Structural biology in drug design: selective protein kinase inhibitors

Giovanna Scapin

Protein kinases have a fundamental role in signal transduction pathways, and aberrant kinase activity has been observed in many diseases. In recent years, kinase inhibition has become a major area for therapeutic intervention and a variety of kinase inhibitor pharmacophores has been described. This review illustrates some of the efforts and results in the field of structure-based design of protein kinase inhibitors. The methods and results discussed here illustrate the power of structure-based design in lead discovery, for example via virtual screening and in guiding the optimization of the pharmacological properties of these molecules.

Giovanna Scapin
Merck Research Laboratories
PO Box 2000, RY50 105
Rahway, NJ 07065, USA
tel: +1 732 594 8429
fax: +1 732 594 5042
e-mail:
giovanna_scapin@merck.com

▼ The phosphorylation of proteins, triggered in response to extracellular signals, represents a universal mechanism for the cellular control of many different processes, including metabolic pathways, cell growth and differentiation, membrane transport, and apoptosis [1,2]. The reverse reaction of dephosphorylation is catalyzed by specific protein phosphatases [3–5]. Phosphorylation of protein targets is catalyzed by a large family of homologous proteins known as protein kinases (PKs), which catalyze the transfer of the terminal (or γ) phosphate of ATP to specific residues of protein substrates. The protein kinase family comprises two major subfamilies: the protein tyrosine kinases and the protein serine–threonine kinases. Recently, histidine kinases, which phosphorylate an imidazole nitrogen on a histidine residue, have also emerged as signaling enzymes [6]. The protein tyrosine kinases (PTKs) activate numerous signaling pathways within cells, leading to cell proliferation, differentiation, migration and metabolic changes [7]. They include receptor tyrosine kinases (RTKs, such as the insulin-receptor tyrosine kinase, IRK, or the epidermal growth factor receptor kinase, EGFR-K) and non-receptor tyrosine kinases (NRTKs), which include Src, JAKs and Abl, among others. RTKs are transmembrane glycoproteins that are composed of three major domains: an extracellular receptor domain, a membrane-spanning linker domain and a cytoplasmic domain, which contains the catalytic kinase activity. They are typically activated following the binding of a specific ligand to the receptor domain. The NRTKs are cytoplasmic proteins and are integral components of the signaling cascades triggered by RTKs and other cell surface receptors, such as G-protein coupled receptors and T-cell receptors [8]. In addition to the PTK family, a large number of serine–threonine kinases have been identified. One well-characterized example is the family of the mitogen-activated protein kinases (MAPKs [9]). To date, more than 530 kinase-related sequences have been identified in the human genome. For a comprehensive list of protein kinases, including sequence alignments, three-dimensional (3D) structural information and links to the genome, see the Protein Kinase Resource website at http://pkr.sdsc.edu/html/index.shtml.

Structure of protein kinases

Overall structure

All protein kinases contain a structurally conserved catalytic domain, which was first elucidated for the cyclic AMP-dependent kinase (PKA [10]). Several crystal structures of protein kinases have been reported since then [the number of structures in the Protein Data Bank (PDB) as of October 2001 is 170 and includes structures of catalytic and regulatory domains] and the organization of the catalytic domain is now well established [11]. It is composed of two major domains (N- and C-terminal domains; Fig. 1), that are further subdivided into 11 subdomains (color-coded in Fig. 1). The two domains are connected through a single polypeptide strand (the linker region), which acts as a hinge about which the two domains

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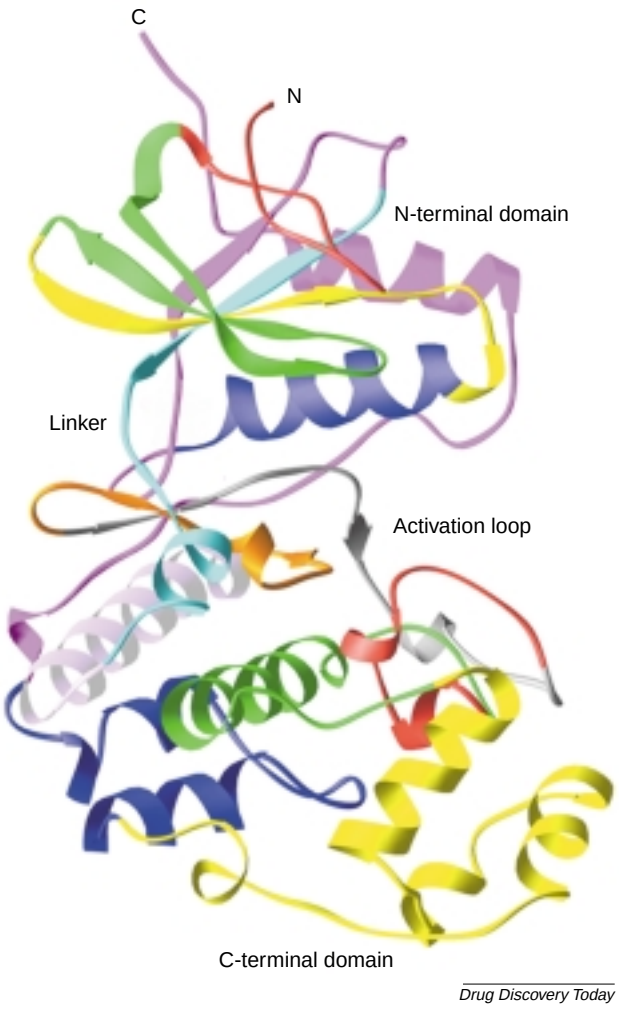

Figure 1. Ribbon diagram showing the catalytic domain of extracellular-signal-regulated kinase (ERK2) (Protein Data Bank code ERK1). The 11 subdomains that comprise the catalytic domain are shown in different colors.

can rotate with respect to one other upon binding of ATP and/or substrate, and without the disruption of the kinase's secondary structure. All protein kinases contain an aspartate residue (D166 in PKA) which has been implicated in the catalytic mechanism: this residue is located in the C-terminal domain in a loop termed the catalytic loop.

Many protein kinases, but not all, require phosphorylation on one or more of the serine, threonine or tyrosine residues that are located in a segment running from the conserved DFG motif to the conserved APE motif (activation- or T-loop; Fig. 1) [12]. Phosphorylation of residues in the activation segment causes conformational changes in the protein that lead to the correct positioning of substrate binding residues and catalytic residues, and relief of steric blocking to enable access of substrates to the catalytic site. Protein kinases that are not regulated by phosphorylation have other mechanisms for localization of their activation segments, without the requirement of posttranslational modification [2]. Although activation-loop phosphorylation is one way in which kinases are regulated, other control mechanisms recognized to date include: (1) control by additional subunits or domains that might function in response to second messengers (e.g. cyclic AMP binding to the regulatory subunit of PKA, or Ca^{2+}–calmodulin binding to calmodulin-dependent protein kinase); (2) control by additional subunits whose level of expression varies depending on the functional state of the cell (e.g. cyclin regulation of the cyclin-dependent protein kinases, CDKs); (3) control by additional subunits that target the kinases to different molecules or subcellular localizations [e.g. the Src homology 2 and 3 (SH2 and SH3) domains of SRC kinases]; and (4) control by additional domains that inhibit kinase activity by autoregulatory processes [e.g. myosin light chain kinase (MLCK) or Src kinases].

ATP-binding site

ATP binds in a cleft between the two major domains and is anchored to the enzyme via hydrogen bonds between its adenine moiety and residues of the linker region, and the ribose ring and residues at the start of the C-terminal domain. The triphosphate group is coordinated by two metal ions that are ligated by Asp and/or Asn residues located in the DFG motif and the catalytic loop. In addition, polar interactions with several residues of the glycine-rich loop, the conserved DFG motif and the catalytic loop further stabilize the phosphates and the transition state that is generated during the phosphotransfer reaction. In contrast to the well-defined ATP-binding site, the substrate-binding regions of kinases are shallow surface depressions, whose structures are poorly understood and have not been used as targets for inhibitor binding. Substrate recognition and high-affinity binding rely partly on local residues, but in many cases it has become apparent that short peptide sequences do not exploit the complete binding capacity offered by the protein kinases. This suggests that kinases might use distal recognition elements that are likely to be important for efficient phosphorylation [13].

It is known that several diseases can arise from, or involve, faulty protein kinase activity. For example, in humans, PTKs have a significant role in the development of many disease states, including diabetes and cancer, and have been also linked to a wide variety of congenital syndromes [14]. Many different tumor types have been related to dysfunctional RTK, with consequent overactivity of the RPTK system. The overexpression or aberrant activity of NRTKs can also lead to alterations in signal transduction pathways: for example, Src kinases have been implicated

in the pathology of tumors, osteoclast-mediated bone resorption and disorders associated with T-cell proliferation. Serine–theronine kinases also represent a large class of kinases for drug discovery opportunities: for example, MAP kinase p38 activation by proinflammatory cytokines and environmental stress has been associated with inflammatory processes, such as the initiation and progression of rheumatoid arthritis [15]. Therefore, modulation of protein kinase activity represents an attractive area for the design of new therapeutic agents.

Kinase inhibition

Although the catalytic core of protein kinases has been evolutionarily conserved [11], the mechanisms by which the inhibition of each kinase is achieved might vary considerably. Physiologically, at least three inhibition mechanisms have been identified: a pseudo-substrate mechanism, an adenine mimetic mechanism and a mechanism that involves locking the enzyme into an inactive conformation by using surfaces other than the active site [16]. Chemically, the field of protein-kinase inhibition has been highly active in the past 10 years and, to date, several synthetic pharmacophores have been identified as potent kinase inhibitors. Given the different nature of the nucleotide- and substrate-binding sites outlined previously, much of the synthetic effort has been devoted to the ATP-binding site, and the great majority of reported inhibitors are adenine mimetics [17–22], although several examples of inhibitors directed to noncatalytic domains of PTKs exist [23–26]. By contrast, the protein substrate-binding site has not been extensively exploited for inhibitor design [21], even though compounds that block the enzyme function by substrate competition might have an advantage over ATP mimetics, that for several reasons, including the fact that the high intracellular concentration of ATP could reduce the efficacy of ATP mimics, and that substrate-competitive inhibitors are likely to be more specific and selective. Recently, small substrate-competitive inhibitors of the insulin-like growth factor-1 (IGF-1R) and insulin receptor (IR) kinases have been described [27,28].

Several excellent reviews covering the chemistry and biology of kinase inhibitors have been published in the past few years [17,22,29,30]. Here, we will briefly describe the specific efforts that have resulted in the structure-based design of PK inhibitors; for example, the design or optimization of a small-molecule inhibitor based on the knowledge of the protein structure and specific protein–ligand interactions. It is worth noting that, although all the structures reported here are referred to as being obtained by structure-based design efforts, they are never solely the product of this approach, but rather a combination of crystallography, modeling, screening and medicinal chemistry efforts. As such, it might sometimes be more appropriate to define them as structure-assisted designs.

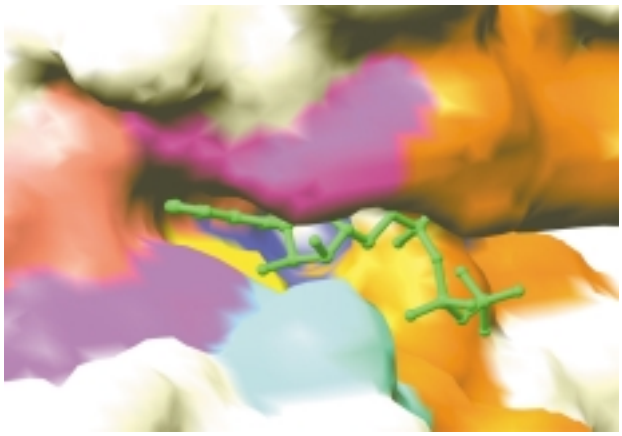

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Figure 2. The ATP-binding cleft in human lymphocyte specific kinase (LCK; Protein Data Bank code 3LCK). The regions composing the binding site are: (1) the adenine binding region, yellow; (2) the sugar pocket, aqua; (3) the phosphate binding region, orange; (4) the hydrophobic region 1, behind the adenine ring, blue; and (5) the hydrophobic region 2, a cleft or a tunnel adjacent to the ribose pocket, magenta.

ATP-competitive inhibitors

A great majority of the kinase inhibitors presented in this and other reviews are ATP-competitive. Therefore, selectivity becomes an important issue in the development of safe drugs because protein kinases share common sequences and structural homology in their ATP-binding site. In addition, there are many other proteins encoded by the human genome that rely on purine-based co-factors. Nevertheless, several ATP-site directed and highly selective inhibitors are currently in various stages of drug development. With regards to kinase specificity, only a fraction of all known kinases are screenable at present; for this reason, and assuming that it is virtually impossible to design a completely selective ATP mimic, the concept of a ‘kinase profile’ rather than specific kinase inhibition has recently been used in drug discovery as a better way to obtain a pharmacological response [31]. The numerous structures of complexes with ATP, its analog AMP.PNP, or small-molecule inhibitors bound to different protein kinases have provided a clear description of the ATP-binding cleft and of the similarities and differences that exist within the binding region. It is now clear that there are regions within the binding cleft that are not occupied by ATP, and that these regions (namely, the hydrophobic regions 1 and 2; Fig. 2) show structural diversity between members of the kinase family [18].

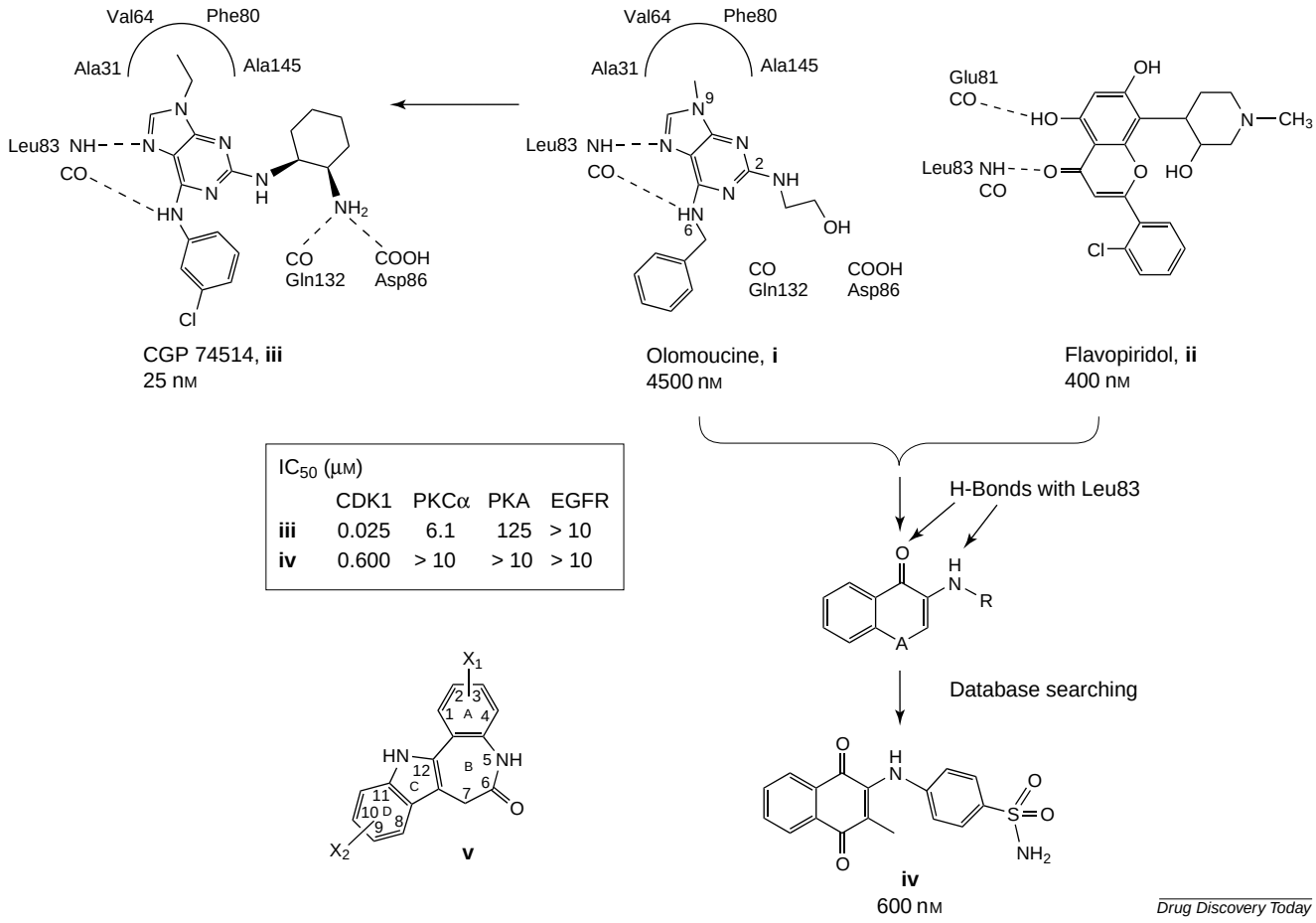


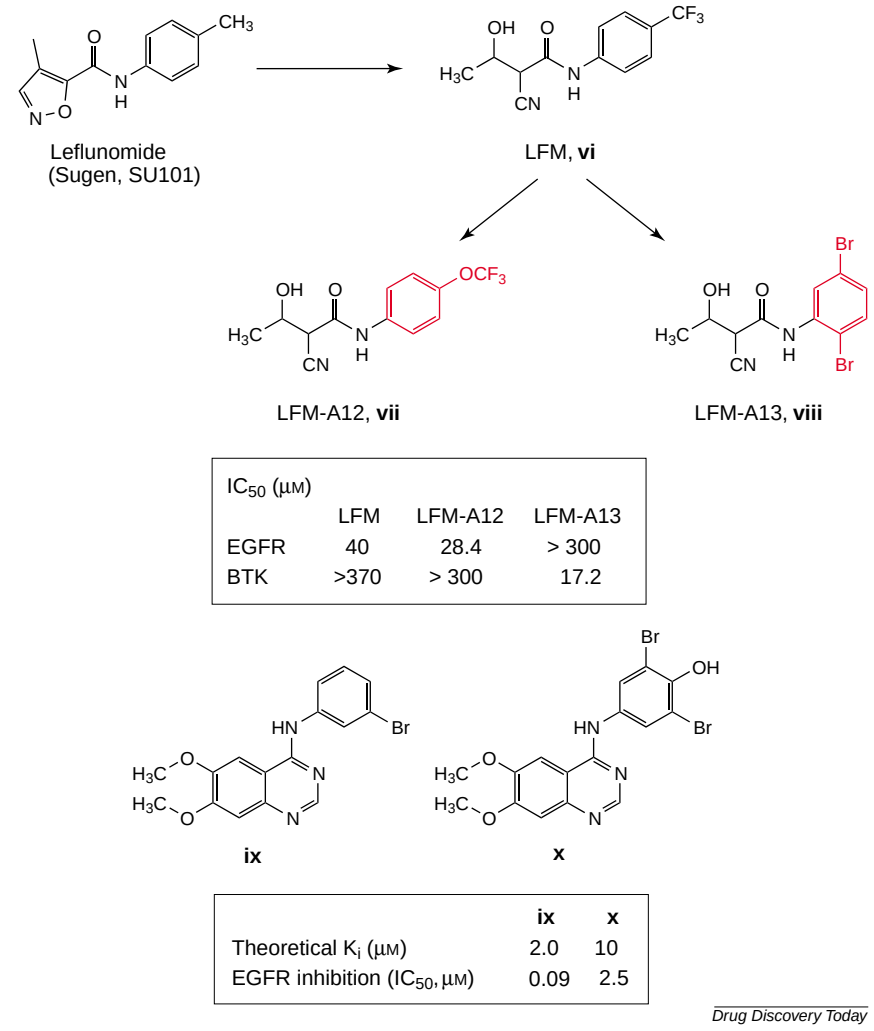
Figure 3. Small-molecule inhibitors of cyclin-dependent protein kinases (CDKs). Analysis of the binding mode of **i** lead to the design and synthesis of the more potent compound **iii**, in which the interactions with the hydrophobic pocket (Ala31, Val64, Phe80 and Ala145) and the side chains of Asp86 and Gln132 are optimized. Comparison of the binding mode of **i** and **ii** led to the design of a scaffold that facilitates the identification of new, potent inhibitors of CDK1, exemplified by **iv**, which retains the potency of **ii** and the selectivity profile of **i**. Molecular modeling and mechanical calculations were used to modify the paullone scaffold **v**: this approach resulted in the discovery of a 9-nitropaullone with nanomolar potency. Abbreviations: PKA, ATP-dependent protein kinase; PKCα, protein kinase Cα; EGFR, epidermal growth factor receptor.

In several cases, the structural biology efforts clearly show that compounds already known to be selective for a specific kinase (e.g. p38 [32]) or kinase class (e.g. the Src family kinases [33–35]), targeted the poorly conserved areas of the ATP-binding site, thus providing a structural basis for the observed selectivity. By contrast, the conserved nature of the kinase core makes it an excellent candidate for homology modeling, in which the sequence of the conserved core of a new kinase can be modeled onto a 3D template. As more structures are determined, the accuracy of the modeling and the possibility of using this approach for inhibitor design will improve. Although the efforts in exploiting this structural knowledge for the design of novel and selective inhibitors have been somewhat limited (for example, compared to medicinal chemistry efforts), structure-based design approaches have played an important role in the identification of new lead structures and in the successful derivatization of 'classical kinase templates'.

Cyclin-dependent kinases

Cyclin-dependent kinases have a crucial role in regulating the cell cycle and constitute potential targets in anticancer drug research [36,37]. Small molecules of different chemical types have been reported to inhibit CDKs, for example, olomoucine (Fig. 3; **i**) and flavopiridol (Fig.3; **ii**); flavopiridol is a semisynthetic flavonoid that emerged from an empirical screening program and is undergoing clinical evaluation [38]. Crystal structures of CDK2 with both compounds bound have been determined [39,40]. Using these data, Furet *et al.* [41,42] developed a structure-based drug design approach to improve the potency of olomoucine-derived compounds and to find different

structural classes of CDK1 inhibitors. Based on CDK2 structures, they constructed a 3D model of the ATP-binding pocket of CDK1 bound to olomoucine. This model was used to design modifications at the synthetically accessible N9, N6 and C2 positions (Fig. 3). This approach resulted in the design of compounds (e.g. **iii**; Fig. 3), which are several orders of magnitude more potent than olomoucine and retained the selectivity profile displayed by olomoucine. Analysis of the binding mode of olomoucine and flavopiridol to CDK2 enabled the design of an ATP-site directed ligand scaffold that facilitated the identification of new, potent inhibitors of CDK1 in subsequent database searching. Testing of the newly discovered naphthoquinone compounds (Fig. 3; **iv**) showed that they were at least equipotent to **i** and **ii**, and that they were characterized by some degree of selectivity for CDK1 over a panel of other kinases (CDK4, PKCα, PKA and EGF-R). One of the compounds was also shown to be equipotent to flavopiridol in inhibiting the proliferation of human bladder carcinoma T24 cells *in vitro*. The structure of one analog of **iv** bound to CDK2 was also determined, confirming that the binding mode was the one predicted by the design hypothesis for naphthoquinone inhibitors. A similar homology modeling approach, together with quantum mechanical calculations, was used by Gussio *et al.* [43] in the modification of a paullone scaffold (Fig. 3; **v**) for the design of CDK inhibitors. The electronic properties derived from the calculations helped direct synthetic chemistry efforts to produce ligands that promote better charge transfer and strengthen hydrogen bonding as facilitated by resonance stabilization. This approach resulted in the discovery of a 9-nitropaullone with nanomolar potency in enzyme inhibition and a favorable anti-proliferative activity profile in living cells.



IC_{50} (μM)

	LFM	LFM-A12	LFM-A13
EGFR	40	28.4	> 300
BTK	>370	> 300	17.2

	ix	x
Theoretical K_i (μM)	2.0	10
EGFR inhibition (IC_{50}, μM)	0.09	2.5

Figure 4. Small-molecule inhibitors of epidermal growth factor receptor (EGFR) and Bruton's tyrosine kinase (BTK) kinases. Compound **vii** and **viii**, although derived from the same parent compound (**vi**), are remarkably selective for their target. The K_i for EGFR inhibitors 4-anilinoquinazolines (**ix**, starting compound, and **x**, best compound identified) were estimated by modeling studies; the IC_{50} values were measured by transfilter cell-invasion assays [50]. These compounds were shown to inhibit the proliferation and *in vitro* invasiveness of EGFR-positive human breast-cancer cells. Abbreviation: LFM, leflunomide.

Another way to exploit available crystallographic information, which has been used in the design of CDK2 inhibitors, is the structure-based design of combinatorial libraries, representing an effective way to either improve inhibitor discovery or improve potency of already known inhibitors. From structural studies, it was shown that olomoucine binds to CDK2 with the purine ring rotated by ~160° with respect to the adenine ring in ATP. Based on this comparison, three purine sites (positions 2, 6 and 9) were identified, the modification of which could potentially lead to improved binding affinity and selectivity. The best inhibitors identified from the libraries were approximately three orders of magnitude more potent than the parent compound [44,45]. Ikuta and co-workers [46] took the homology modeling concept one step further and synthesized a CDK4-mimic CDK2 protein in which the ATP-binding pocket of CDK2 was replaced by the one

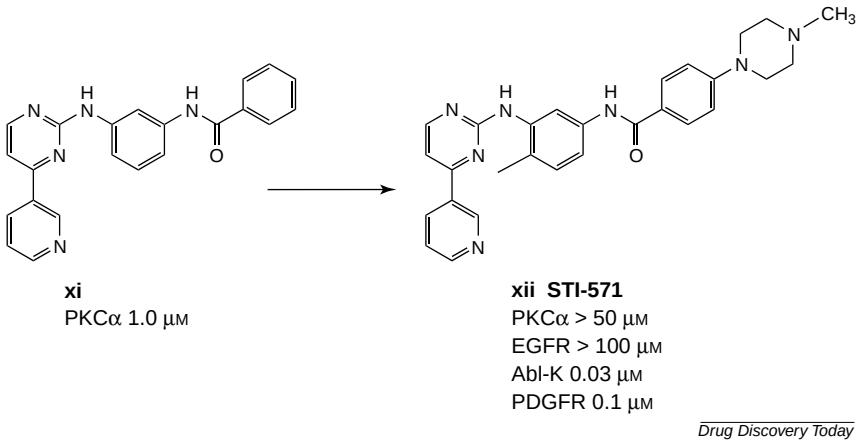


Figure 5. STI571 is a very selective Abl kinase inhibitor. This class of compounds has been approved by the FDA for treatment of chronic myelogenous leukemia (CML). Abbreviations: EGFR, epidermal growth factor receptor; PKCα, protein kinase Cα; PDGFR, platelet-derived growth factor receptor.

in CDK4 by mutating three non-conserved residues. The mutant protein was crystallized in both the ligand-free and ligand-bound form, and the derived structural information was used to design and synthesize a CDK4-specific inhibitor with essentially no CDK2 activity.

ErbB/EGFR kinase

Members of the EGFR family are transmembrane proteins whose overexpression in cancer cells has been associated with excessive proliferation and metastasis [47]. The EGFR is often used as a tumor prognostic marker, because its overexpression has been correlated with a poor prognosis in several tumor types. As well as being an ideal and viable target in cancer drug discovery programs, the role of EGFR in cell proliferation makes it an attractive target in other hyperproliferative disorders, such as psoriasis. Furet *et al.* [48] published the first data of a pharmacophore model for inhibitors competing for the ATP-binding site of the EGFR kinase. This pharmacophore model was used successfully for the design and synthesis of 4-phenylamino-pyrrolo[2,3-*d*] pyrimidines, 4-phenylaminopyrazolo[2,3-*d*]pyrimidines, substituted isoflavones and 3-phenyl-4(1*H*)-quinolones. These compounds have EGFR-kinase inhibitory activity with IC_{50} values of 1–5 nM [49]. In a similar approach, a homology model of the EGFR-kinase catalytic domain was built based on the structural alignment of the EGFR sequence with the sequences of known crystal structures of several other protein kinases; this model was then used in combination with molecular docking methods to identify analogs of the active metabolite of leflunomide (LFM; **vi**, Fig. 4) with potent and specific inhibitory activity against EGFR (LFM-A12; **vii**, Fig. 4) [50]. Interestingly, the same lead compound (**vi**) was used as a starting point to design Bruton's tyrosine kinase (BTK) inhibitors, which were then evaluated using a 3D homology model for the BTK domain and molecular docking procedures. This process resulted in the identification of LFM-A13 (**viii**; Fig. 4), which, although very similar to LFM-A12, is a remarkably selective inhibitor of BTK and does not show any activity compared with the EGFR kinase [51]. The model of the EGFR-binding pocket was also used in combination with docking procedures to estimate K_i values (based on the binding interaction between the inhibitor and the catalytic pocket of EGFR) and design substitutions for another class of EGFR inhibitors, the 4-anilinoquinazolines (Fig. 4; **ix** and **x**). Both LFM- and quinazoline-derived compounds inhibited the proliferation and *in vitro* invasiveness of EGFR-positive human breast-cancer cells. However, unlike the LFM-derived compounds, the quinazoline compounds were not specific for EGFR [50].

Using a slightly different approach, selective, potent and irreversible inhibitors of the erbB receptor subfamily were designed using an extensive comparative analysis of sequence conservation between the Ser–Thr and Tyr kinases in the context of the structure of the cAMP dependent Ser–Thr kinase. This enabled the identification of a conserved Cys residue in the nucleotide-binding site of the erbB receptor family and the design of a thioadenosine that covalently inactivates erbB-1 (or epidermal growth factor receptor, EGFR [52]).

Janus kinase 3

Members of the Janus family of tyrosine kinases (JAKs) activate a family of DNA-binding proteins known as STATs (signal transducers and activators of transcription) [53], that have a dual role as signaling molecules and transcription factors. JAKs are abundantly expressed in primary leukemic cells from children with acute lymphoblastic leukemia and represent a potential target for treatment of leukemias with hyperactive JAK–STAT signaling machinery [54]. The structure-based design of inhibitors of JAK3 is described by Sudbeck and co-workers [55]. After constructing a homology model for the kinase domain of JAK3, modeling and docking studies were used to predict how well potential inhibitors could fit into and bind to its catalytic site. The modeling studies suggested that 4-(phenyl)-amino-6,7-dimethoxyquinazoline compounds with an OH group at

the 4′ position of the phenyl ring would most strongly bind to JAK3 because of the added interactions with Asp967. These compounds inhibited JAK3 in immune complex kinase assays in a dose-dependent fashion, with K_i values between 0.6 and 2.3 μM, while compounds lacking the 4′-OH did not show any significant JAK3 inhibition. Furthermore, the lead dimethoxyquinazoline compound did not inhibit JAK1, JAK2, SYK (spleen tyrosine kinase), BTK, LYN (LCK-related kinase) or the insulin receptor kinases. The same compound was shown to inhibit the clonogenic growth of JAK3-positive leukemia cell lines DAUDI, RAMOS, LC119, NALM6, MOLT3 and HL60 (but not JAK3-negative BT20 breast cancer, M24-MET melanoma or SQ20B squamous carcinoma cell lines) in a concentration-dependent fashion.

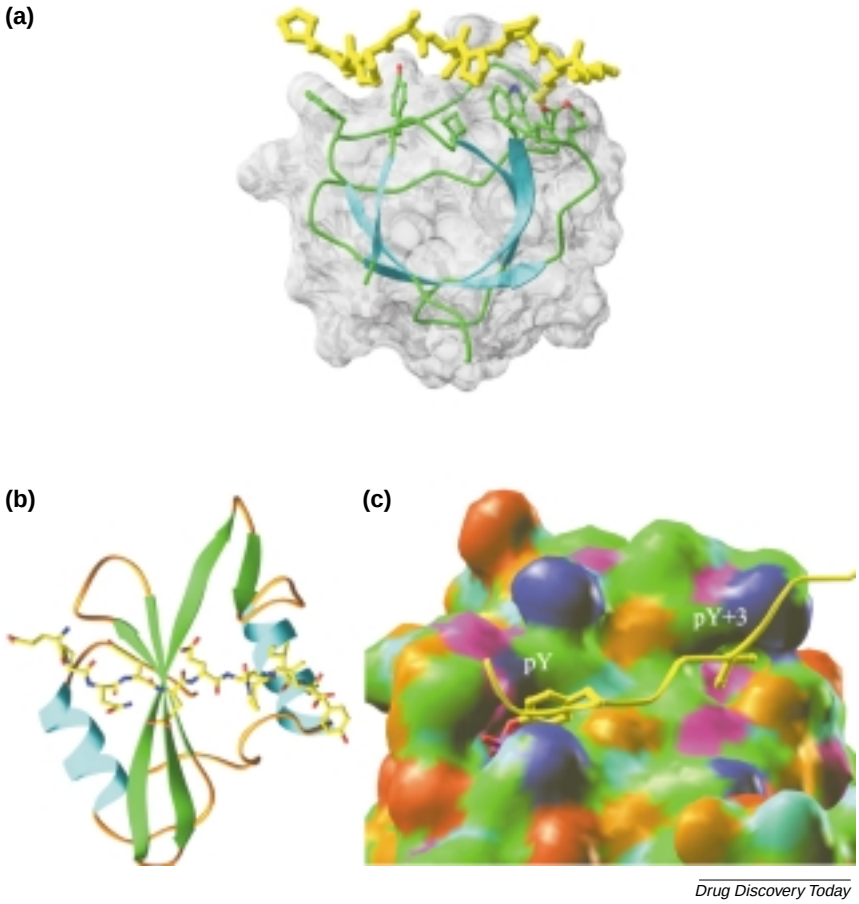


Figure 6. (a) Ribbon diagram of the c-CRK SH3 domain in complex with the C3G peptide [Pro-Pro-Pro-Ala-Leu-Pro-Pro-Lys-Lys-Arg; Protein Data Bank (PDB) code 1CKA]. The surface of the protein is shown in grey. The proline-rich peptide adopts a left-handed type II polyproline helix configuration, in which one residue in every three faces the SH3 domain. At the surface of the SH3 domain these residues interact with a recognition platform consisting of highly conserved aromatic residues (Trp, Tyr, Phe) and a proline. Other ionic interactions have an important role in the recognition process, for example, those with the acidic cluster represented by the two glutamates in the figure. **(b)** Ribbon diagram of human p56-LCK in complex with an 11-residue phosphotyrosyl peptide (PDB code 1LCJ). **(c)** Binding surface for the p56-LCK SH2 domain: for clarity, only the phosphotyrosine (in position pY) and the residue in position pY+3 of the bound peptide are shown.

Abl kinase

Random-screening identified a phenylamino-pyrimidine compound (Fig. 5; **xi**) as an unselective, submicromolar inhibitor against PKCα. Simple modifications of this lead compound led to compounds that completely lost the PKCα activity, but were dual Abl–PDGFR kinase inhibitors. This eventually led to the development of CGP57148, or STI571 [49] (Fig. 5; **xii**), a potent and selective Abl kinase (Abl-K) inhibitor, which has been recently approved by the FDA for the treatment of chronic myelogenous leukemia [caused by the inadvertent activation of the Abl-K by fusion of the corresponding gene (*Abl*) to a segment of the downstream *bcr* gene]. The structure of an STI571 analog bound to the catalytic domain of Abl-K [56] provides a structural mechanism for the selective inhibition of Abl-K. Crucial to the binding of the compound is the adoption by the kinase of an inactive conformation in which the activation loop is not phosphorylated. The conformation of the loop is distinct from that observed in activated PTKs, as well as from that observed in the inactive form of the closely related Src kinase. Although the previous ATP-competitive inhibitor was not identified with the help of a pharmacophore model, it exemplifies the ATP-binding site of protein kinases as an exciting target, and shows that minor modifications of a molecule's structure and its mode of interaction with the target enzyme can lead to major changes in the selectivity profile. These results also suggest that compounds that exploit the distinctive inactivation mechanism of individual protein kinases can achieve both high affinity and high selectivity.

Inhibition of intra-macromolecular interactions

Signaling by PTKs involves a regulated series of protein–protein and protein–membrane interactions mediated by a variety of conserved protein modules, including the Scr SH3 and SH2 domains, the C1A and C1B subdomains of

PKC, as well as a variety of other motifs [57–60]. This type of protein–protein interaction is another potential target for the rational design of inhibitors. Such inhibitors could be used to elucidate the roles of their target molecules in cellular functions, and to evaluate their potential as targets for therapeutic intervention.

SH3 domains

Src homology 3 domains are small (55–70 amino acid) non-catalytic protein modules that mediate protein–protein interactions by binding to proline-rich sequences. The interaction requires the minimal consensus sequence PxxP (Fig. 6a). Originally discovered as a homology domain present in the tryosine kinase v-Src, SH3 domains have now been found in numerous intracellular proteins, including kinases, lipases, GTPases, adapter proteins, structural proteins and viral regulatory proteins. X-ray and/or NMR methods have determined numerous SH3 domain structures and such studies have revealed that the ligand binds to the SH3 domain in a left-handed type II polyproline helix conformation. The ligand can interact with the SH3 domain in one of two orientations, depending on the position (N- versus C-terminal) of its proline-rich sequence [58,61]. Core SH3 ligands are characterized by weak interactions (K_d = 5–100 μM) that show little binding selectivity within SH3 families. Higher affinity, more selective ligands require additional flanking residues that bind to less conserved portions of the SH3 surface. Most of the efforts in designing potent and SH3-selective compounds have been based on the use of combinatorial libraries to target either the N-terminal end of the cognate peptide, or the internal Pro-Pro fragment [62,63]. Although these efforts resulted in the synthesis of peptidomimetics, to date there are no reports of non-peptidyl small molecule SH3 inhibitors.

SH2 domains

Src homology 2 domains are non-catalytic motifs of ~100 amino acids that specifically bind phosphotyrosine residues (pTyr), with an affinity that is dependent on the amino acid sequence C-terminal to the pTyr motif [58,64]. All the SH2 domains determined to date share common structural properties, including a central antiparallel β-sheet core, flanked on each side by an α-helix (Fig. 6b). The phosphorylated ligand binds perpendicular to the β-sheet and typically interacts with two well-defined binding pockets: the pTyr binding pocket and a hydrophobic pocket that interacts with the pY+3 side chain (Fig. 6c). Inhibitors of specific SH2 domains have become important therapeutic targets in the treatment and/or prevention of several diseases, including restenosis, cancers, cardiovascular disease and osteoporosis [25]. In most cases, peptide libraries have

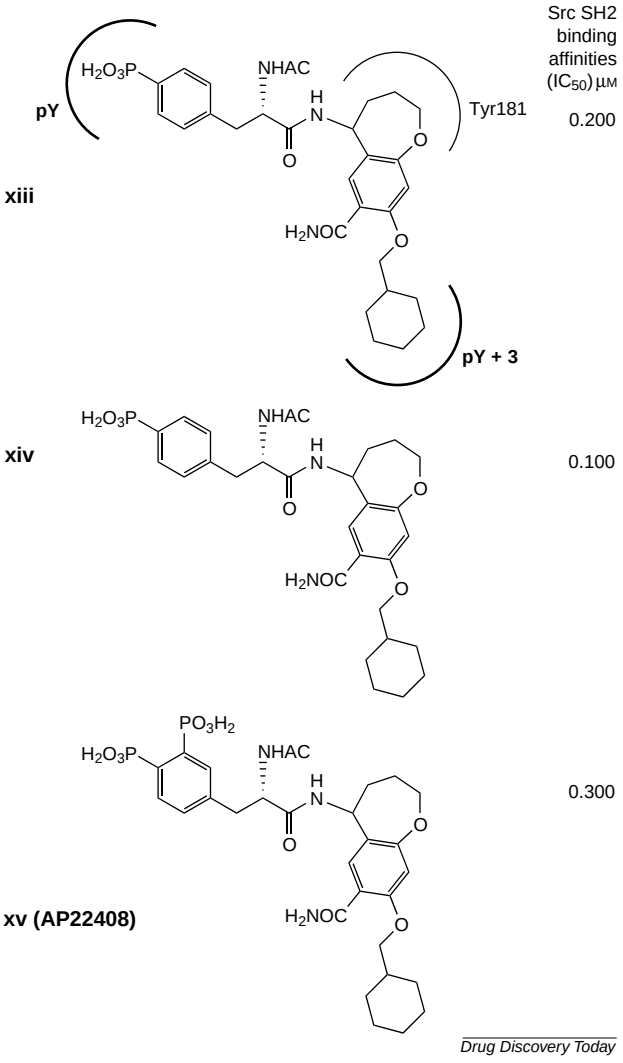


Figure 7. Small molecule, non-peptidic inhibitors of the Src homolgy 2 (SH2) domain with *in vivo* inhibition of osteoclast-mediated bone resorption. The high affinity is achieved by positioning of the seven-member ring over the side chain of Tyr181, which forms the hydrophobic region that separates the pTyr and the pY+3 pockets.

generated the initial ligands; the subsequent use of structural information has been crucial in understanding the mode of binding of these inhibitors and the design of second-generation compounds. Several examples of novel, non-peptide inhibitors of the Scr SH2 domain, designed on the basis of X-ray crystal structures, have been described [65–67]. In each case, the X-ray crystal structure of one or more of the inhibitors bound to the SH2 domain confirmed the interactions targeted but also revealed a unique mode of binding for the pTyr analog, which could be further exploited for optimization of the compounds [65]. Modeling, and the crystal structures of phosphopeptides bound to SH2 domains, helped in the design of second-generation

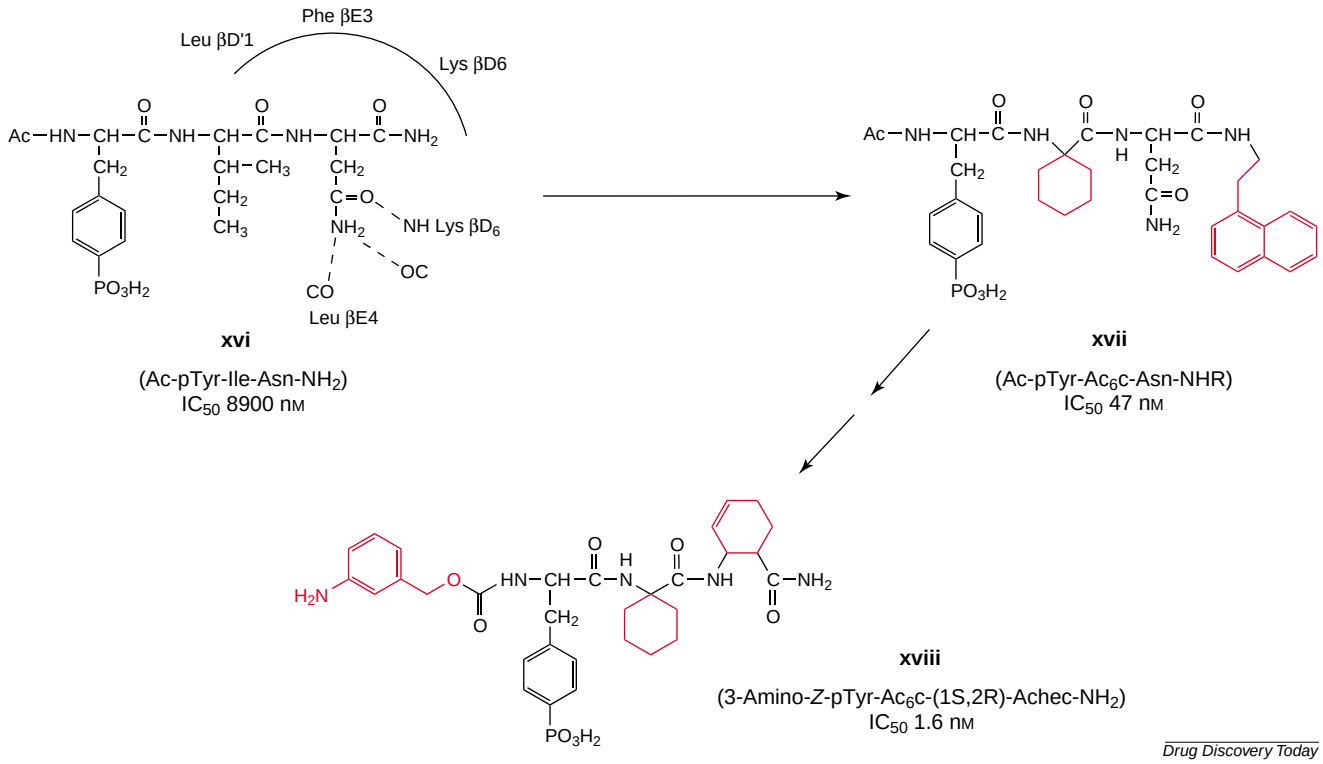


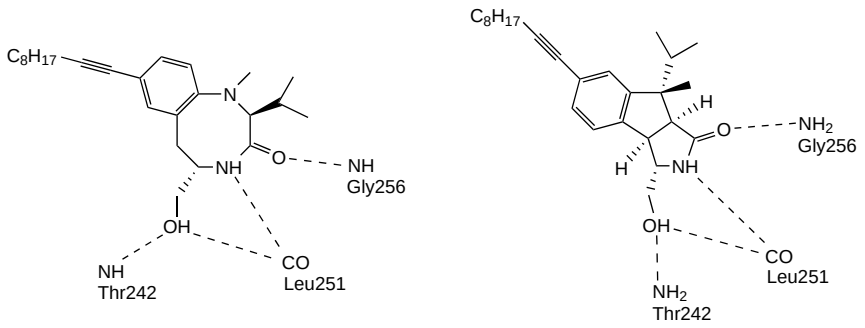
Figure 8. Progress in the design of small molecule, non-peptidic inhibitors of the Grb–SH2 domain. Analysis of the structure of Grb2–SH2 in complex with the phosphopeptide **xvi** revealed the presence of a large hydrophobic region near the C-terminal end of the peptide formed by the side chains of Leu βD′1, Phe βE3 and the hydrocarbon part of the side chain of Lys βD6. This feature was exploited in the design of **xvii**, in which the 3-naphtalen-1-yl-propyl group interacts with this area [69]. The presence of three hydrogen bond interactions made by the ligand asparagine with the main chain atoms of Lys βD6 and Leu βE4, and its position and conformation (I + 2 of a type I β-turn), were used to design queries for a database search that resulted in the replacement of the asparagine with a β-amino acid mimetic (**xviii**, [70])

inhibitors (Fig. 7; **xiii** and **xiv**), which represent some of the tightest binding inhibitors for this SH2 domain [67]. Further modification of this series produced a compound (Fig. 7; **xv,** AP22408) that demonstrates *in vivo* inhibition of osteoclast-mediated bone resorption [68].

Another system for which the structure-based design of SH2 inhibitors produced suitable antagonists is the adaptor protein Grb2. The SH2 domain of Grb2 serves as a crucial link between various growth factor receptors and *ras* proteins. In recent years, intracellular signaling events involving *ras* proteins have been directly implicated in many oncogenic processes, suggesting that Grb2–SH2 domain inhibitors could be useful in cancer research. As reported for the c-Src SH2, the initial leads for the Grb2–SH2 inhibitors were derived from minimal peptide sequences (Fig. 8; **xvi**). The X-ray structure of the Grb2–SH2 domain in complex with a specific phosphopeptide ligand revealed an extended hydrophobic area adjacent to the primary-binding site of the ligand on the SH2 domain. This feature has been exploited to design hydrophobic C-terminal groups that improve the binding affinity of the minimal sequence pTyr-Ile-Asn recognized by the Grb2–SH2 domain. This modification, combined with replacement of the isoleucine residue by 1-aminocyclohexane carboxylic acid to stabilize the β-turn conformation, which is required for recognition by the Grb2–SH2 domain, resulted in the high affinity (47 nM in an ELISA assay) and selective (over a variety of other SH2 domains) phosphopeptide (Fig. 8; **xvii**) [69]. To further reduce the peptidic character of the compound, the Asn residue was successfully replaced by a six-membered ring β-amino acid, designed on the basis of the Asn side conformation and hydrogen-bonding pattern. This new antagonist (Fig. 8; **xviii**) maintained the activity observed for its precursor (3-amino-*Z*-pTyr-Ac_6c-Asn-NH_2) [70].

C1B domain of PKC

PKC is a signaling enzyme that has been proposed to have an important role in carcinogenesis, metastasis and chemotherapy-associated multidrug resistance. A recent review [71] summarizes several lines of evidence suggesting that the expression of constitutive PKCα activity is required for the survival of human prostate cancer cells, thereby proposing



xix
Benzolactam

xx
Pyrrolidone

IC_{50} (nM)	PKCα	PKCβ	PKCγ	PKCδ	PKCε
xix	23	70	43	114	40
xx	580	701	279	1630	469

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Figure 9. Small-molecule protein kinase C (PKC) activators that could be used in the treatment of prostrate cancer. Based on the X-ray structure of PKCδC1b in complex with phorbol 13-acetate [72] and the structure of the potent activator **xix**, with the aid of molecular modeling, a new class of PKC modulators – exemplified by **xx** – was designed [60]. These pyrrolidone analogues maintain the hydrogen-bonding network that is crucial for interaction with PKC.

that PKCα could serve as a novel target in the treatment of prostate cancer. Based upon the structure of the potent PKC activator 8-decynylbenzolactam (Fig. 9; **xix**) and the X-ray structures of the second activator-binding domain of PKCδ (C1B domain) in complex with phorbol 13-acetate, Qiao and co-workers [60] designed a new class of rigidified pyrrolidone-based PKC activators. These compounds maintain the hydrogen-bond network and hydrophobic interactions crucial for binding to C1B, and were found to possess reasonably good affinity for PKC and, in a few cases, some isoenzyme selectivity. One of these pyrrolidone compounds (Fig. 9; **xx**) was shown to induce a dose-dependent induction of apoptosis in LNCaP prostate cancer cells.

Conclusions

Several examples exist in the recent literature of structure-based drug design, which rely on some knowledge of a protein kinase's 3D structure to design and/or optimize specific inhibitors. Most of the studies use homology models, which can only approximate the actual target structure but are becoming more and more accurate as the number of kinase crystal structures are solved. The structure-based design of a new compound is almost never a *de novo* process, but more often a modification of existing leads, both naturally occurring (ATP, peptide substrates, natural products) or found by conventional biochemical screening. One potential pitfall of relying solely on structural considerations to design preclinical candidates is that structure-based drug design might provide inhibitors that are extremely potent and selective *in vitro*, but with limited or no activity in tissue cultures or in animals. Nevertheless, the methods and results summarized in this review clearly illustrate the power of structure-based design in lead discovery (i.e. by virtual screening) and in the process of assisting the optimization of the pharmacological properties of these molecules.

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