Drug Discovery Today • Volume 12, Numbers 15/16 • August 2007

ELSEVIER

With increasing interest in protein kinases as drug targets, attention is shifting towards approaches that identify ATP non-competitive kinase inhibitors as new therapies in the treatment of diseases.

A new paradigm for protein kinase inhibition: blocking phosphorylation without directly targeting ATP binding

Marie A Bogoyevitch[1,2] and David P Fairlie[3]

[1] Cell Signalling Laboratory, Biochemistry and Molecular Biology, School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, Australia
[2] Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, 30 Flemington Road, Parkville, Victoria 3010, Australia
[3] Centre for Drug Design and Development, Institute for Molecular Bioscience, University of Queensland, Queensland 4072, Australia

Protein kinases are now recognised as an important class of drug targets. Whilst many protein kinase inhibitors directly interact with the ATP-binding site, Gleevec is a notable example from a new class of allosteric inhibitors that alter protein kinase conformation to block productive ATP binding. Recently, kinase inhibitors with different mechanisms of action have also been described. Some of these are allosteric inhibitors that alter kinase conformation and prevent protein substrate binding. Other inhibitors directly compete with protein substrate binding. These inhibitors promise exciting therapeutic opportunities by exploiting new mechanisms of action and may thus allow greater specificity in protein kinase inhibition with fewer off-target side effects.

Over 500 human genes encode protein kinases, and these can be grouped into a number of subsets based primarily on sequence and structural similarities [1]. These subsets are shown schematically in Figure 1, and this classification emphasises the similarities, as well as the differences, in this large family of enzymes [1]. In general, protein kinases catalyse the transfer of the terminal phosphoryl group of ATP to specific hydroxyl groups (–OH) of serine, threonine or tyrosine residues of their protein substrates. Thus, protein kinases can be considered broadly to be serine/threonine kinases or tyrosine kinases, or in some instances dual-specificity kinases when they phosphorylate serine/threonine as well as tyrosine residues. Since protein phosphorylation controls a diverse range of cellular and pathogenic processes, even subtle changes in protein kinase activity can lead to a wide variety of diseases, including cancer, inflammatory disorders, diabetes, neurodegeneration and central nervous system diseases. This pivotal role has made protein kinases an important and tractable therapeutic class for drug discovery.

Most small molecule protein kinase inhibitors interact with the conserved ATP-binding site of their target protein kinase. This probably reflects the low ATP concentrations used in the *in vitro* screening conditions as well as the structural features of many of the chemical libraries screened in high throughput by the major pharmaceutical companies in their search for protein kinase inhibitors [2]. These ATP-competitive protein kinase inhibitors must ultimately target the kinase with high affinity to compete with the high intracellular concentrations of ATP, but sometimes they do not discriminate between the ATP-binding sites conserved in protein kinases and other ATP-binding proteins [2]. This lower specificity for their intended target may limit their clinical use, particularly when there are off-target side effects. Thus, there is now increasing interest in identifying new classes of protein kinase inhibitors that do not directly compete with ATP. These new inhibitors may potentially enable the selective regulation of specific protein kinases associated with a particular disease but without affecting other protein kinases involved in normal physiology.

In this review, we consider small molecule inhibitors that do not directly compete for the ATP-binding site of protein kinases. Some of these inhibitors stabilise the inactive kinase conformation and kinetically appear 'ATP-competitive' in their inhibition despite their allosteric mechanism of action to disrupt ATP binding. Others show 'ATP non-competitive' kinetics of inhibition. For this latter group, we describe a range of inhibitors that have been characterised kinetically and/or that have been co-crystallised

Corresponding author: Bogoyevitch, M.A. (marieb@unimelb.edu.au)

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2007.06.008

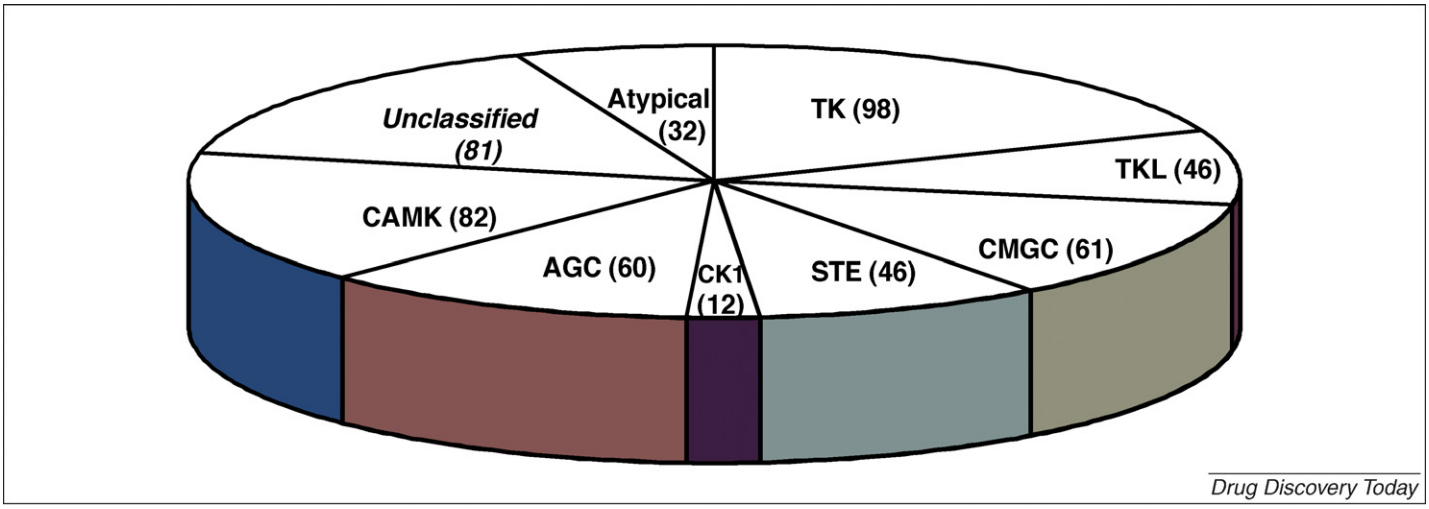


FIGURE 1

An overview of proteins kinases and protein kinase inhibitors. Classification of human protein kinases based on sequence and structure. The figure denotes the different protein kinase subsets, together with the number of protein kinases in each subset as indicated by the numbers in parentheses to indicate the large number of protein kinases, their similarities and their differences. The protein kinase subsets have been defined as follows: TK: tyrosine kinase; TKL: tyrosine kinase like; CMGC: CDK, MAPK, GSK3 and CLK; STE: homologues of Sterile 7, Sterile 11 and Sterile 20 kinases; CK1: casein kinase 1; AGC: PKA, PKG and PKC; CAMK: calcium/calmodulin-dependent protein kinase. The allosteric and protein substrate competitive kinase inhibitors as described in this review act on a range of different protein kinases, specifically Abl, c-Kit, PDGR-R, VEGF-R, IGF-R1 (TK); B-Raf (TKL); GSK3β and MAPKs (CMGC); MEK1/2 (STE); Akt (AGC); and polo-like kinase and IKK-1/2 (unclassified). It is likely that further research efforts will identify additional inhibitors for protein kinases in these and other protein kinase subsets.

with their target kinase and shown to interact outside the ATP-binding site of their target kinase. This highlights the general mechanisms being exploited by this new generation of protein kinase inhibitors to act as allosteric inhibitors or to target the protein substrate binding site of the kinase and thus be protein substrate competitive inhibitors. These new inhibitors offer exciting therapeutic opportunities by exploiting different inhibition mechanisms.

Allosteric kinase inhibition through altered ATP binding: Gleevec, BIRB796, BAY43-9006, AAL-993

Gleevec

Gleevec (imatinib mesylate, STI571, CGP57148 (Figure 2A)) is a potent allosteric inhibitor of several tyrosine kinases. These kinases include Bcr-Abl, platelet-derived growth factor receptor (PDGF-R) and stem cell factor receptor (c-Kit)). As these kinases are important for the phenotypic transformation and progression of a number of specific forms of cancer, Gleevec has been successfully used in the treatment of these cancers [3,4]. The success of Gleevec has now launched substantial new efforts in kinase inhibitor design and development. Thus, additional compounds have been described that bind the inactive conformation of their target kinases.

In considering the discovery and characterisation of Gleevec, it should be noted that the initial results of kinetic analyses to define its actions and ability to inhibit Bcr-Abl suggested its actions as an ATP-competitive inhibitor. However, further structural analyses have revealed the binding of Gleevec near the ATP-binding site of the inactive Abl without directly competing with ATP binding. The structure of the Abl:Gleevec complex has been extensively reviewed (for examples, the reader is referred to references [5,6] and the Protein Data Bank entry 1IEP). Briefly, these structural studies have shown that Gleevec stabilises an inactive conformation of Abl; crystal structures have shown its binding between the N-terminal and C-terminal kinase lobes causing a large conformational change of the kinase active site motif Asp-Phe-Gly (DFG) [7]. In this inactive 'DFG-out' conformation, the Phe residue cannot occupy hydrophobic groove between the two lobes of the kinase structure.

Gleevec also inhibits c-Kit by binding its inactive 'DFG-out' conformation [8], and this mechanism is also likely to underlie its inhibition of the PDGF-R. The importance of this 'DFG-out' conformation extends beyond the actions of Gleevec as it resembles that first described for the inhibited insulin receptor. This suggests that this conformational change, the 'DFG-out' conformation, is a common underlying feature in inhibited protein kinase structures.

Strategies that facilitate the development or stabilisation of this conformation are being explored in the development of new protein kinase inhibitors. These additional inhibitors include BIRB796 that binds p38 mitogen activated protein kinase (MAPK), BAY43-9006 that binds B-Raf and AAL-993 that binds the vascular endothelial growth factor-receptor (VEGF-R). In the following sections, we consider the discovery and subsequent use of these three protein kinase inhibitors.

BIRB796

BIRB796 (Doramapimod (Figure 2B)) [9–12] was developed from a series of diaryl ureas identified by high-throughput screening as inhibitors of the serine/threonine kinase, p38 MAPK. Structural features, as revealed by X-ray crystallography of the inhibitor-kinase complex, showed an inactive p38 MAPK conformation with the reorientation of the DFG motif, the phenylanine residue shifting 10 Å away from the hydrophobic-binding pocket. This created a new hydrophobic-binding pocket adjacent to the ATP-binding site for insertion of the *tert*-butyl substituent of BIRB796.

Although BIRB796 selectively inhibited the p38α MAPK isoform, blocked TNFα release *in vitro* and inhibited collagen-induced

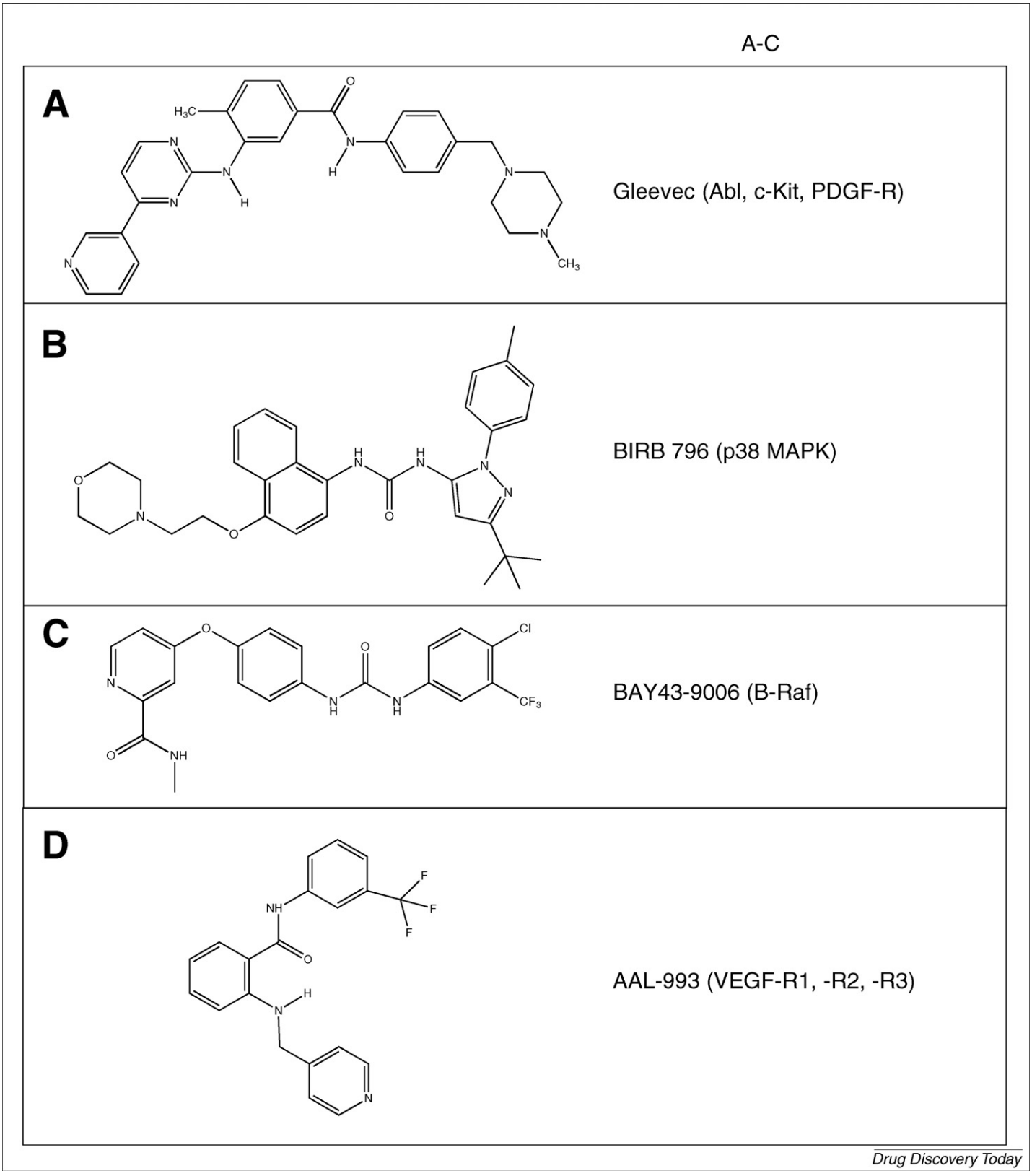


FIGURE 2

Selected allosteric inhibitors that disrupt the protein kinase ATP binding site. **(A)** Gleevec, an inhibitor of the tyrosine kinases Abl, c-Kit and PDGF-R. **(B)** BIRB796, an inhibitor of the p38 MAPKs. **(C)** BAY43-9006, an inhibitor of B-Raf. **(D)** AAL-993, an inhibitor of vascular endothelial growth factor-receptor (VEGF-R) 1, 2 and 3.

arthritis, its use in Phase II clinical trials for rheumatoid arthritis, psoriasis and Crohn's disease has been compromised by its liver toxicity [13]. Nevertheless, BIRB796 has been useful in probing kinase conformational plasticity and the molecular features underlying the recognition of p38 MAPK by both upstream kinases and downstream substrates [14,15]. Other p38 MAPK inhibitors also act by perturbing the kinase conformation [16] with up to 1000-fold selectivity for p38α MAPK over the related MAPKs, the extracellular signal regulated kinases (ERKs) and the c-Jun N-terminal kinases (JNKs), as well as the tyrosine kinase Lck. These compounds will be useful in further exploring the therapeutic potential of p38 MAPK inhibition as well as delineating whether the liver toxicity of BIRB796 is an off-target side effect or a specific effect following p38 MAPK inhibition.

BAY43-9006

Another compound in this group of allosteric inhibitors is BAY43-9006 (Sorafenib or Nexavar® (Figure 2C)), discovered as a Raf inhibitor through combined medicinal and combinatorial chemistry approaches [17]. The co-crystallisation of the kinase domain of B-Raf with BAY43-9006 has also revealed the inactive 'DFG-out' conformation for the inhibitor-bound kinase [18].

The mechanism of action of BAY43-9006 has important consequences, both positive and negative for its possible use in the treatment of cancer patients. Specifically, BAY43-9006 inhibits VEGF-R2, VEGF-R3, FLT-3, PDGF-R, p38 MAPK and c-Kit [19], and this broad target spectrum is likely to contribute to its *in vivo* efficacy through inhibition of angiogenesis in addition to the pro-proliferative cell signalling of the tumour cells [20,21]. Conversely, the oncogenic-activated forms of B-Raf, such as the V599E mutant, have been shown to be less sensitive to BAY43-9006 [18]. Thus, BAY43-9006 may show compromised inhibition in those cells with the oncogenic B-Raf mutants. Additionally, there remains the possibility of BAY43-9006-induced resistant mutations of B-Raf arising during chronic BAY43-9006 treatment [18].

AAL-993

AAL-993 (Figure 2D), an anthranilic acid derivative of interest in the treatment of cancer, inhibits angiogenesis through its inhibition of VEGF-R1, VEGF-R2 and VEGF-R3. AAL-993 targets the inactive 'DFG-out' conformation of these VEGF-Rs [22]. AAL-993 has shown good biopharmaceutical properties and oral availability in animal models, with demonstrated anti-tumour efficacy in an orthotopic animal model [22]. The results from further *in vivo* testing should reveal its efficacy in additional specific cancer models.

Other ATP non-competitive allosteric inhibitors disrupt protein substrate interactions

As described in the preceding section, the allosteric inhibitors BIRB796, BAY43-9006 and AAL-993 disrupted ATP-binding of their target kinases and showed ATP-competitive inhibition kinetics. Protein kinases typically act on different protein substrates and so the specificity in targeting particular protein kinases might be achieved more readily by targeting more remote sites such as the protein substrate binding regions. These allosteric protein kinase inhibitors binding at sites remote from the substrate-binding (either ATP or protein substrate) active site of the kinase show non-competitive kinetics. In this section, we consider examples of ATP-non-competitive allosteric inhibitors.

PD09859 and other MEK inhibitors

The MAPK pathway provides a key example in the discovery of ATP-non-competitive allosteric inhibitors. The ERK MAPK pathway is an important regulator of cell growth, and numerous screening efforts have been directed towards discovery of inhibitors of components in this pathway. A number of ATP-non-competitive inhibitors have been identified for MEK, the dual-specificity kinase immediately upstream of ERK1/2 in the ERK MAPK pathway. The most commonly studied MEK inhibitor has been PD098059 (Figure 3A), presumably because this was the first commercially and widely available inhibitor of any kinase within the ERK MAPK pathway. Subsequently, U0126 (Figure 3B) was identified in a screen for an inhibitor of the ERK MAPK pathway using a reporter gene assay as a high-throughput readout [23]. Kinetic analysis has subsequently revealed that PD098059 and U0126 are non-competitive both with respect to ATP and their protein substrate ERK [23]. PD098059 and U0126 bind to a common or two overlapping sites on MEK [23]. Similarly, PD184352 identified in a small molecule screening approach for direct inhibitors of MEK showed an ATP and protein-substrate non-competitive inhibition of MEK. PD184352 has been subsequently used *in vivo* as a lead anti-cancer agent [24].

Key features of the interactions of these non-competitive allosteric inhibitors with MEK have been revealed by MEK1/2 crystallised with either PD184352 or the closely related analogue PD318088 (Figure 3C) [25]. Notably, the PD318088-MEK1 structure included Mg^{2+} and ATP, confirming that PD318088 did not disrupt ATP binding. It also revealed an interaction with two residues, methionine and lysine, of the MEK1 ATP binding site. Unexpectedly, this structure showed MEK1 in the 'closed' conformation typical of an activated protein kinase. However, binding to PD318088 caused marked changes in the conformation of the kinase activation loop and N-terminal lobe resulting in inhibition rather than activation of the kinase. A similar structure was observed for MEK2 in a complex with Mg^{2+}, ATP and PD318088 [25]. Whether this mode of action explains the specificity noted for PD184352-like MEK inhibitors is unknown. In addition, coumarin derivatives G8935 and G0328 (Figure 3D) have been identified as allosteric inhibitors of MEK. Whilst the structural basis for interaction with MEK remains to be determined, docking studies suggest that they bind to the same allosteric site of MEK as PD098059 and UO126 [26].

CMPD1

High-throughput screening identified CMPD1 (Figure 3E) as an inhibitor of the p38 MAPK mediated phosphorylation of the downstream substrate MK2 [27]. An unexpected finding was that the p38 MAPK mediated phosphorylation of two other substrates, the ATF2 transcription factor and the general kinase substrate myelin basic protein, was not affected. Additional experiments confirmed that CMPD1 interacted with p38 MAPK, rather than substrate, making CMPD1 an example for a small group of substrate selective protein kinase inhibitors [27]. Deuterium exchange mass spectrometry suggested that CMPD1 bound close to the active site of p38α MAPK resulting in local perturbation of three

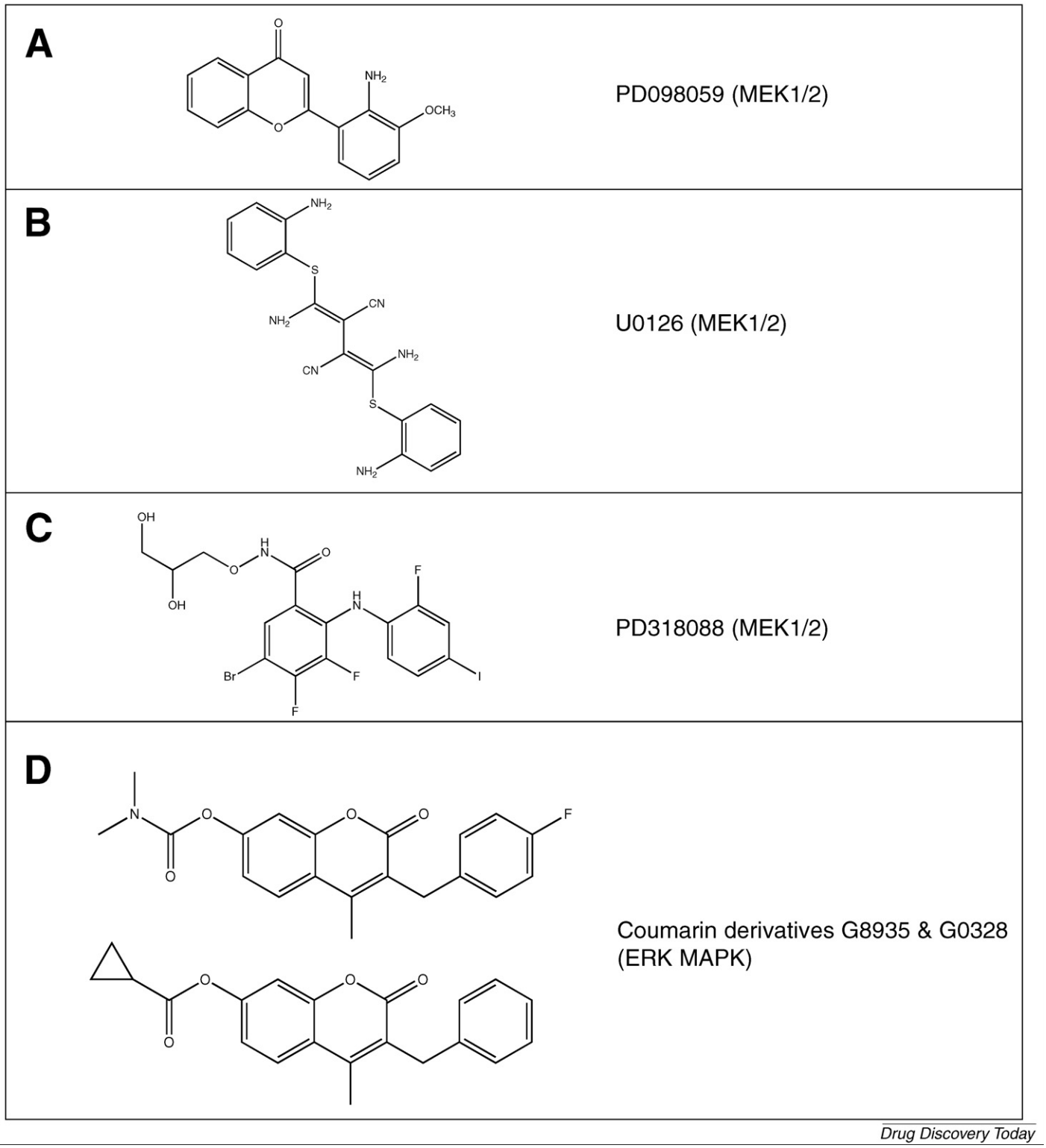


FIGURE 3

Selected allosteric inhibitors that disrupt the protein kinase protein substrate binding site. **(A)** PD098059 an inhibitor of the dual-specificity kinases MEK1/2. **(B)** U0126, an inhibitor of MEK1/2. **(C)** PD318088, an inhibitor of MEK1/2. **(D)** Coumarin derivatives G895 and G0328, inhibitors of the extracellular signal regulated kinase (ERK) mitogen-activated protein kinases (MAPKs). **(E)** CMPD1, an inhibitor of the ERK MAPKs. **(F)** API-2, an inhibitor of Akt. **(G)** Amino-functionalised quinoxaline compound 5, an inhibitor of Akt. **(H)** Pyrazinone derivative 14f, an inhibitor of Akt. **(I)** Akt-I-1, an inhibitor of the ERK MAPKs. **(J)** Derivatised quinoline regioisomer 5, an inhibitor of the Akt isoforms 1 and 2. **(K)** The thiadiazolidines, inhibitors of glycogen synthase kinase 3-β. **(L)** Chloromethyl thienylketone compound 17, an inhibitor glycogen synthase kinase 3-β. **(M)** BMS-345541, an inhibitor of IκB kinase. **(N)** GNF-1 and GFN-2, inhibitors Abl.

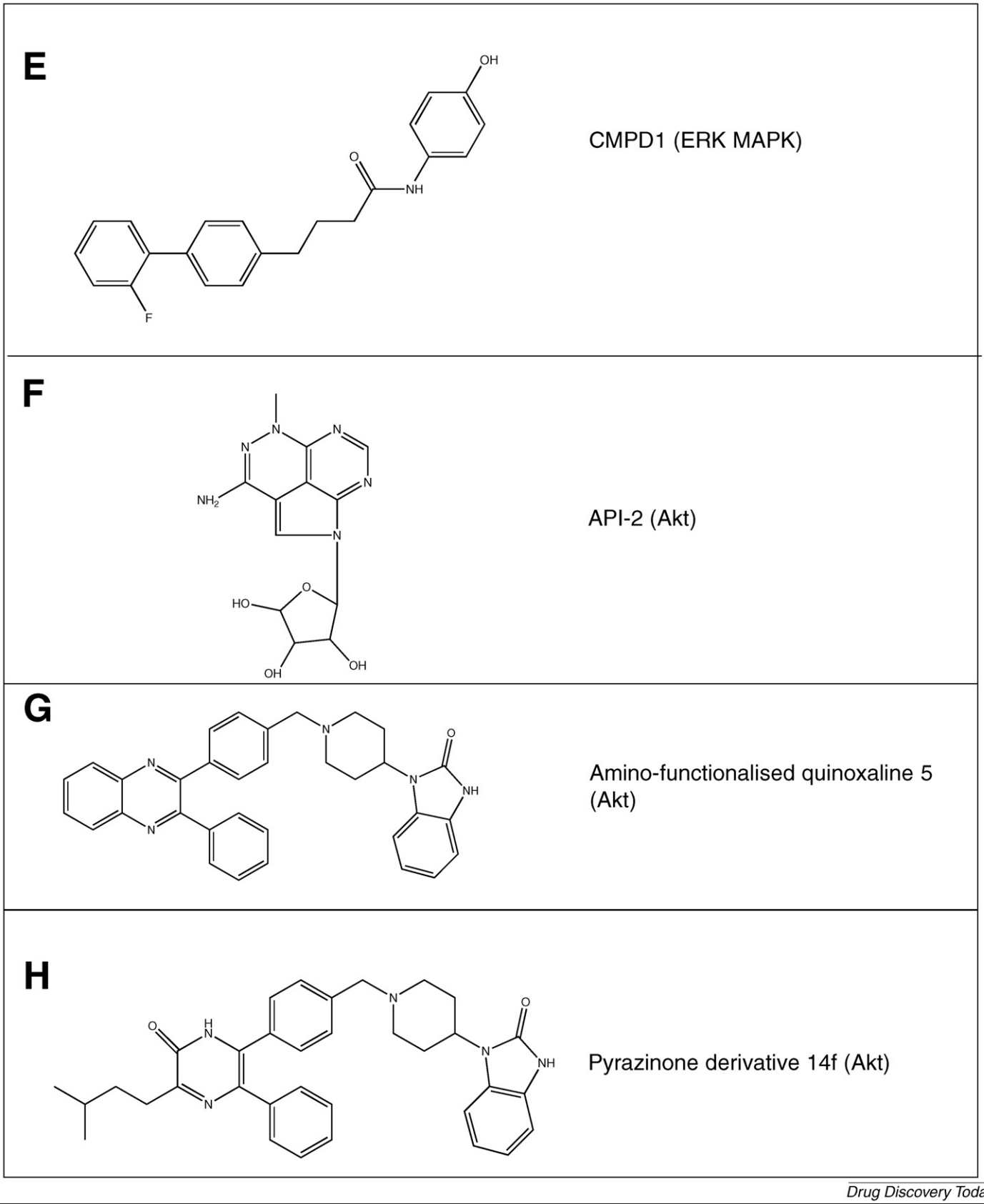
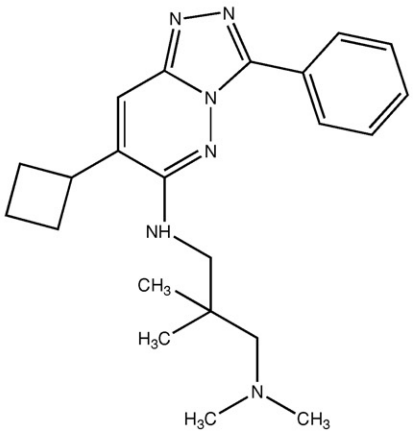


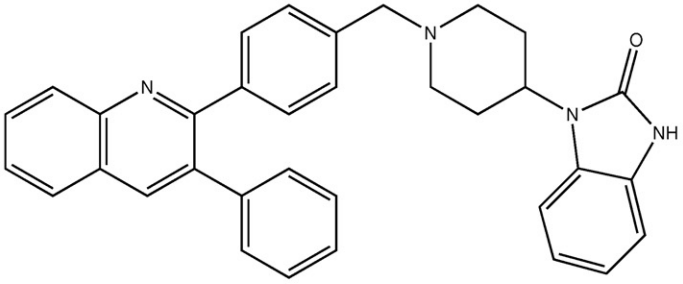
FIGURE 3 (*Continued*)

I



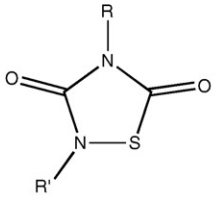
Akt-I-1 (Akt1)

J



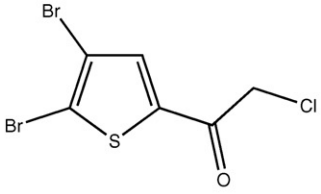
Derivatised quinoline regioisomer 5 (Akt1&2)

K



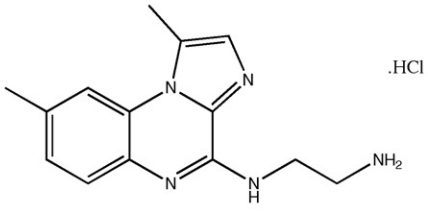
Thiadiazolidines (Glycogen synthase kinase 3-β)

L



Chloromethyl thienylketone 17 (Glycogen synthase kinase 3-β)

M



BMS-345541 (IκB kinase)

Drug Discovery Today

FIGURE 3 (*Continued*)

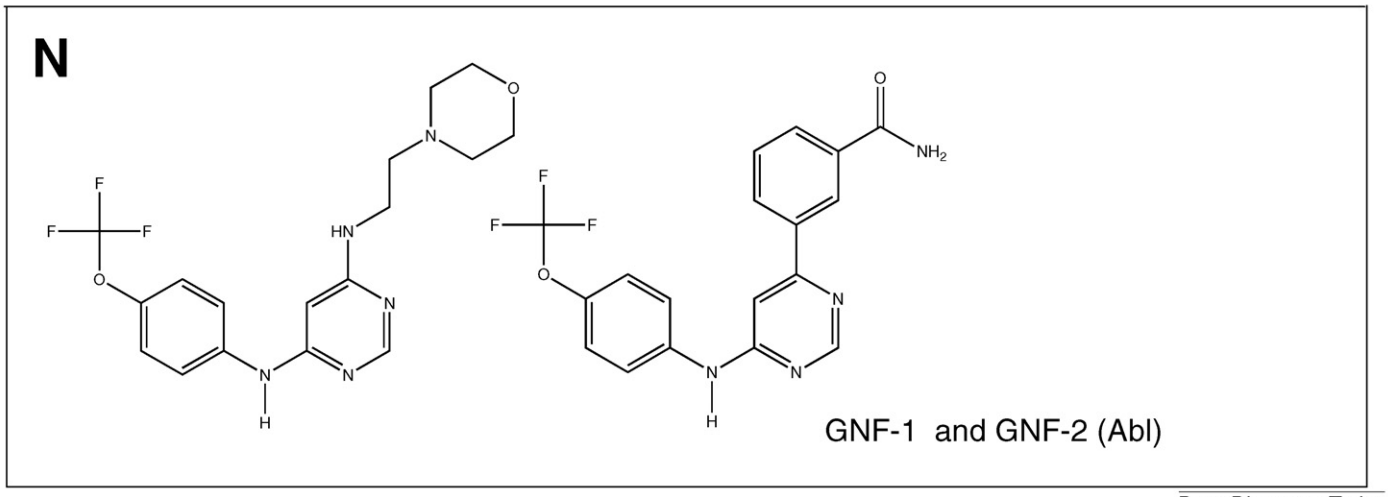


FIGURE 3 (*Continued*)

important regions—the ATP-binding site, the protein substrate docking site and the Mg^{2+} cofactor binding region [27]. Thus, suboptimal positioning of substrates and cofactors during catalysis appeared to result in enzyme inhibition. It is, however, unclear how the observed selectivity is achieved.

Akt Inhibitors

In addition to the members of the MAPK pathway, other serine/threonine protein kinases have attracted attention as possible anticancer agents and have been shown to be inhibited by ATP non-competitive allosteric inhibitors. As one example, Akt (protein kinase B/PKB), a phosphatidylinositol $(3,4,5)P_3$ binding protein kinase, plays a key role in the regulation of cell survival, proliferation and growth, and screening approaches have been used to identify selective inhibitors [28]. Whilst the first identified inhibitors have been shown to be ATP-competitive in their actions, additional classes have since been described that include peptidomimetic pseudosubstrate inhibitors [28], as well as phosphatidylinositol analogues that block phosphatidylinositol $(3,4,5)P_3$ binding and Akt translocation and activation [29]. One heterocyclic nucleoside analogue, API-2 (Figure 3F), inhibited growth of Akt2-expressing cells [30]. Whether this inhibitor directly targets Akt must be specifically evaluated.

Novel series of potent and isoform-selective Akt inhibitors based on a 2,3-diphenylquinoxaline or a pyrazinone core also has been reported [31]. An amino derivatised quinoxaline (compound 5 as shown in Figure 3G) showed potency towards Akt1, lesser effects on Akt2 and did not inhibit Akt3 but was compromised in its further use by poor solubility and lack of cellular activity [31]. By contrast, the pyrazinone derivative 14f shown in Figure 3H showed selectivity towards Akt2 [31], whereas the compound Akt-I-1 (Figure 3I) identified in another screen showed selectivity towards Akt1 [31]. Kinetic analyses have indicated ATP non-competitive and protein-substrate non-competitive inhibition, consistent with an allosteric mode of inhibition dependent on the presence of the Akt pleckstrin homology (PH) domain [31,32]. Considering the close sequence homology of the three Akt isoforms, this observed isoform-selectivity has been initially surprising as any inhibitor directed to one of the Akt isoforms would be expected to recognise all three isoforms equally [32]. These isoform-specific inhibitors provide an opportunity to evaluate the contribution of Akt isoforms to downstream signalling events [32] and the relevance of each of the Akt isoforms in diseases such as cancer. However, the poor physical properties of the 2,3 diphenylquinoxaline core have prompted modifications through incorporation of a more basic nitrogen atom in the core ring structure. This leads to a series of 2,3,5-trisubstituted pyridine derivatives including the regioisomer 5 shown in Figure 3J that inhibit both Akt1 and Akt2 but not Akt3 [33]. Interestingly, inhibition of both Akt1 and Akt2 appears to result in greater sensitisation of cancer cells to apoptotic stimuli [34], and so the *in vivo* characterisation of these pyridine derivatives will be of great interest.

Glycogen synthase kinase-3β inhibitors

In addition, ATP-non-competitive inhibitors of glycogen synthase kinase-3β (GSK-3β) have been described on the basis of a thiadiazolidinone scaffold (Figure 3K) as the new drug leads for the treatment of Alzheimer's Disease, diabetes, chronic inflammatory disorders and central nervous system disorders [35]. Structure–activity relationships and modelling studies have suggested two binding sites on GSK-3β for these inhibitors [36]. Interestingly, one of these sites is in the vicinity of the activation loop of GSK-3β whilst the other is the ATP-binding pocket [36]. The latter appears incompatible with the idea of ATP-non-competitive inhibition, and further structural work is required to confirm the binding site. Other GSK-3β inhibitors identified in library screening were thienyl and phenyl α-halomethyl ketones with the thienylketone demonstrated to show ATP-non-competitive inhibition (Figure 3L) [37]. Again, further structural analysis will be required to reveal the binding site of these inhibitors and to establish whether it is shared with the thiadiazolidinone inhibitors.

BMS-345541

Additional efforts are being directed towards finding serine/threonine kinases involved in diverse cellular responses. As an example,

the IκB kinases IKK-1 and IKK-2 regulate NFκB transcriptional activity by phosphorylation of IκB. This leads to subsequent degradation of IκB and nuclear translocation of the transcription factor NFκB. BMS-345541 (Figure 3M) has been identified as an inhibitor of IKK-1 and IKK-2 [38]. Kinetic analysis has suggested an allosteric mechanism of inhibition. Although the exact binding site must be identified, BMS-345541 showed selective inhibition of the IKKs when compared with a panel of 14 other protein kinases. The therapeutic potential of IKK inhibition is emphasised by the efficacy of these inhibitors *in vivo* to inhibit lipopolysaccharide-induced TNFα production, to reduce in joint destruction and inflammation in a collagen-induced arthritis model, to reduce inflammation in ischaemic brain damage and to induce apoptosis of melanoma cells [38–41].

GNF-1 and GFN-2

Lastly, a new class of allosteric inhibitors of Bcr-abl has been recently reported [42]. GNF-1 and GNF-2 (Figure 3N) are examples of 4,6-pyrimidines identified in a screen of 50 000 compounds against non-transformed and Bcr-abl-transformed cells [42]. Modelling, competition and site-directed mutagenesis studies suggested that they bind to the myristoyl-binding pocket in the C-terminal lobe of Bcr-abl [42]. Interestingly, these compounds do not inhibit *in vitro* kinase activity suggesting that important cell-context-specific interactions are required.

Inhibitors that compete with protein substrates

An additional strategy to inhibit protein kinase activity targets protein substrate interaction. Key recent examples are considered in this section, beginning with the targeting of the MAPKs.

MAPK Inhibitors

Previous studies on substrate recognition by the MAPKs have shown their requirement for specific substrate-docking domains. on searching for new classes of ERK inhibitors, the ERK2 substrate-docking site has been specifically targeted [43]. *In silico* screening of >800 000 compounds identified 80 candidate compounds for testing as inhibitors of the growth factor stimulated phosphorylation of ERK substrates, the downstream kinase Rsk1 and the transcription factor Elk-1. The best inhibitor (compound 76, (Figure 4A)) shows promise in inhibiting cell growth and thus as a new drug for the treatment of cancer. As predicted from its interaction with the ERK substrate docking site, compound 76 did not prevent phosphorylation of docking domain-independent ERK substrates such as myelin basic protein [43]. The specificity of these inhibitors for ERKs remains to be fully investigated. Co-crystallisation of ERK2 with compound 76 is now required to confirm the predicted mode of interaction with the substrate-docking site and further direct inhibitor optimisation.

The stress-activated and cytokine-activated JNKs interact with phosphorylate and enhance activity of a range of transcription factors, such as c-Jun, p53, Elk-1, ATF2 and NFAT, and appear to be crucial players in numerous apoptotic and inflammatory disorders. Since JNKs require protein substrate docking domain interactions for their activity, it should be feasible to use ATP-non-competitive inhibitors directed towards this domain for their inhibition. This discovery and design process should be facilitated by co-crystallisation of JNK with a JNK inhibitory peptide previously shown kinetically to be substrate-competitive despite its derivation from the JNK pathway scaffold protein JNK interacting protein (JIP) rather than specific JNK substrates such as c-Jun [44–46]. Small-molecule inhibitors of the substrate-docking domain may be able to selectively inhibit phosphorylation of selected JNK substrates. This may be an important advantage, given the wide range of possible therapeutic applications being evaluated for JNK inhibitors.

ON01910 and ON012380

Other small-molecule inhibitors of protein kinases outside the MAPK family have also shown direct competitive inhibition with the protein substrate. For example, ON01910 (Figure 4B) is a substrate competitive inhibitor of polo-like kinase-1 [47]. As the function of this kinase is crucial during mitosis and maintenance of genome stability, ON01910 is a new lead for cancer therapy in combination with, or instead of, ATP-competitive polo-like kinase-1 inhibitors such as BI2536, or inhibitors such as Scytonemin that are characterised kinetically as mixed inhibitors [48]. However, higher concentrations of ON01910 inhibit other tyrosine kinases, such as PDGF-R, Abl, FLT-1, Src, Fyn and polo-like kinase-2 as well as the serine/threonine cyclin-dependent kinase-1 [47]. Whether the efficacy of ON01910 in inhibiting tumour growth is the direct result of inhibiting polo-like kinase-1 or other protein kinases therefore remains an issue to be explored in greater detail. Similarly, a substrate-competitive inhibitor of Bcr-abl, ON012380 (Figure 4C), which is active against Gleevec-resistant cancer cells, has been reported by the same researchers [49].

AG538

Tyrosine kinases have also been shown to be targeted by substrate-competitive non-ATP competitive inhibitors [50]. AG538 (Figure 4D) has been identified as a protein substrate competitive inhibitor of the insulin-like growth factor receptor 1 (IGF-R1) and has been further modified by replacing the catechol moiety with benzoxazolone groups on either side of the molecule to enhance its stability in cells [51]. The ability of these derivatives to block growth of prostate and breast cancer cells *in vitro* has led to the suggestion that these substrate competitive inhibitors may prove useful as anti-neoplastic agents [51], thus joining the growing arsenal of tyrosine kinase inhibitors being tested for their use as anticancer drugs [52,53].

Future challenges

Despite the success of some kinase inhibitors, such as Gleevec, in the treatment of disease, important challenges for kinase inhibitor development remain. These include (1) to better understand the complex functions of kinases in biological systems so that appropriate kinases are selected as targets for therapeutic intervention; (2) to better understand structure-function relationships, particularly the conformational flexibility of kinases and the influence of residues remote from the substrate binding sites on kinase activity and specificity; and (3) to identify the impact of kinase overexpression and mutations on drug resistance.

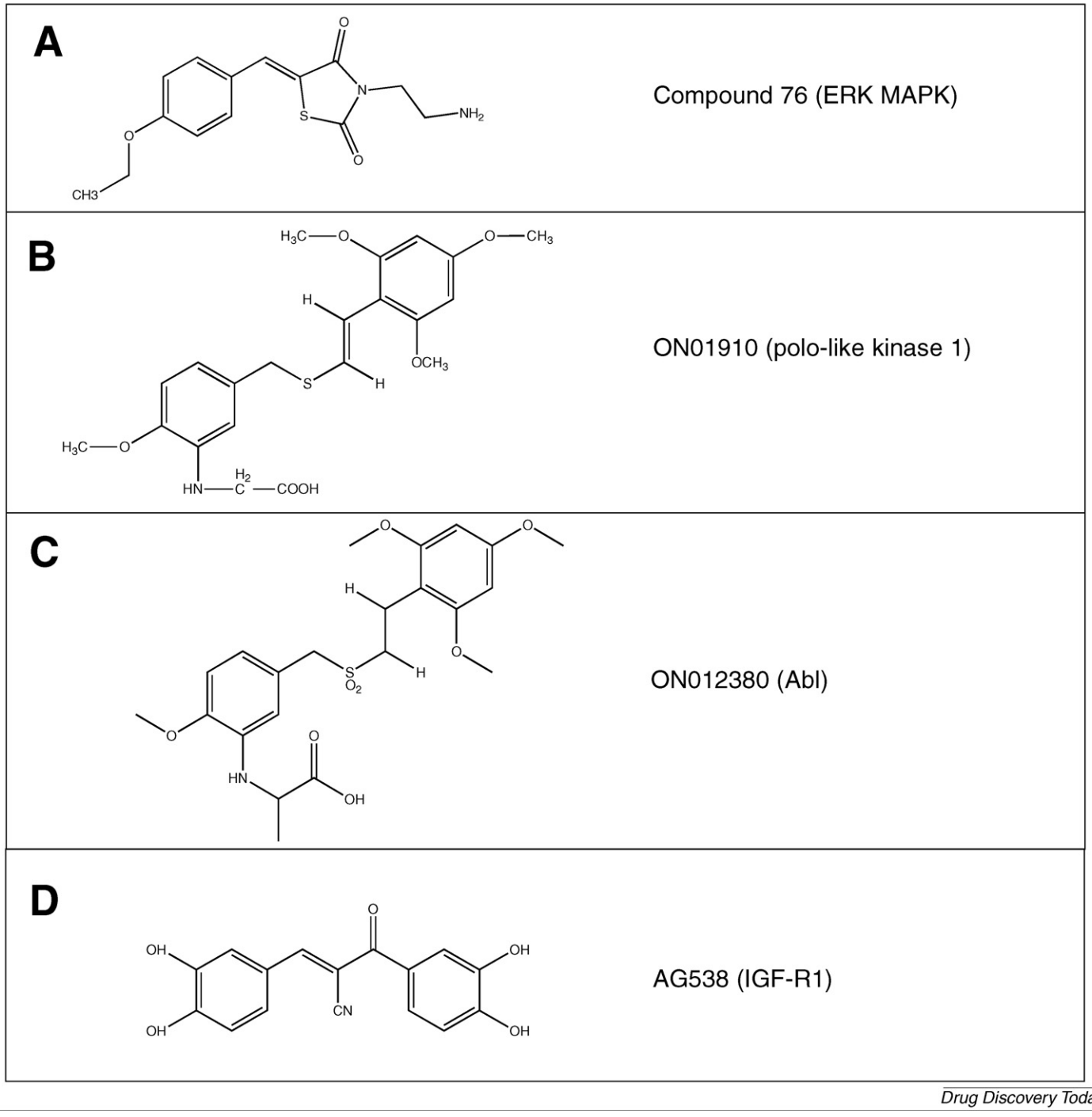


FIGURE 4

ATP-non-competitive inhibitors that disrupt the protein kinase interaction with protein substrates. **(A)** Compound 76, an inhibitor of the extracellular signal regulated kinase (ERK) mitogen-activated protein kinases (MAPKs). **(B)** ON01910, an inhibitor of polo-like kinase 1. **(C)** ON012380, an inhibitor of Abl. **(D)** AG538, an inhibitor of the insulin-like growth factor-receptor 1 (IGF-R1).

In addition, some issues could be more thoughtfully considered during inhibitor design. These include (a) an evaluation of the merits of reducing inhibitor off rates to enhance overall binding affinities, as observed for the ATP-competitive inhibitor GW572016 of epidermal growth factor receptor [54]; (b) an examination of whether chronic inhibitor administration as required in the treatment of prolonged disease states including cancer and inflammatory disease requires greater or lesser kinase selectivity; (c) whether broader spectrum kinase inhibitors could be advantageous for combating resistance; (d) whether *in vitro* selectivities are relevant to *in vivo* conditions as the concentrations of protein kinases and inhibitors in specific tissues may exceed concentrations tested *in vitro*, leading to unexpected inhibition of multiple kinases *in vivo* and loss of selectivity profiles painstakingly optimised *in vitro* [55]; and (e) whether regulating kinase function might be more effectively addressed at the level of the nucleus through directly controlling

either kinase genes via gene silencing approaches or genes for endogenous kinase inhibitors via approaches to enhance their levels.

Conclusions

The selective inhibition of a protein kinase or protein kinases associated with a specific disease, without affecting protein kinases involved in normal physiology, remains an important goal in the design and use of protein kinase inhibitors as drugs. Off-target side effects of protein kinase inhibitors [56–58] have diverted efforts from targeting the ATP-binding pocket in order to produce inhibitors that have the potential to be more kinase specific. Progress has been made towards addressing such specificity problems through the use of combinations of high-throughput screening, virtual *in silico* screening, structure-based drug design and combinatorial chemistry including fragment-based libraries, resulting in the discovery and development of potent and selective inhibitors. New directions have included generating allosteric inhibitors that alter kinase conformation to block productive binding of substrate (either ATP or protein). However, the difficulty in predicting the conformational plasticity of protein kinases [59] may thwart rational computer-based discovery and optimisation of allosteric kinase inhibitors [60]. The requirement for selective phosphorylation of specific protein substrates by each kinase suggests that rational substrate-based design might be a promising alternative approach for the development of kinase inhibitors.

Acknowledgements

We thank the Australian Research Council (ARC) and the National Health and Medical Research Council (NHMRC) for financial support.

References

1 Manning, G. *et al.* (2002) The protein kinase complement of the human genome. *Science* 298, 1912–1934
2 Fischer, P.M. (2004) The design of drug candidate molecules as selective inhibitors of therapeutically relevant protein kinases. *Curr. Med. Chem.* 11, 1563–1583
3 Druker, B.J. (2004) Imatinib as a paradigm of targeted therapies. *Adv. Cancer Res.* 91, 1–30
4 Wong, S. and Witte, O.N. (2004) The BCR-ABL story: bench to bedside and back. *Annu. Rev. Immunol.* 22, 247–306
5 Noble, M.E. *et al.* (2004) Protein kinase inhibitors: insights into drug design from structure. *Science* 303, 1800–1805
6 Liu, Y. and Gray, N.S. (2006) Rational design of inhibitors that bind to inactive kinase conformations. *Nature Chem. Biol.* 2, 358–364
7 Nagar, B. *et al.* (2002) Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res.* 62, 4236–4243
8 Mol, C.D. *et al.* (2004) Structural insights into the conformational selectivity of STI-571 and related kinase inhibitors. *Curr. Opin. Drug Discov. Dev.* 7, 639–648
9 Pargellis, C. *et al.* (2002) Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat. Struct. Biol.* 9, 268–272
10 Regan, J. *et al.* (2002) Pyrazole urea-based inhibitors of p38 MAP kinase: from lead compound to clinical candidate. *J. Med. Chem.* 45, 2994–3008
11 Regan, J. *et al.* (2003) Structure–activity relationships of the p38alpha MAP kinase inhibitor 1-(5-*tert*-butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl]urea (BIRB 796). *J. Med. Chem.* 46, 4676–4686
12 Regan, J. *et al.* (2003) The kinetics of binding to p38MAP kinase by analogues of BIRB 796. *Bioorg. Med. Chem. Lett.* 13, 3101–3104
13 Lee, M.R. and Dominguez, C. (2005) MAP kinase p38 inhibitors: clinical results and an intimate look at their interactions with p38alpha protein. *Curr. Med. Chem.* 12, 2979–2994
14 Kuma, Y. *et al.* (2005) BIRB796 inhibits all p38 MAPK isoforms *in vitro* and *in vivo*. *J. Biol. Chem.* 280, 19472–19479
15 Sullivan, J.E. *et al.* (2005) Prevention of MKK6-dependent activation by binding to p38α MAP kinase. *Biochemistry* 44, 16475–16490
16 Gill, A.L. *et al.* (2005) Identification of novel p38α MAP kinase inhibitors using fragment-based lead generation. *J. Med. Chem.* 48, 414–426
17 Wilhelm, S. *et al.* (2006) Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat. Rev. Drug Discov.* 5, 835–844
18 Wan, P.T. *et al.* (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 116, 855–867
19 Wilhelm, S.M. *et al.* (2004) BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res.* 64, 7099–7109
20 Adnane, L. *et al.* (2005) Sorafenib (BAY 43-9006, Nexavar((R))), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. *Methods Enzymol.* 407, 597–612
21 Hahn, O. and Stadler, W. (2006) Sorafenib. *Curr. Opin. Oncol.* 18, 615–621
22 Manley, P.W. *et al.* (2004) Advances in the structural biology, design and clinical development of VEGF-R kinase inhibitors for the treatment of angiogenesis. *Biochim. Biophys. Acta* 1697, 17–27
23 Favata, M.F. *et al.* (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273, 18623–18632
24 Sebolt-Leopold, J.S. *et al.* (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. *Nat. Med.* 5, 810–816
25 Ohren, J.F. *et al.* (2004) Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. *Nat. Struct. Mol. Biol.* 11, 1192–1197
26 Han, S. *et al.* (2005) Identification of coumarin derivatives as a novel class of allosteric MEK1 inhibitors. *Bioorg. Med. Chem. Lett.* 15, 5467–5473
27 Davidson, W. *et al.* (2004) Discovery and characterization of a substrate selective p38α inhibitor. *Biochemistry* 43, 11658–11671
28 Barnett, S.F. *et al.* (2005) The Akt/PKB family of protein kinases: a review of small molecule inhibitors and progress towards target validation. *Curr. Top. Med. Chem.* 5, 109–125
29 Castillo, S.S. *et al.* (2004) Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues. *Cancer Res.* 64, 2782–2792
30 Yang, L. *et al.* (2004) Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res.* 64, 4394–4399
31 Lindsley, C.W. *et al.* (2005) Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. *Bioorg. Med. Chem. Lett.* 15, 761–764
32 Barnett, S.F. *et al.* (2005) Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem. J.* 385, 399–408
33 Zhao, Z. *et al.* (2005) Discovery of 2,3,5-trisubstituted pyridine derivatives as potent Akt1 and Akt2 dual inhibitors. *Bioorg. Med. Chem. Lett.* 15, 905–909
34 DeFeo-Jones, D. *et al.* (2005) Tumor cell sensitization to apoptotic stimuli by selective inhibition of specific Akt/PKB family members. *Mol. Cancer Ther.* 4, 271–279
35 Martinez, A. *et al.* (2002) First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. *J. Med. Chem.* 45, 1292–1299
36 Martinez, A. *et al.* (2005) SAR and 3D-QSAR studies on thiadiazolidinone derivatives: exploration of structural requirements for glycogen synthase kinase 3 inhibitors. *J. Med. Chem.* 48, 7103–7112
37 Conde, S. *et al.* (2003) Thienyl and phenyl alpha-halomethyl ketones: new inhibitors of glycogen synthase kinase (GSK-3beta) from a library of compound searching. *J. Med. Chem.* 46, 4631–4633
38 Burke, J.R. *et al.* (2003) BMS-345541 is a highly selective inhibitor of IκB kinase that binds at an allosteric site of the enzyme and blocks NF-κB-dependent transcription in mice. *J. Biol. Chem.* 278, 1450–1456
39 McIntyre, K.W. *et al.* (2003) A highly selective inhibitor of I kappa B kinase, BMS-345541, blocks both joint inflammation and destruction in collagen-induced arthritis in mice. *Arthritis Rheum.* 48, 2652–2659

40 Herrmann, O. *et al.* (2005) IKK mediates ischemia-induced neuronal death. *Nat. Med.* 11, 1322–1329
41 Yang, J. *et al.* (2006) BMS-345541 targets inhibitor of kappaB kinase and induces apoptosis in melanoma: involvement of nuclear factor kappaB and mitochondria pathways. *Clin. Cancer Res.* 12, 950–960
42 Adrian, F.J. *et al.* (2006) Allosteric inhibitors of Bcr-abl-dependent cell proliferation. *Nat. Chem. Biol.* 2, 95–102
43 Hancock, C.N. *et al.* (2005) Identification of novel extracellular signal-regulated kinase docking domain inhibitors. *J. Med. Chem.* 48, 4586–4595
44 Heo, Y.S. *et al.* (2004) Structural basis for the selective inhibition of JNK1 by the scaffolding protein JIP1 and SP600125. *EMBO J.* 23, 2185–2195
45 Barr, R.K. *et al.* (2004) The critical features and the mechanism of inhibition of a kinase interaction motif-based peptide inhibitor of JNK. *J. Biol. Chem.* 279, 36327–36338
46 Barr, R.K. *et al.* (2002) Identification of the critical features of a small peptide inhibitor of JNK activity. *J. Biol. Chem.* 277, 10987–10997
47 Gumireddy, K. *et al.* (2005) ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. *Cancer Cell* 7, 275–286
48 Strebhardt, K. and Ullrich, A. (2006) Targeting polo-like kinase 1 for cancer therapy. *Nat. Rev. Cancer* 6, 321–330
49 Gumireddy, K. *et al.* (2005) A non-ATP-competitive inhibitor of BCR-ABL overrides imatinib resistance. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1992–1997
50 Levitzki, A. and Mishani, E. (2006) Tyrphostins and other tyrosine kinase inhibitors. *Ann. Rev. Biochem.* 75, 93–109
51 Blum, G. *et al.* (2003) Development of new insulin-like growth factor-1 receptor kinase inhibitors using catechol mimics. *J. Biol. Chem.* 278, 40442–40454
52 Arora, A. and Scholar, E.M. (2005) Role of tyrosine kinase inhibitors in cancer therapy. *J. Pharmacol. Exp. Ther.* 315, 971–979
53 Baselga, J. (2006) Targeting tyrosine kinases in cancer: the second wave. *Science* 312, 1175–1178
54 Wood, E.R. *et al.* (2004) A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res.* 64, 6652–6659
55 Rishton, G.M. (2005) Failure and success in modern drug discovery: guiding principles in the establishment of high probability of success drug discovery organizations. *Med. Chem.* 1, 519–527
56 Davies, S.P. *et al.* (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 351, 95–105
57 Bain, J. *et al.* (2003) The specificities of protein kinase inhibitors: an update. *Biochem. J.* 371, 199–204
58 Fabian, M.A. *et al.* (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* 23, 329–336
59 Huse, M. and Kuriyan, J. (2002) The conformational plasticity of protein kinases. *Cell* 109, 275–282
60 Frembgen-Kesner, T. and Elcock, A.H. (2006) Computational sampling of a cryptic drug binding site in a protein receptor: explicit solvent molecular dynamics and inhibitor docking to p38 MAP kinase. *J. Mol. Biol.* 359, 202–214