Recent Progress in Development of Non-ATP Competitive Small-Molecule Inhibitors of Protein Kinases

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Abstract: The majority of marketed and late stage development kinase inhibitors are reported to be ATP-competitive. As a result, many promising drug candidates display non-specific activity that results in undesired physiological effects. There is growing interest towards non-ATP competitive kinase inhibitors, as they are expected to yield highly specific and efficacious molecules devoid of non-mechanistic toxicity. Recent developments in this area are summarized in our review.

Keywords: Protein kinases, Tyrosine kinases, Serine/threonine kinases, Non-ATP competitive inhibitor, Allosteric binding, Library design.

ATP- VERSUS NON-ATP COMPETITIVE INHIBITION

Protein kinases play a key role in virtually all physiological processes including proliferation, angiogenesis, migration, cell cycle, *etc*. It is now recognized that abnormal phosphorylation of proteins mediated by kinases may result in diseases including cancer, diabetes, rheumatoid arthritis and hypertension [1]. To address the need for effective therapeutics against these pathologies, a large number of small-molecule protein kinase inhibitors have recently been developed [2]. With several compounds on the market, such as Imatinib and Gefitinib, and numerous drug candidates in late-stage clinical trials (for example, see [3]), small-molecule kinase inhibitors continue to attract attention.

As binding of ATP is essential for kinase activity, the discovery of small molecule ligands competing for the ATP binding site has been the main source for new kinase inhibitors [1]. Due to the structural conservation of ATP binding sites in more than 500 protein kinases in the human genome [4], molecules that compete with ATP binding often cross-react with many different kinases [5,6]. Despite the fact that relatively specific ATP competitive inhibitors have been described [7,8], the lack of structural diversity at the ATP binding sites generally prevents identification of selective agents. As an example, highly homologous Akt isoenzymes can be mentioned, which have identical binding sites but different biological functions [9].

Table 1. Non-ATP Competitive Inhibitors of Protein Kinases in Clinical Trials*

Agent	Target kinase enzyme	Development phase	Disease	Developer	Ref.
BIRB 796	p38 MAP kinase	Phase III	Inflammation, rheumatoid arthritis, Crohn's disease	Boehringer Ingelheim	[10]
U 0126	MEK1/2	Phase I	cancer	Promega	[11]
PD 184352	MEK1/2	Phase II	cancer	Pfizer	[12]
ARRY-142886	MEK1/2	Phase I	cancer	Array BioPharma/ Astra Zeneca	[13]
ON 01910	polo-like kinase 1	Phase I	cancer	Temple University School of Medicine	[14]

* The shown data reflect the status of a survey completed in mid-2005.

In recent years appreciation has grown that non-ATP-site directed approaches to modulating kinase function are also viable and offer alternative approaches to down-regulating kinase pathway function. The considerable effort in developing non-ATP competitive agents led to several agents that entered clinical trials (Table **1**). The growing track record of success suggests the great potential benefits associated with this class of protein kinase inhibitors. In our review, we describe several successful programs that yielded non-ATP competitive kinase antagonists. We also discuss current strategies for identification of non-ATP competitive kinase inhibitors, including mechanism of action and perspectives of this approach.

NON-ATP COMPETITIVE KINASE INHIBITORS

The non-ATP competitive approach was successfully applied to the discovery of specific inhibitors for mitogen-

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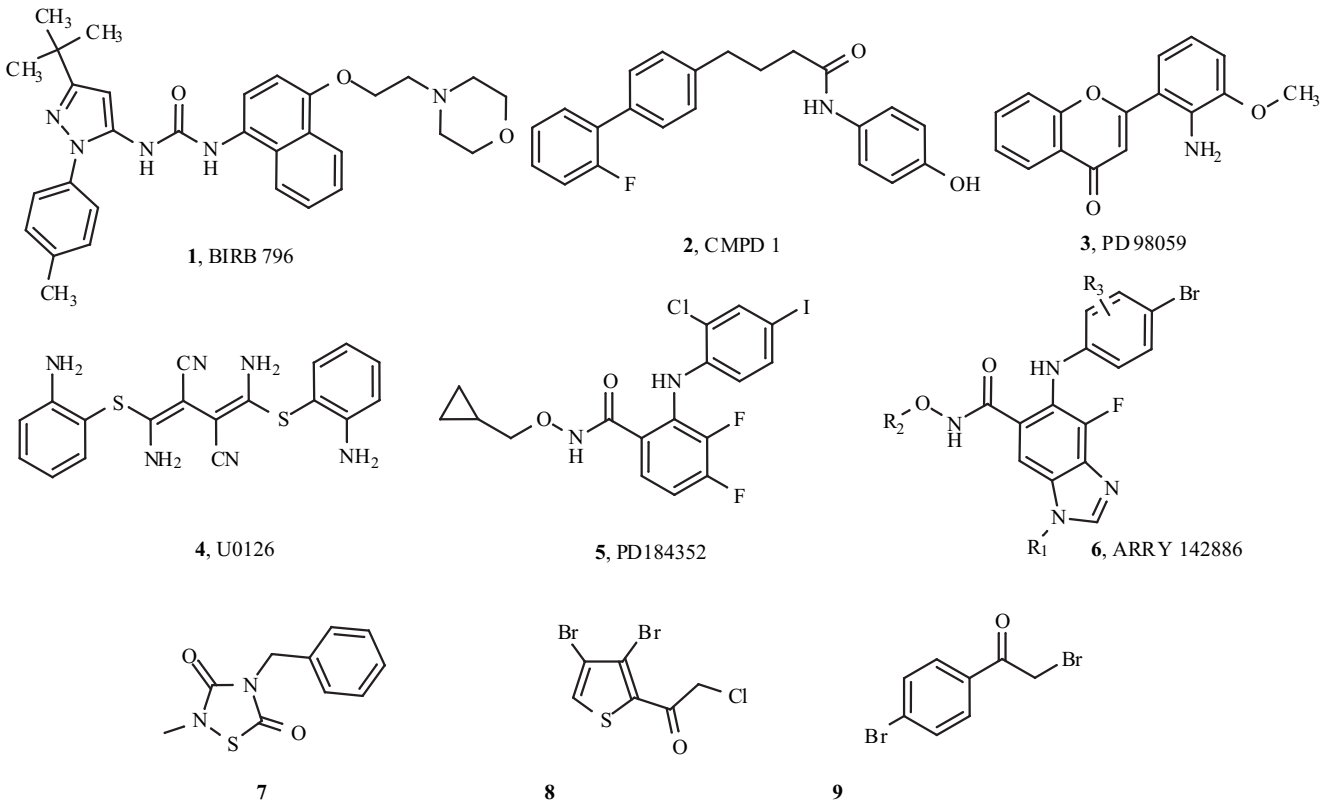

Fig. (1). Non-ATP competitive protein kinase inhibitors. Compounds **1** and **2** – inhibitors of MAPK; compounds **3-6** – inhibitors of MEK1/2; compounds **7-9** – inhibitors of GSK3β.

activated protein kinases (MAPKs), which control complex cellular programs, such as embryogenesis, differentiation, proliferation and cell death, in addition to short-term changes required for homeostasis and acute hormonal responses [15,16]. p38 Ser/Thr MAP kinase, an example of this class of enzymes, is reported to be a critical component of the signal transduction cascade leading to the production of proinflammatory cytokines, such as TNF and IL-1 [17]. Blocking p38 is expected to yield efficacious anti-inflammatory agents. In a study by Pargellis *et al.*, structural data were used to design a potent p38-MAPK inhibitor [10]. Analysis of the binding mode for the early HTS hit demonstrated that this molecule induced conformational changes in the enzyme. Subsequent medicinal chemistry efforts yielded the optimized non-ATP competitive inhibitor BIRB796 **1** (Fig. **1**). This molecule afforded a 12,000-fold increase in binding affinity compared to the initial lead in addition to high selectivity over a panel of 11 kinases.

Recently, a novel inhibitor of p38 MAPK, CMPD1 (structure **2**), was identified by HTS [18]. This inhibitor is substrate selective and non-competitive with regard to ATP. In steady-state kinetics experiments, CMPD1 was observed to specifically prevent p38-dependent phosphorylation (K_i = 330 nM) of the splice variant MAPK-activated protein kinase 2 (MK2a); at the same time, it did not affect phosphorylation of ATF-2 (K_i > 20 μM) [18].

Inhibiton of MAPK/ERK kinases (MEK's), dual specificity kinases that phosphorylate MAPK, is an alternative approach in modulating MAPK signaling. Enhanced MEK1 and MEK2 activity was detected in a significant number of primary human tumor cells [19, 20]. Compound PD98059 **3** (Fig. **1**) was characterized as a non-ATP competitive inhibitor of MEK. It prevented activation of MAPK kinase 1 and reversed the phenotype of Ras-transformed cell lines [21]. Although PD 98059 was useful in dissecting the MAPK signaling cascade, it was not sufficiently potent or soluble to be useful *in vivo*. A more potent inhibitor of MEK1 (U0126, structure **4**) has been identified [11]. Radiolabelled U0126 competes for binding with PD 98059 suggesting that both compounds have a similar binding site on the kinase. U0126 also blocks IL-2 synthesis and T-cell proliferation [22]. Its clinical utility as an anticancer agent is currently being evaluated in Phase I trials.

A second-generation allosteric MEK1/2 inhibitor, PD 184352 **5**, works via an allosteric mechanism. [12]. It maintains kinases in an inactive state by preventing their phosphorylation by upstream activating kinases such as Raf. PD 184352 has an IC_{50} value below 20 nM against both kinases. In addition, it shows enhanced bioavailability [12]. In a functional assay, elevated ERK1 and ERK2 activity in colon 26 carcinoma cells was inhibited by PD 184352 with an IC_{50} of 120 nM. The compound inhibited growth of tumors derived from these cells when given orally to mice [12]. PD 184352 is under evaluation in Phase I clinical oncology trials [23].

Researchers at Array BioPharma described compound ARRY-142866 (AZD-6244) **6** (exact structure is not disclosed) as a potent (IC_{50} = 12 nM), selective non-ATP competitive inhibitor of MEK1 [13]. ARRY-142886 produced significant dose-dependent inhibition of tumor

growth in the human colon carcinoma HT-29 xenograft model in mice. The observed effect was well correlated with reduced levels of phosphorylated ERK in tumors [24]. ARRY-142886 is currently being investigated in a Phase I clinical trial.

Glycogen synthase kinase-3 (GSK-3) is an attractive metabolic target. A specific isoform of this enzyme, GSK-3β is believed to play a key role in various diseases including neurodegeneration, type II diabetes, bipolar disorders, stroke, cancer, chronic inflammation and Alzheimer's disease [25]. The majority of reported GSK-3β inhibitors are ATP-competitive and provide limited selectivity (for example, [26-28]). In an attempt to improve selectivity, a series of non-ATP competitive inhibitors were developed (for example, thiadiazolidinone **7**, Fig. **1**) [29]. Subsequently, the same group introduced several allosteric inhibitors of this kinase based on α-halomethyl ketones (**8, 9**) [30]. Compounds **7**, **8** and **9** inhibited this enzyme with IC_{50} = 2, 0.5 and 0.5 μM, respectively. The presence of a strong alkylating moiety in these molecules is expected to severely limit their utility.

Natural lactone Sporostatin **10** (Fig. **2**) isolated from a fungus of *Sporormiella sp*. was initially reported as an inhibitor of c-AMP phosphodiesterase [31]. It was subsequently found to be a specific inhibitor of EGFR kinase [32] with IC_{50} = 0.38 μM *in vitro*. The compound showed impressive specificity against other members of ErbB-family of receptors including ErbB2 (IC_{50} = 11 μM) and other kinases (> 100 mM for PDGFR, v-src and PKC). Kinetic analyses revealed that inhibition of EGFR was non-competitive with regard to both substrate and ATP. This result was further confirmed in a cell-based assay.

Imidazo[1,2-a]quinoxaline, BMS-345541 **11** (Fig. **2**) was introduced as a highly selective inhibitor of I kappa B (IκB) kinase [32] active *in vivo* [33]. This compound was described as a potent, allosteric inhibitor of the catalytic subunit IKK-2 (IC_{50} ~300 nM). The molecule exhibited ~10-fold greater selectivity over IKK-1 (IC_{50} ~ 4.0 μM) and no activity towards IKKε and a panel of 15 additional kinases at concentration > 100 μM. In addition, BMS-345541 inhibited cellular IκBα phosphorylation (IC_{50} = 4 μM in THP-1 cells) and LPS-induced cytokine production both *in vitro* (IC_{50} = 1-5 μM in THP-1 cells) and *in vivo* (IC_{50} = 10 mg/kg in mice). This agent also effectively blocked inflammation and joint destruction in a murine arthritis model [35, 36]. The compound exhibited a promising PK profile in mice, with 100% oral bioavailability.

The aberrant activation of polo-like kinase1 (Plk1) has been found in many human tumors [37]. Researchers from Temple University School of Medicine examined the effects of Plk1 inhibition on tumor growth in several animal models [14]. They found that compound ON01910 **12** is a

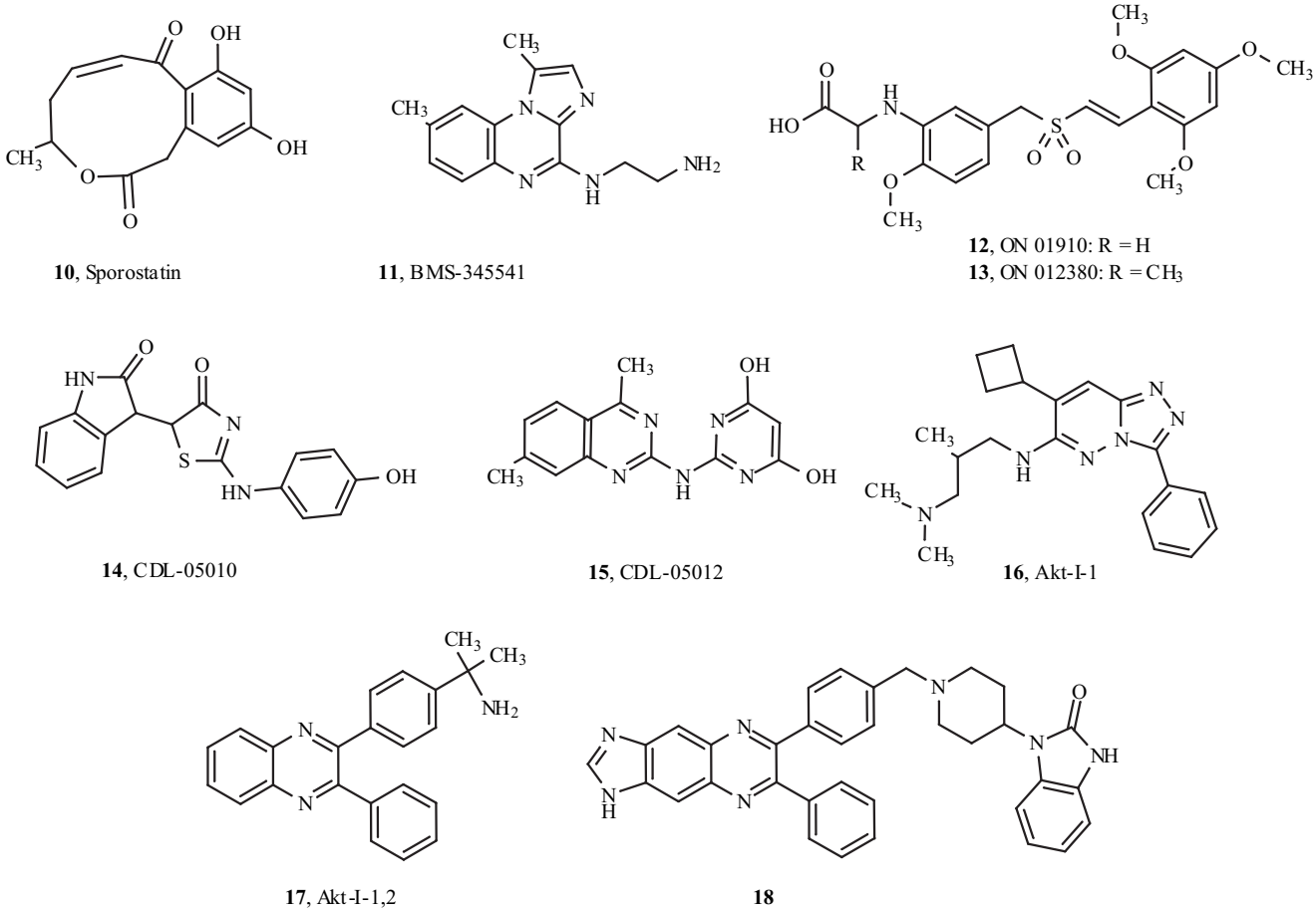

Fig. (2). Non-ATP competitive protein kinase inhibitors. Compound **10** – inhibitor of EGFR; compound **11** – inhibitor of I kappa B kinase; compound **12** – inhibitor of polo-like kinase1; compounds **13-15** – inhibitors of Bcr-Abl kinase; compounds **16-18** – inhibitors of Akt kinase.

potent inhibitor of Plk1 activity. It induced mitotic arrest of tumor cells as evidenced by spindle abnormalities leading to cell death *via* apoptosis. This compound was not ATP-competitive, but competed for the substrate binding site of the enzyme. ON01910 significantly inhibited a variety of human tumors, including liver, breast, and pancreatic cancers in murine models. Normal cells were not affected. Currently, this molecule is undergoing Phase I clinical trials.

Several ATP-competitive inhibitors of bcr-Abl, including PD180970 and CGP76030, have been shown to be marginally effective in the treatment of Imatinib™-resistant chronic myelogenous leukemias (CML) [42-45]. A mechanistically similar Src kinase inhibitor, BMS-354825, was also shown to inhibit bcr-Abl [46]. However, none of these ATP-competitive compounds addressed Imatinib™ resistance caused by all the documented mutations within the bcr-Abl kinase domain. Compound ON012380 (**13**, Fig. **2**) has been described as a broad-spectrum inhibitor of bcr-Abl kinase mutants that show resistance to ATP-competitive agents [38], including Imatinib™ [39-41]. Kinetic studies demonstrated that this compound is substrate-competitive. It showed synergy with Imatinib™ in inhibiting a wild-type bcr-Abl as well.

In the course of a discovery campaign, which included iterative cycles of virtual and high-throughput screening of large chemical libraries [47], researchers from ChemDiv described a series of novel non-ATP competitive bcr-Abl kinase inhibitors exemplified in Fig. **2** (structures **14** and **15**). These compounds demonstrated good inhibitory activity (IC_{50} 0.44 and 1.1 μM, respectively) and selectivity against ErbB and PDGFR families of kinases. Kinetic studies of **14** and **15** showed that the rate of substrate phosphorylation in the presence of these inhibitors was constant for increasing ATP concentrations. Both IC_{50} and K_m values remained unchanged as well, suggesting that these compounds are not ATP-competitive inhibitors of the kinase. When the same experiments were performed with Imatinib, an opposite result was obtained in which ATP effectively competed with the inhibitor as suggested by an increased IC_{50} with increasing concentration of ATP, which is in accordance with the published data [48].

A serine/threonine kinase Akt (Protein kinase B/PKB) received considerable attention due to its role as a regulator of the cell apoptotic machinery. Akt was described to have a critical downstream effect on multiple growth factors and receptors involved in tumorigenesis [49, 50]. All three isozymes of human Akt (Akt1, Akt2, and Akt3) are commonly over-expressed or are constitutively active in a large number of human cancers. Despite the high level of homology (>85%), neither the function nor the expression patterns of the Akt isoforms are identical [51, 52]. Notably, it has been reported that the residues in the ATP-binding pocket of all three enzymes are identical [9]. Based on this evidence, identification of isoform-specific inhibitors of Akt poses considerable challenge.

Barnett *et al.* screened approx. 270,000 compounds for their ability to inhibit Akt1, Akt2 or Akt3. Two compounds exhibited isoenzyme specificity (structures **16** and **17**) [53]. The first compound (Akt-I-1) inhibited only Akt1 (IC_{50} 4.6 μM) while the second compound (Akt-I-1,2) inhibited both Akt1 and Akt2 with IC_{50} values of 2.7 and 21 μM, respectively. Neither compound inhibited Akt3 or mutants lacking the PH (pleckstrin homology) domain at concentrations of up to 250 μM. It has been suggested that Akt-I-1 and Akt-I-1,2 inhibit the enzymes by directly interacting with the PH domain and/or binding to a site that is only formed in the presence of the PH domain. The same group of researchers described a series of potent and selective allosteric Akt inhibitors that display high selectivity for either Akt1, Akt2 or both Akt1/Akt2 [54]. For example, compound **18** displayed high potency (IC_{50}'s: Akt1 = 58 nM, Akt2 = 210 nM, and Akt3 = 2119 nM), solubility and cell permeability. Interestingly, in a cell-based assay, this compound was found to possess a similar pattern of inhibition for Akt1 and Akt2 (IC_{50}s: Akt1 = 305 nM, Akt2 = 2086 nM) while having no inhibition of Akt3 (IC_{50} > 25,000 nM).

MECHANISMS OF NON-ATP COMPETITIVE INHIBITION

Review of the literature suggests that there are at least two mechanisms for non-ATP competitive inhibition of kinases. Specifically, compounds can compete with the substrate for a particular binding site. Alternatively, inhibition can proceed *via* an allosteric mechanism. Allosteric inhibitors do not directly compete for binding with the substrate, but they bind to a part of the enzyme, which is distinctly separated from the active site, and change the ability of the ATP- or substrate binding site to function properly. Substrate-competitive mechanism offers better structural insight into a respective binding site and an opportunity for a rational design of inhibitors. For example, the ability of different MAPK groups to interact with specific substrates is mediated by unique docking sites, so-called common docking (CD) domains that are distal from the ATP binding site [55]. These domains are well defined spatially (up to 18 amino acids). They contain several well-defined recognition elements that could be targeted by small molecules, namely a conserved acidic functionality and a specific surface topography [55].

Several advantages to the use of allosteric modulators of receptor activity have been noted [56]. These include: (1) receptor saturation leading to a well-defined efficacy for a drug; (2) ability to selectively tune responses in specific tissues; and (3) potential for greater receptor subtype selectivity. These attributes could be extrapolated to the allosteric modulators of kinases.

Gunasekaran *et al.* argued in a recent review [57] that all dynamic proteins are potentially allosteric. This phenomenon is derived from the redistribution of the dynamic conformational ensembles. A successful allosteric drug is expected to considerably change the properties of the active site, effectively altering its specificity, as exemplified for the p38 MAP kinase [10]. This study demonstrated that BIRB796 inhibits p38 MAP kinase by stabilizing a conserved Asp-Phe-Gly (DFG) motif in the active site, which renders the enzyme conformationally incompatible with ATP binding ("DFG-out"). In the "DFG-out" conformation, the Phe side chain moves by ~10Å to a new position, which prevents ATP binding. For illustration, (Fig. **3**) shows the structure of human p38 MAP kinase in complex with BIRB796 [10,58].

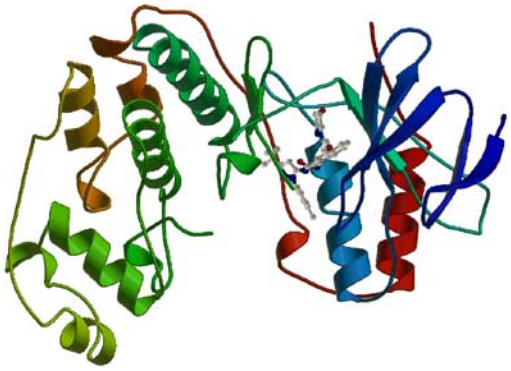

Fig. (3). The structure of human p38 MAP kinase in complex with BIRB796.

Such a conformational variability of the DFG motif may be a general phenomenon in Ser/Thr and Tyr kinases, and thereby may play an important regulatory role. For example, the inactive insulin receptor tyrosine kinase exists in "DFG-out" conformation, which interferes with the binding of ATP [59]. A complex of bcr-Abl with an ATP-competitive inhibitor Imatinib™ was observed in the "DFG-out" conformation [60]. The same principle was recently used in the design of selective inhibitors of GSK-3 (*vide supra*) [61].

DISCOVERY CONCEPTS

It is worth noting that HTS of large diversity-based libraries is still the most popular strategy for identification of non-ATP competitive kinase inhibitors. Examples of successful leads resulting from these HTS campaigns can be found in the literature (for example, [10,21,29,30,47,53, 54]). Two recent reviews [62,63] focus on the development of small molecule protein kinase inhibitors, with emphasis on a detailed description of the various assay formats currently being employed for high throughput screening.

Molecular diversity alone cannot be considered a sufficient library design criterion. In the past few years, a significant medicinal chemistry component has been introduced in the design of focused compound libraries for bioscreening. Various ligand- and target-based design strategies, which can be implemented in kinase-targeted library design, are extensively reviewed in the literature (for some recent reviews, see [64-68]). The existence of similar allosteric binding sites for diverse kinases suggests the possibility of a common theme among the respective binders [65]. In our opinion, modern docking algorithms hold great promise for the discovery of small molecule non-ATP competitive kinase ligands, although their potential is currently underutilized. Information on the primary structure of protein kinases has become increasingly accessible from their sequencing. 3D Molecular models of the allosteric binding sites can be developed based upon homologous X-ray structures available from the Protein Databank [58]. For example, in order to use the conformational variability of the DFG motif, the allosteric site of GSK-3 has been modeled from apo-GSK-3 using homology modeling [61]. Both sequence profile and structural comparison of apo-structures indicated that GSK-3 may undergo conformational change similar to p38 upon compound binding. Consequently, a series of known allosteric p38 inhibitors were employed to validate the model of the GSK-3 allosteric site. This structural model could be further used as a starting point for the rational design of allosteric GSK-3 inhibitors. Typical challenges and pitfalls of target structure-based virtual screening for kinase targets are illustrated in a series of recent reviews [69-71].

Unfortunately, in most cases the topology and/or location of allosteric sites are unknown. Several specific types of structure-based analysis were developed for identifying potential allosteric binding sites and the effects of the ligands binding on the active sites. For example, Luque and Freire [72,73] have performed a structure-based analysis of stability of binding sites using the COREX algorithm, which detects small-scale motions in proteins [74]. Larger motions (e.g., hinge bending) can be investigated by molecular dynamics simulations (for example, [75,76]). Lockless and Ranganathan [77] and Suel *et al*. [78] devised a sequence-based method to quantitatively map global networks of amino acid interactions in a protein. Analysis of three structurally and functionally distinct protein families revealed a subset of conserved residues that link distant functional sites in the tertiary and quarternary structures. These could be probed as "hot spots" for the design of allosteric inhibitors.

Identification of potent kinase inhibitors across different subfamilies of the kinase enzymes can also be based on the systematic analysis of structural genomics data [79,80]. The latest human genome initiatives allow for establishing the relationships between kinase ligands and targets. This, in turn offers the potential to use the knowledge obtained in the screening experiments for "target hopping". In a relevant study, selectivity data were employed by researchers from Lilly to define the chemogenomic kinase dendrogram for 43 kinases [81]. This information was further used as a guideline for making decisions about target selection and identification of inhibitors for chemogenomically related targets. A review by ter Haar *et al.* summarized the application of the chemogenomic approach to kinases [82].

Modern chemogenomics approaches can successfully complement ligand and target structure-based design. As an illustration, a novel methodology of identification of "druggable" protein ligand binding sites has been developed by researchers from MolSoft. The methodology is based on the new "pocketome" concept [83]. The pocketome is a collection of all currently known "druggable" pocket shapes for a given organism derived from the three-dimensional structures deposited in the Protein Data Bank [58] as well as some validated homology models for known drug targets. Researchers from MolSoft and the Scripps Research Institute have recently completed a large-scale classification of the identified envelopes according to their shape and properties (Totrov M., Abagyan, R. personal communication). This approach can be used in the design of biologically diverse and representative chemical libraries targeted against non-ATP binding sites of protein kinases.

CONCLUSIONS

The majority of marketed and late-stage development kinase inhibitors are ATP-competitive. Despite their

demonstrated clinical efficacy, these compounds may have non-specific activity due to the relatively conserved structure of ATP-binding site in kinases. In addition, these may bind to other ATP-dependent proteins (for example, ABC-cassette proteins [84]). In order to address this issue, several drug discovery programs targeting non-ATP-binding sites were initiated in the past few years, resulting in a series of advanced clinical candidates and promising leads.

Both substrate-competitive and allosteric approaches are considered in the design of non-ATP competitive inhibitors. The former is valid for kinases with considerable structural differences in the substrate-binding domain. Most of the reported non-ATP competitive ligands inhibit kinase enzymes *via* allosteric mechanisms, specifically by stabilizing the inactive conformation of the kinase. Several developmental candidates have been unequivocally shown to inhibit kinases by this route. The described advantages of allosteric agents include potency, remarkable selectivity and well-defined target pharmacology. Unfortunately, this approach is not universal as the location of the allosteric site(s) and the functional effect of their modulation are typically unknown. A possible solution in this case is the application of specific types of structure-based analysis for identifying potential allosteric binding sites. Once the binding site is identified or a quality 3D homology model is generated, sophisticated docking algorithms can help select better candidates for synthesis and biological testing. At the same time, rapid and conceptually simple ligand-based strategies can still be very useful as pre-screening procedures in cases where the structure of a target cannot be easily established.

It must be realized that, given the vast number of protein kinases in the human genome and their sequence and structural similarities, added to the inability to test the promising molecules against all kinases, specificity remains a main concern with the non-ATP competitive agents. Nevertheless, this class of compounds offers a great deal of promise to significantly broaden the horizon of modern kinase-directed drug discovery.

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