines, AMN107 was found to be 10-25 fold more potent than Imatinib for reduction of both autophosphorylation and proliferation [48]. It has been suggested that the enhancer potency of this inhibitor compared with Imatinib is due to its higher affinity to the ABL tyrosine kinase pocket [48]. Like Imatinib, AMN107 is also an inhibitor of the tyrosine kinases PDGFR-α, PDGFR-β and c-kit as demonstrated in A31 and GIST 882 cells [48]. AMN107 has made its way into clinical testing. Dasatinib is a novel ABL-targeted small molecule inhibitor (Figure 4) that also shows activity towards Src kinases. It has up to 100 fold increased activity against the ABL kinase compared to Imatinib and retains activity against 14 of the 15 Imatinib-resistant BCR-ABL mutants *in vitro* [50]. Dasatinib binds to the ATP-binding site in a position similar to Imatinib. The central cores of Dasatinib and Imatinib share overlapping regions, the difference being that they extend in opposite directions. Unlike Imatinib and AMN107, Dasatinib is able to bind to the active as well as the inactive conformation of ABL kinase. Furthermore, Dasatinib makes fewer contacts with ABL than Imatinib or AMN107 and places less stringent conformational requirements on the kinase [51].

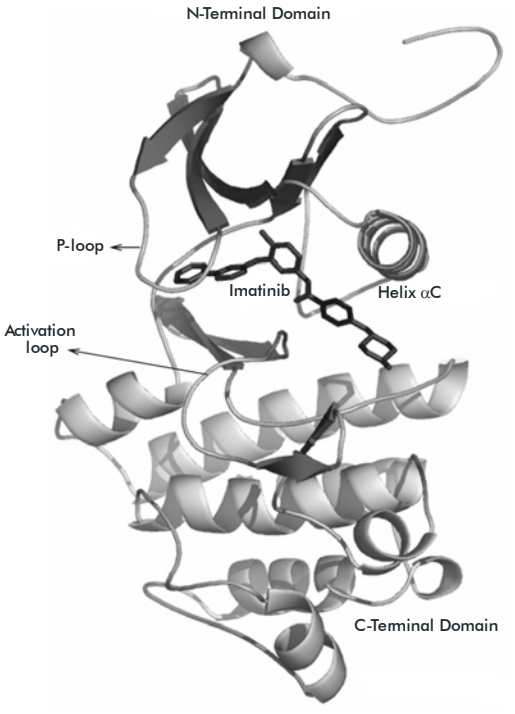

Figure 5. Ribbon representation of the catalytic domain of ABL-BCR tyrosine kinase (inactive) in complex with Imatinib, 1IEP [49].

FDA has granted accelerated approval of Dasatinib in the last year for the treatment of adults in all phases of CML (chronic, accelerated, and myeloid or lymphoid blast phase) with resistance or intolerance to prior therapy, including Imatinib and also for the treatment of adults with acute lymphoblastic leukemia (Ph + ALL) with resistance or intolerance to prior therapy. Dasatinib is the first approved oral tyrosine kinase inhibitor predicted to bind to multiple conformations of the ABL kinase based on modelling studies. At nanomolar concentrations, Dasatinib inhibits BCR-ABL, Src family (Src, Lck, Yes, Fyn), c-kit, Epha2, and PDGFR-β. By targeting these kinases, Dasatinib inhibits the overproduction of leukemia cells in the bone marrow of patients with CML and Ph + ALL and allows normal red cell, white cell, and blood platelet production to resume. Another strategy to inhibit ABL tyrosine kinase has been to target the substrate binding site as shown below.

As it was demonstrated in the design of the second generation of ABL tyrosine kinase inhibitors (AMN 107, Dasatinib and ON012380) the analysis of crystallographic kinase-inhibitors complexes clarifies the manner in which inhibitors bind within the catalytic domain, constituting a powerful information source to design novel inhibitors and improve potency of already known ones. Several examples drew into perspective the use of bioinformatics approaches to exploit structural information obtained from ligands or targets on protein kinase inhibitor design [52-56].

A possible approach is to use chemical scaffolds designed *in silico* or obtained by experimental assays, which make favourable interactions with the parts of the targeted binding site that are known to be critical for ligand affinity. These scaffolds can then lead to the synthesis of prototype molecules or serve as substructure queries in compound database search.

This strategy was used to discover novel furanopyrimidine and pyrrolopyrimidine ATP inhibitors of the human Chk1 kinase [52] and it is described here. A lead compound inhibitor of Chk1 (IC_{50} of 15.4 μM) identified during a medium throughput screening of compounds provided by Chemical Diversity database (ChemDiv) [57] was used to recognize other compounds with similar chemical core using the substructure search algorithm implemented in MDL ISIS/Base software [58]. Four analogues compounds we-

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re identified in the Vernalis electronic catalogue of commercially available compounds [59] and other four were synthesized in-house corresponding to furanopyrimidine compounds. All analogs were assayed and they had an $IC_{50} > 100\mu M$. In order to explain the loss of activities of the chemical analogues, the lead compound was docked into the kinase catalytic domain using Ribo- Dock program [60]. Fortunately, the two binding modes predicted by docking were corroborated because they could solved the crystal structures of the lead and two other less potent inhibitors, which clarified the importance of critical interactions for an effective binding and inhibition of Chk1. The analysis of the crystallographic information suggested that changing a furan moiety to a pyrrole in the lead compound, would contribute to the formation of an additional hydrogen bond between the ligand and the kinase. Further experimental analysis proved that in fact, this substitution translated into a 10-fold increase in affinity [52].

Another example was the identification of aminoimidazo [1, 2-α] pyridines as a new class of ATP site direct CDK inhibitor. This new scaffold was identified using the crystallographic complex of CDK with its already known inhibitor, staurosporine. Several initial ligand orientations were generated in the target protein's active site using DoMCoSAR methodology [61], followed by MD-based simulated annealing and finally refinement by minimization. These models were used to design modifications in the ligands and finally resulted in the identification of aminoimidazo [1, 2-α] pyridine scaffold. Co-crystal structures of CDK2 in complex with the novel inhibitors containing the aminoimidazo (1, 2-α) pyridine core confirms their attachment to ATP pocket. Chemical modification of the substituents at certain position greatly improves the potency and selectivity [53].

Another way to exploit the information of known ligands for inhibitor design is using kinase frequent hitters, instead of attempting to predict specificity of ligands for select targets (as described before). This method chose to profile those molecular features that tend to coincide with a propensity for nonselective inhibitor of multiple kinases [62]. This strategy led to the identification of a five-point pharmacophore for kinase frequent hitters using the X-ray structural information of four promiscuous inhibitors of PKA, Src, CDK-2, Erk2 and GSK-3. This pharmacophore is able to discriminate between frequent hitters and selective ligands, therefore it could be used for rapid virtual screening of compound libraries for molecules with a potential for non selective inhibition of kinases.

Target structures had also been widely used for inhibitor design; structural based virtual screening methods were successfully applied in order to discover ATP competitive compounds inhibiting Akt1, BCR-ABL tyrosine kinase and CK-II protein kinase. In the searching for novel Akt1 inhibitors, two different strategies using FlexX [63], GOLD [64] and CSCORE [65] programs were applied and the 100-200 top-scoring compounds docked from a total of 50 000 compound library provided by Chembridge database were experimentally tested. The first strategy consisted in simply relying on results provided by the scoring functions, and the second took into account a consensus between scoring functions and H-bonding patterns similar to those observed in the crystal structure of Akt1 in complex with AMP-PNP. The hit rate obtained with the first strategy was only slightly superior to the expected one from a random approach (0.01-0.5%) in contrast with the second strategy, which achieved a remarkable 10% hit rate. Finally, two low-micromolar inhibitors for Akt1 protein kinase were identified [54].

In the case of BCR-ABL tyrosine kinase, the start up was a database containing 200 000 commercially available compounds provided by ChemDiv, the top 1 000 compounds with the best DOCK [66] energy score were selected and subjected to activity assays against K562 cells with IC_{50} value ranking from 10 to 20 μM and two lead compounds were selected [55] for further drug design and optimization.

In the last example, a virtual screening targeting the ATP binding site of a homology model of human casein kinase 2 (CK-II) enzyme and a database of 400 000 compounds using DOCK [66] led to the discovery of the most potent and selective CK-II ATP-competitive inhibitor, a 7-substituted indoloquinazoline (5-oxo-5,6-dihydroindolo (1,2-α)-quinazolin-7-yl)acetic acid) inhibiting the enzymatic activity of CK-II with an IC_{50} value of 80 nM [56].

Targeting the kinase catalytic domain at the Non-ATP binding site

Certainly, one drawback of ATP competitors is that they frequently fail to provide a cure because tumours almost invariably acquire mutations in the ATP binding pocket that interfere with drug binding and cause resistance, as in the case of Imatinib. Another two disadvantages are that these inhibitors must compete with high intracellular ATP concentrations and are likely to hit other proteins kinases causing side effects due to the ubiquitous nature of ATP binding sites.

Great hope is placed on compounds binding to other parts of protein kinases's catalytic domain rather than the ATP binding site; such binding would confer more specificity than binding to the ATP pocket and might also overcome resistance from compounds that are ATP competitive.

Recently, a non-ATP competitive inhibitor of BCR-ABL tyrosine kinase, ON012380 (Onconova Therapeutics) (Figure 6) has been described [67]. Although it's exact binding site on the kinase has yet to be reported, ON012380 targeting site differs from Imatinib because ATP fails to compete with ON012380 and in contrast Crk, a natural substrate, readily competes with ON012380 and interfere with its ability to inhibit BCR-ABL kinase. Furthermore, Imatinib and ON012380 were found to synergistically inhibit wild-type BCR-ABL, suggesting that they bind to different sites on the enzyme. ON012380, is very effective at inhibiting all the Imatinib-resistant mutants of BCR-ABL already known and it is 10-fold more potent than Imatinib. Another important feature of ON012380 is the very desirable safety profile that is not often seen in conventional chemotherapeutic agents; ON012380 is currently in preclinical phase [67].

Another non-ATP-competitive inhibitor, ON01910 (Onconova Therapeutics) (Figure 6) has entered Phase I clinical trial for cancer therapy. It inhibits Plk1 at

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9-10 nM concentration. Plk1 plays an essential role in cell cycle progression and it is overexpressed in many tumour cells [68-70]. It is likely that this compound binds to Plk1 at or near the substrate binding domain, since substrates of Plk1 compete for the inhibitory activity of ON01910. In addition to Plk1, ON01910 was also inhibitory to Abl, Flt-1, and PDGFR kinases at low nanomolar concentrations. At approximately 10- to 20-fold higher concentrations, inhibition of Src, Fyn, and Plk2 kinases was also observed [30]. This compound, which exhibits low toxicity, was found to be a potent inhibitor of tumour growth in a wide variety of human cancers, and showed a high degree of synergism with several chemotherapeutic agents currently used in cancer therapy, often inducing complete regression of tumours [30].

In 2004, structural studies confirmed the existence of a novel non-catalytic binding site for PD184352-like MEK inhibitors (Pfizer) (Figure 6). PD184352 inhibitor was identified as a highly selective, potent inhibitor of MEK1 and MEK2 that was non-competitive for both ATP and MAPK [71]. MEK1 and MEK2 are closely related DFKs found in the MAPK signalling pathway and constitutive activation of MEK1 results in cellular transformation [71]. The PD184352-like MEK inhibitors, have proven to be exceptionally specific for MEK1 and MEK2, and do not inhibit other known S/TKs [72], even within the MEK family, where they do not inhibit the activity of MKK3, MKK4, MKK6 or MKK7, although it has shown to be a weak inhibitor of MKK5 [73]. Compared with previously described ATP-competitive inhibition, the non-competitive MEK inhibitors bind a novel allosteric binding pocket, adjacent to but not overlapping with the ATP binding site [74-76]. Notably, the MEK inhibitor binding pocket is located in a region where the sequence homology to other protein kinases is quite low and distinct from the highly homologous ATP binding site. The inhibition of the kinase activity of MEK1 and MEK2 by PD184352-like inhibitors is the result of the stabilization of a naturally occurring inactive conformation of the protein kinase which involves activation loop and helix C, and it might also causes conformational changes in the binding pocket precluding enzymatic activity.

The discovery of this series of inhibitors opened the door to find similar allosteric kinase inhibitors for several other protein kinases, which could form a similar pocket.

Targeting the kinase extracellular domain

Other attractive site for the kinase activity inhibition is the extracellular domain of the receptor protein kinase, which constitutes an excellent target for antibodies. Monoclonal antibody (MAb) blockade of EGFR represents a new and exciting direction in cancer therapy. In fact, Cetuximab (Erbitux; Imclone**,** Bristol-Myers Squibb), a chimeric MAb and a recombinant one, Vectibix (Panitumumab; Amgen), were designed to specifically inhibit EGFR [32]; and had been approved for the treatment of colorectal cancer in 2004 and 2006 respectively [12]. Several other antibodies that bind to EGFR are in various stages of clinical development, including the fully humanized ABX-EGF (Abgenix) [77].

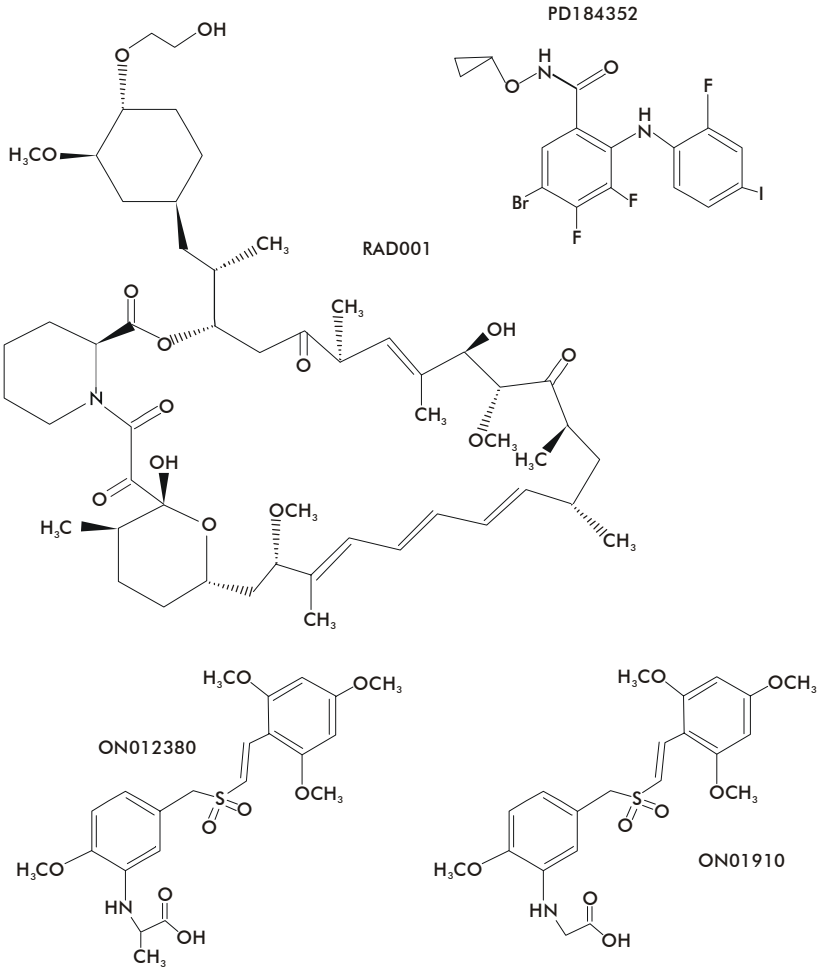

Figure 6. Chemical structures of some protein kinase non-ATP competitive inhibitors.

Targeting the kinase substrates

Other ways to block protein kinase action are through the targeting of its substrate protein. This mechanism sounds promissory allowing for the selective prevention of some actions of the kinases whilst leaving others intact. Protein kinase inhibitory peptides were successfully identified by screening a random cyclic peptide phage display library with a target peptide based on the CK-II substrate sites from the HPV-16 E7. Peptide derived from the strongest inhibitory phage corresponded to P15 (*WMSPRHLGT*). P15 fused with cell-permeable peptide sequence (HIV-Tat protein) impairs CK-II phosphorylation of HPV-16 E7 at a final concentration of 100 μM. When P15 was applied to cultures of TC-1 cells, it activated caspase-3 and prevent tumour growth following TC-1 cell implantation [78]. As CK-II is frequently elevated in proliferating and transformed tissues, including leukemia and solid tumours [79], these results suggested that P15 could be a peptide-based drug for some cancer types, alone or co-administered with conventional chemotherapy.

Other ways to inhibit kinase activity

There are other ways to inhibit protein kinase activity without targeting catalytic domains. For example, Rapamycin (RAPA, sirolimus, Rapamune; Wyeth-Ayerst

57. http://www.chemdiv.com.

58. http://www.mdli.com.

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Laboratories) is a natural product of the bacteria *Streptomyces hygroscopicus* with antiproliferative effects, mediated through the formation of an active complex. The complex, composed of Rapamycin and the immunophilin FK506-binding protein 12 (FKBP12), inhibits the mammalian target of rapamycin (mTOR), which is a kinase responsible for mitogen-induced cell proliferation/survival signalling and its activation leads to a cell-cycle progression from G1 to S phase. mTOR and pathways upstream of this kinase were found to be frequently upregulated in neoplastic diseases [80]. Rapamycin was approved as immunosuppressant agent for clinical use in 1999 but in spite of the significant antitumour activity, Rapamycin programme was abandoned and it could not be further developed as a cancer therapy drug, leading instead to the synthesis of three analogues: CCI-779, RAD001 and AP23573 [80]. RAD001 (40-O-[2-hydroxyethyl]-rapamycin, everolimus, Novartis Pharma AG) is an orally bioavaliable derivative of RAPA, currently under development as an antiproliferative agent (Figure 6).

Targeting kinase packing defects

Interestingly, a new strategy for the generation of protein kinase inhibitors is related with the use of kinase packing defects [81]. These defects consist of solvent exposed backbone hydrogen bonds and they are termed dehydrons, since they promote their own dehydration as a mean to strengthen and stabilize the underlying electrostatic interaction. Dehydration is supported by attracting nonpolar groups, or excluding water molecules. The useful of dehydrons in the design or improvement of protein kinases inhibitors resides in their lack of conservation across proteins with common ancestry [82, 83]. Consequently, targeting these features by turning drugs into protectors or "wrappers" (water-excluders) of packing defects may control cross reactivity. The possibility of achieving specificity by designing a wrapping drug has been tested experimentally [84-87]. Fernández et al [85] redesigned the commercially available anticancer drug Imatinib with the introduction of wrapping modifications to enhance its specificity towards its primary target, the ABL tyrosine kinase. They used 3D structure of the alternative targets and made a structural alignment to investigate the microenvironment conservation for these intramolecular interactions. They improved Imatinib selectivity for ABL-BCR by targeting dehydrons not conserved across paralogs. These results demonstrated that the knowledge of non-conserved kinase packing defects may be a suitable way to ensure a successfully inhibition and it should be incorporated as a mandatory step during the computer-assisted rational design of kinase inhibitors.

Conclusions

The success of some protein kinase inhibitors currently introduced into clinical practice for treatment of several cancers has proved that targeting protein kinase constitutes an effective option for cancer therapy. However, there is still much to learn concerning kinase inhibition. The high structural conservation of kinases causes selectivity problems in most drug inhibitors, often resulting in dangerous side effects. This is often the reason for kinase inhibitor failure at the stage of clinical trials. Even so, multi-target inhibition could be a useful approach if the major drug targets are protein kinases involved in cancer signaling pathways. Simultaneous hitting of some these target proteins could overcome many of the often overlapping, biological pathways that tumor use to grow, resist death, and spread. Furthermore, combination therapy with two different inhibitors having the same target could also be used to preclude appearance of resistance.

Most inhibitors already approved or undergoing clinical trials compete for ATP binding site. Nevertheless, non-ATP competitive inhibitors are becoming very attractive and promise to be more potent and/or safe. Moreover, the substrate targeting approach also represents a good choice, especially for inhibition of phosphorylation mediated by constitutively active kinases and/or kinases having a huge repertory of different substrates.

The massive amount of crystallographic structural information of protein kinase and kinase-inhibitor complexes nowadays available could help to overcome the current problems with existing antikinase cancer therapies. The design or redesign of more selective and potent protein kinase inhibitors in a shorter time absolutely demands the analysis of these structural data. In this sense, redesign of already known ATP-binding site inhibitors targeting non-conserved packing defects offers a novel route to rescue old inhibitors previously discarded because of their highly cross-reactivity. Therefore, protein kinase inhibitors either alone or in combination with classical chemotherapy seem to bring new hopes in the field of cancer research and therapy in the coming future.

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