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Insights for the development of specific kinase inhibitors by targeted structural genomics

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Many protein kinases are validated intervention points for drug development, however active site similarities often lead to a lack of selectivity and unwanted side effects in the clinic. To address this issue, it is desirable to increase the number of high resolution crystal structures and complexes with non-adenosine ligands available for the rational design of more selective inhibitors. Recent progress in protein crystallography and biotechnology has enabled structural genomics projects to target challenging proteins successfully, including protein kinases. As we discuss here, this effort has resulted in a considerable increase in the number of available high resolution structures and inhibitor complexes and has identified novel structural motifs that are available for drug development.

Introduction

Structural data that are available in the public domain are a valuable foundation for structure-guided drug discovery projects. In recent years, structures of human proteins determined by structural genomics organizations has contributed significantly to the number of human protein structures that are available for structure-based design projects [1]. In particular, high-throughput structural biology laboratories that focus on protein families have contributed to the structural description of several human protein families, and provide valuable structural and chemical information for use in the design of bioactive compounds. In addition, established expression and crystallization conditions have generated essential reagents, methodologies and technologies that will facilitate research projects in academia and drug discovery programs in industry. To date, four main large-scale projects have focused at least part of their efforts on human targets. These are the Protein Structure Initiative (http://www.nigms.nih.gov/psi/), Riken Structural Genomics Initiative (http://www.gsc.riken.go.jp/indexE.html), Structural Proteomics in Europe (SPINE, http://www.spineurope.org/) and the Structural Genomics Consortium (SGC, http://www.thesgc.com/).

Here, we summarize the impact of structures determined by structural genomics in the area of human protein kinases, an area that constitutes a large fraction of all targets currently selected by pharmaceutical companies for the development of novel drugs [2].

Protein kinases as drug targets

Protein kinases have a crucial role in most, if not all, signalling pathways and regulate diverse cellular functions, such as cell-cycle progression, apoptosis, metabolism, differentiation, cell morphology and migration, and secretion of cellular proteins. Deregulation of signalling pathways is a key factor in the development of human disease and, thus, modulation of kinase activity by small molecules is an attractive strategy for drug discovery [3–5]. A recent review reported 113 'mature' chemical compounds that have been developed to inhibit protein kinases. Most of the 101 developed inhibitors are low molecular-weight compounds, of which 76 are currently being pursued actively in clinical trials [2].

Oncological indications account for almost 75% of all targeted applications for kinase inhibitors, with rheumatoid arthritis and other inflammatory indications constituting ~20% of the clinical interest. Most of the efforts to develop low molecular-weight inhibitors that have entered clinical programs are focused on ATP-competitive compounds. Therefore, we have limited this review to summarizing recent developments in structural biology in the area of kinase catalytic domains. Several reviews and original papers regarding efforts on noncatalytic protein kinase domains have been published recently [6–9].

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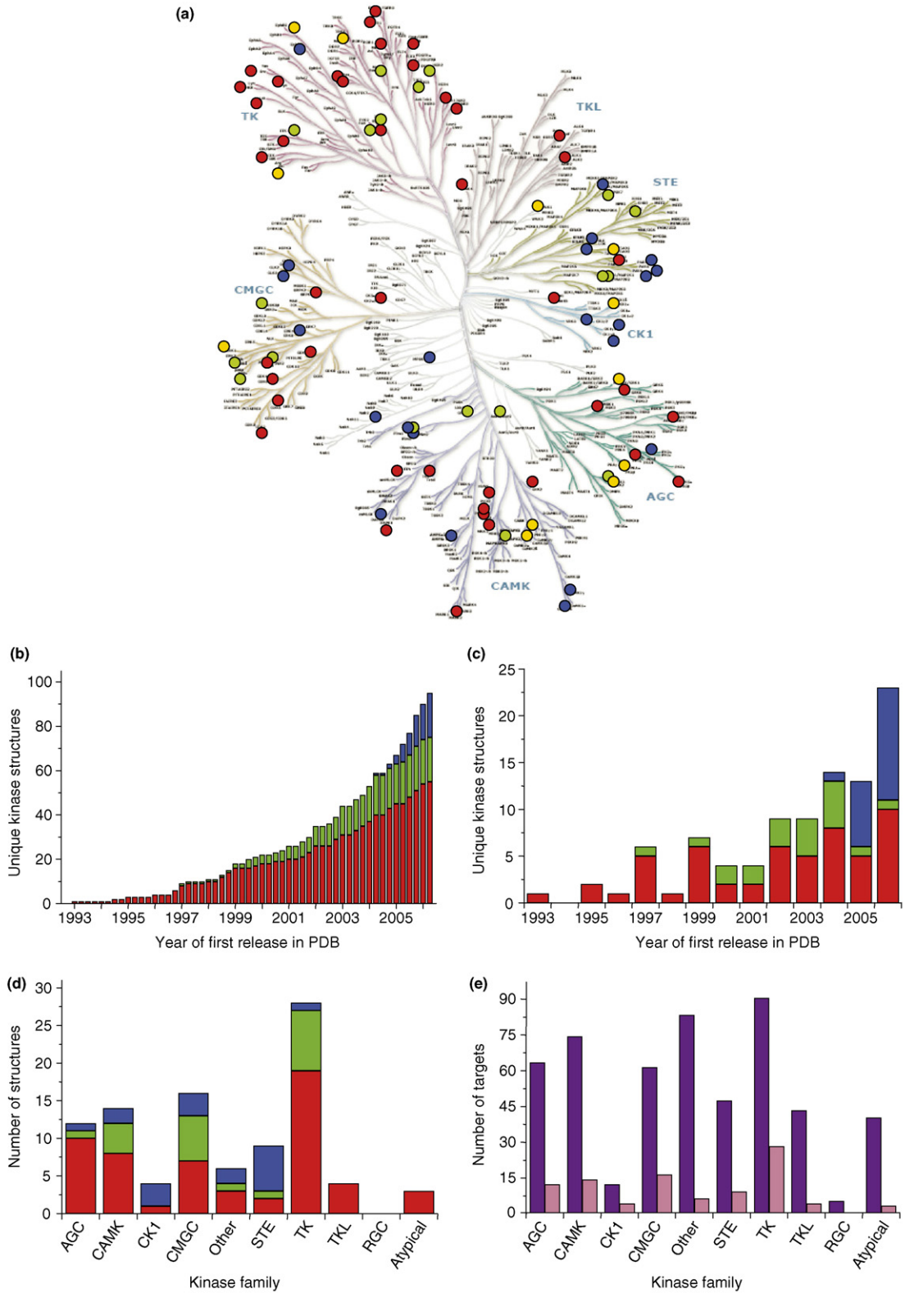

FIGURE 1

Structural coverage of the kinome. **(a)** Phylogenetic tree of the kinome. Only structures released to the protein databank (http://www.rcsb.org/pdb/home/home.do) with a resolution better than 3.0 Å have been considered. Structures released by academia are highlighted in red, industry in green and structural genomics in blue. Structures determined from other organism with >95% sequence ID to the human protein are shown in yellow. Colour-coding is identical throughout (a–d). **(b)** Cumulative number of unique kinase structures deposited per year by academia, industry and structural genomics. **(c)** Total number of novel kinases structures deposited per year. **(d)** Total number of novel structures released per kinase family. **(e)** Structural coverage of kinase families (purple bars,

Structural coverage of the kinome

The completion of the human genome sequence identified 518 human protein kinases, which can be grouped into ten major families [10]. The human kinome is one of the largest protein families, and constitutes 1.7% of all genes. Protein kinases are the result of divergent molecular evolution and it is likely that they are derived from a single ancestral protein. Consequently, all kinases share a relatively high degree of sequence similarity, with conserved, key, regulatory elements, a similar overall three-dimensional structure, and the same catalytic mechanism of gamma phosphate transfer from ATP to a serine, threonine or tyrosine residue on a protein substrate.

High-resolution structural information that covers representative members of this family is important for the rational design of selective inhibitors. During the past three years, the field of kinase structural biology has also developed rapidly because of a large contribution by structural genomics efforts. In 2004, the structures of 38 human protein kinases (7% of all kinase targets) were available publicly and only 12 of these contained non-adenosine chemotypes [5]. Before 2005, the rate of structure determination was linear, resulting in an estimated time of ~100 years to determine the structures of the entire human kinome [5]. During the past three years, structural genomics has contributed the structures of >20 novel kinases. Including structures that have been determined from other species that are at least 95% identical in sequence to the human protein, 96 unique kinase catalytic domain structures were known by the end of 2006 (Figure 1; Table 1). During 2005 and 2006, structural genomics efforts determined >50% of all novel protein kinase catalytic domain structures. In contrast to many protein families, for which initial structures have been determined by academic structural-biology groups, the rate of structure determinations of human protein kinase catalytic domains has not diminished, but has instead remained stable at approximately seven structures per year, reflecting a sustained interest in this class of enzymes.

Researchers in structural genomics have released structures for many members of kinase families that were previously devoid of structural information. These include the first catalytic domain structure from the 'never in mitosis' family, NEK2 [11], the first Numb-associated kinase (NAK) family member myristoylated and palmitoylated serine threonine kinase (MPSK1, PDB code 2BUJ) and the first CDC2-like kinases CLK1 and CLK3 (PDB codes 1Z57 and 2EUD). With, to date, 19 released high resolution structures, our laboratory has, been the primary source of novel kinase catalytic domain structures deposited in the Protein Structure Data Bank (PDB, http://www.rcsb.org) (Table 1). Our main focus has been on the Ser/Thr kinase family, which represents 80% of all kinases in the human kinome and is currently under-represented in the PDB (Figure 1d,e). Representative structures of the catalytic domains of several other subfamilies are yet to be determined, including the receptor guanylate cyclase group, the polo-like kinase family, the UNC-51-like kinase family and many of the smaller subfamilies.

Many inhibitors of Ser/Thr kinases, such as Aurora kinases, CDKs and mitogen-activated protein kinases are undergoing clinical testing [5] and it is likely that more, less well-characterised kinases will also be targeted in the near future. For example, a recent systematic study reveals that a change in expression of Ser/Thr kinases occurs frequently in human tumours, which indicates that other Ser/Thr kinases might become drug targets pending further validation studies [12]. The Ser/Thr subfamily is also interesting from a structural biology viewpoint. This large family contains 39 pseudokinases that lack at least one of the conserved catalytic residues and are, therefore, predicted to be catalytically inactive [10,13]. Many of these pseudokinases are essential, but not all have been characterized functionally, and it is expected that some will be catalytically active [13]. High-resolution structural information is therefore essential to determine how catalytically important motifs can be replaced functionally in these proteins. However, the three-dimensional structure of a pseudokinase domain has not yet been reported.

Discovery of new structural signature motifs

Many novel structural motifs have been identified in the structures determined by the use of structural genomics, some of which could be utilized to develop more specific inhibitors. For example, a small, helical, secondary-structure element (helix αT) has been identified in the active site of the kinase NEK2 following the DFG motif (Figure 2) [11]. In NEK2, the DFG motif and the five C-terminal residues constitute an extended motif (DFGLARIL) that is largely conserved in NEK-family members. This small helix is similar to structural elements that have been described in inactive cyclin dependent kinase CDK2 [14], in the inactive conformation of epidermal growth factor receptor [15] and in the tyrosine kinases Src and Hck [16,17]. The inhibitor SU11652, which has been co-crystallized with NEK2, makes several contacts with hydrophobic residues in this motif – an interaction that could be utilized for the development of more potent, specific NEK inhibitors. Several other interesting structural motifs have been discovered in the vicinity of active sites of kinase structures that have been determined by structural genomics organizations. The structure of MPSK1 (PDB code 2BUJ) reveals a new activation-segment architecture with a large helical insert that is likely to be conserved within this small subfamily. This structural feature could be explored for the development of selective inhibitors that also target the substrate-binding site [18]. Furthermore, a long β hairpin has been identified in CLK1 and CLK3, which folds over a shallow groove between the lower-lobe helices αD and αE (PDB codes 1Z57 and 2EUD). This hairpin insertion, which is located opposite the hinge region, is structurally conserved in the CLK family but with fairly low sequence homology. The function of this motif is, however, currently unknown.

targets; pink bars, structures). Abbreviations: AGC, protein kinase A (PKA), PKG and PKC family; CAMK, calcium calmodulin-dependent protein kinases; CK, casein kinases; CMGC, containing CDK (cyclin dependent kinases), MAPK (Mitogen-activated kinase), GSK3 (Glycogen synthase kinase) and CLK (Cdc2 like kinase) families; RGC, receptor guanylate cyclases; STE, homologue of yeast sterile kinases; TK, Tyrosine kinases; TKL, tyrosine kinase-like. (a) adapted from http://www.sciencemag.org/cgi/data/298/5600/1912/DC2/1 with the permission of Cell Signaling Technology and Science.

TABLE 1

Protein kinases structures determined by structural genomics[a]

Protein	PDB ID	Resolution (Å)	Inhibitor	Institute	Protein family
CLK1	1Z57	1.70	Hymenialdisine	SGC-Oxford	CMGC
CLK3[b]	2EU9	1.53	None	SGC-Oxford	CMGC
CK1γ1	2CMW	1.75	Compound 52	SGC-Oxford	CK1
CK1γ2	2C47	2.40	5-Iodotubercidin	SGC-Oxford	CK1
CK1γ3[c]	2CHL	1.95	Triazolodiamine 1	SGC-Oxford	CK1
ERK3	2I6L	2.25	None	SGC-Oxford	CMGC
ASK1	2CLQ	2.30	Staurosporine	SGC-Oxford	STE
NEK2	2JAV	2.10	SU11652	SGC-Oxford	Other-NEK
PAK4[d]	2CDZ	2.40	Cdk1 Inhibitor	SGC-Oxford	STE
PAK5	2F57	1.80	Cdk1 Inhibitor	SGC-Oxford	STE
PAK6	2C30	1.60	None	SGC-Oxford	STE
PIM1[b]	1XWS	1.80	BIM I, HB1	SGC-Oxford	CAMK
PIM2	2IWI	2.80	HB1	SGC-Oxford	CAMK
SLK[a,e]	2J51	2.10	Triazolodiamine 1	SGC-Oxford	STE
MPSK1	2BUJ	2.60	Staurosporine	SGC-Oxford	Other-NAK
STK10	2J7T	2.0	SU11274	SGC-Oxford	STE
DAPK3	2J90	2.0	Pyridone 6	SGC-Oxford	CAMK
cAMPKa2	2H6D	1.85	None	SGC-Toronto	CAMK
EphA3	2GSF	1.77	None	SGC-Toronto	TK
PKCiota	1ZRZ	3.0	BIM I	SPINE	AGC
CAMK1G	2JAM	1.7	SU11652	SGC-Oxford	CAMK
CAMK1D	2JC6	2.5	GSK inhibitor XIII	SGC-Oxford	CAMK

[a] Detailed descriptions of structures of targets solved in Oxford are available in form of iSee datapacks, which are free from (http://www.sgc.ox.ac.uk/structures/KIN.html).
[b] CLK3 also deposited as phosphorylated protein, PDB code 2EXE.
[c] CK1γ3 with inhibitors 2CHL, 2IZR, 2IZS, 2IZU and 2IZT.
[d] PAK4 also deposited as an apo-structure in two different spacegroups, 2BVA and 2J01.
[e] Structures with different inhibitors and substrate: PIM1, 2BIK, 2BZH, 2BZI, 2BZJ, 2BZK, 2C3I, 2BIL and 2J2I; SLK and 2JA0.

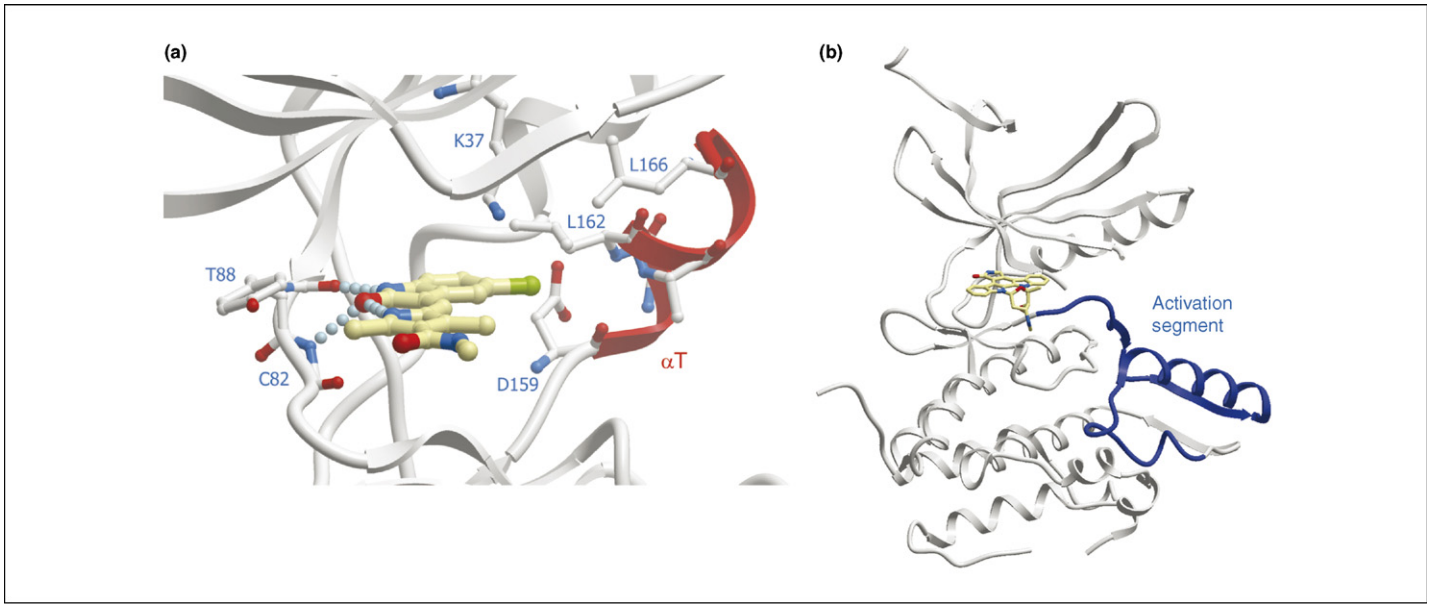

FIGURE 2

Novel structural motifs. **(a)** The active site of NEK2 in complex with SU11652. The N-terminus of the activation segment of this kinase forms a small helix in this inactive conformation of NEK2, which might be explored further for the development of inhibitors. **(b)** The structure of MPSK1 reveals a novel architecture of its activation segment (blue) that contains a large helical insert instead of the extended conformation usually present in other kinases.

The dynamic nature of the kinase catalytic domain

Protein kinases are dynamic, flexible molecules that might occupy a large conformational space in solution [19]. For many kinases, the dynamic nature of the two kinase lobes in the catalytic domain and structural elements in the active site are essential for the regulation of catalysis. The active state of the catalytic domains is characterized by a closed conformation of the two kinase lobes, a well-structured activation loop that is suitable for recognition of the substrate, and a firmly anchored αC helix that forms an ion pair with the conserved lysine residue at the active site, so in a conformation enabling co-factor binding. By contrast, crystal structures of inactive kinases reveal a large diversity of conformations and, often, at least one of the key regulatory elements is either displaced or disordered [20]. This dynamic nature renders crystallization of kinases difficult and, often, inhibitors are used to stabilize the enzyme in a particular conformation. Usually, the binding of either ATP or an ATP-mimetic ligand results in closure of the two kinase lobes, which stabilizes a catalytically competent conformation. However, enzymatically active kinases might also crystallize in catalytically incompetent conformations. Indeed, of the 22 kinases released by structural genomics initiatives, 17 have been crystallized in the presence of ATP-mimetic inhibitors, and the availability of high-affinity inhibitors has been essential to crystallize at least nine of the 19 kinases crystallized in our laboratory.

Inhibitor data generated by structural genomics

As well as increasing the success of crystallization experiments, using inhibitors to generate co-crystal structures has at least three benefits: first, inhibitor complexes might represent more suitable starting points for drug-discovery efforts by computational chemists who, ideally, need the structure of the 'most relevant' conformation that recognizes an ATP-mimetic ligand; second, screening data from identical assays against many targets gives valuable insights into the selectivity of inhibitors and might indicate novel applications for existing inhibitors; and third, the determination of co-crystal structures identifies the binding mode of either novel or known inhibitors to a particular target [21]. The SGC, therefore, systematically screens most proteins that are targeted for structure determination against a small library of potential inhibitors to increase crystallization success [22]. To avoid the time-consuming set-up of an individually adapted screening assay for individual targets, a generic temperature-shift (stabilization) assay is used. This assay can be applied to any target and utilizes the increase in thermal stability of a protein in the presence of a ligand, which is monitored either by a fluorescent probe or by light scattering [23,24]. Protein stability-shift data determined in this way correlate well with ligand affinities and inhibition data generated by enzyme kinetic assays [24–27]. Most information on inhibitor potency that is available generally has been generated using either a single target or a small set of related proteins, and the heterogeneity of the screening assays used in different laboratories often makes direct comparison difficult. By contrast, the availability of inhibitor data on a large family of targets generated using a single standardized assay provides quantitatively comparable inhibition data (Figure 3a). SGC screening data for kinases with deposited structures are publicly available in PubChem (http://pubchem.ncbi.nlm.nih.gov/).

Co-crystal structures with several diverse inhibitors increases the probability of successful drug design

The high degree of domain plasticity of the active site, which is sandwiched between the two mobile kinase lobe domains, poses several challenges for computational chemists, but provides also opportunities to design selective inhibitors. Several, highly specific, clinically successful, so-called, type II inhibitors that target an inactive conformation of kinases, have been developed utilizing the binding pocket generated by the 'DFG out' conformation [28,29]. In this unique conformation, the phenylalanine residue of the conserved activation loop DFG motif moves ~10 Å from its position in the active form. For example, the anti-leukemia drug Imatinib (Gleevec) selectively inhibits the receptor kinases cAbl and Kit, but not the closely related kinase Src [30]. In addition to Imatinib, protein crystallography has confirmed seven additional type II inhibitors, BIRB796 [31], Sorafenib [32], AAL993 [33], diaryl urea [31], indole amide [34], anilinoquinazoline [35] and 4-aminopyrimidinoquinazoline [36], which target four diverse kinases. Currently, it is not possible to predict which kinases adopt a 'DFG out' conformation, which would allow the development of potentially more specific type II inhibitors. Recently, several crystal structures have been determined in which the entire activation segment, including the substrate-binding site with the conserved P1 loop and APE motif, adopts a 'flipped out' conformation. This largely extended conformation leaves the DFG motif in an 'in' position but exchanges the activation segment of these, usually dimeric, kinases to form an active kinase in a trans-conformation. This activation segment conformation was seen first in the check-point kinase CHK2 [37] and later in the STE20 family members STK10 (PDB code 2J7T) and SLK (PDB code 2J51), and in the CAMK DAPK3 (PDB code 2J90). This indicates that this activation-loop conformation is adapted by many diverse kinases, which offers a new type of activation segment conformation for the development of selective inhibitors.

However, the development of conventional, ATP-mimetic, type I inhibitors is also expected to benefit from the availability of structures that give insight into the plasticity of the active site. For example, the recent determination of the structures of all active family members, in apo- and inhibitor complex-forms, of the group II family of p21 activating kinases (PAK4, PAK5 and PAK6), reveals a domain plasticity that involves reorganization of the functionally important helix αC and the identification of six diverse inhibitor scaffolds that might be explored for the further development of inhibitors (Figure 3b) [38]. Differences in dynamic parameters of catalytic domains might also be responsible for the selectivity between closely related family members. For example, several inhibitors targeting the PIM members of the Ser/Thr kinase subfamily showed ~100-fold selectivity for PIM1 and PIM3 versus PIM2 for several identified inhibitor scaffolds. This is despite high conservation of active-site composition with only one conservative exchange (Val126 to Ala in PIM2) within a radius of 6 Å in the active sites, which makes it difficult to explain this isoform selectivity. A pronounced isoform selectivity is also observed for the CLK TG003 [39]. By contrast, group II PAKs and CK1-gamma family members have similar inhibitor profiles, which is expected from their high sequence and structural homology.

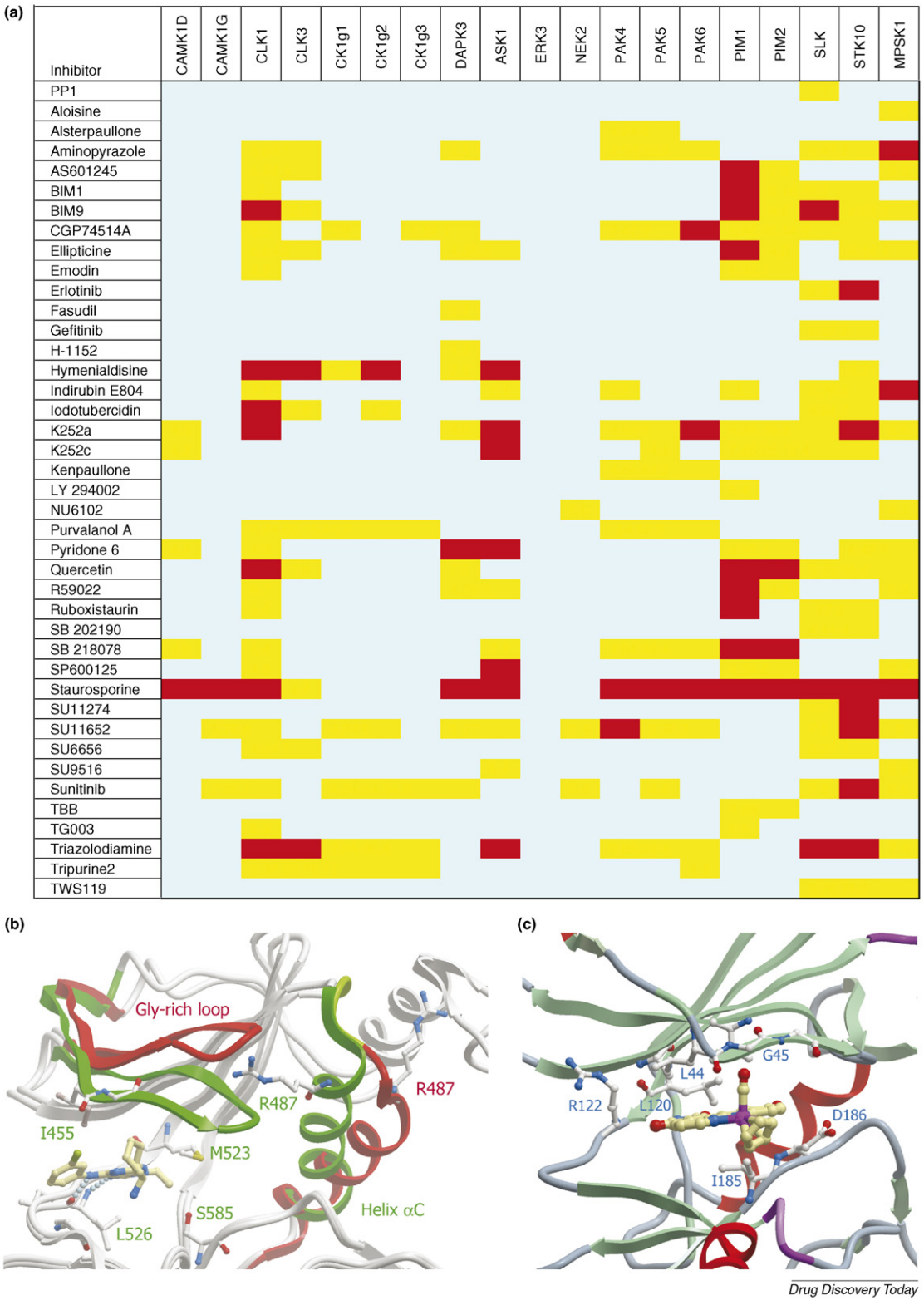

FIGURE 3

Inhibitor screening data, conformation changes upon ligand binding as well as structure of novel inhibitor types. **(a)** Small subset of inhibitor data generated at the SGC. Enzyme stability-shift data measured for kinases that have been determined structurally are shown. Red indicates a temperature shift of >8 °C and yellow a shift of 4–8 °C, which indicate low-μM and nM inhibitors, respectively. All assay data (~600 inhibitors) generated for these targets can be assessed through either PubChem or the SGC website (http://www.sgc.ox.ac.uk/structures/KIN.html) by downloading the structure annotation (iSee) data packs. **(b)** Domain plasticity detected by comparing the PAK5 apo- and inhibitor-complex [38]. Large conformational changes in the glycine-rich loop as well as in helix αC are evident

Extending chemical space

Collaborations between structural genomics and academic medicinal chemistry groups have led to the development of strategies to develop entirely new types of inhibitors. A recent initiative has explored the extension of chemical space using inert metal complexes that function as a 'hypervalent carbon' with largely extended structural opportunities [40–43]. The three-dimensional structures of such novel inhibitor complexes with PIM1 and PIM2 reveal that this inhibitor class has the potential to match the shape of the ATP-binding site more closely than analogous, carbon-based inhibitors (Figure 3c). This concept has led to the development of inhibitors with picomolar potency for PIM kinases [44] and glycogen synthase kinase 3 (GSK-3) [42–45].

Dissemination of data

In most academic laboratories the level of interest in a scientific field is measured by the number of papers published in that area and the number of subsequent citations. However, high-impact publications often require additional functional data from research disciplines that are often not compatible with a high-throughput environment. Consequently, the coordinates of many structural genomics structures are deposited into the PDB without an associated publication, which deprives the community of potentially invaluable data and interpretations. Recognizing these issues, the SGC has developed an intuitive, integrated tool (iSee) to disseminate expertly annotated, three-dimensional structures, along with information regarding the materials and methods for generating the protein structure, literature and database information and associated compound-screening data [46]. This resource is targeted towards the understanding of three-dimensional information about proteins by a diverse scientific audience outside structural biology, such as medicinal chemists, molecular biologists and clinicians [46]. All human protein kinase structures determined by the SGC have been annotated in this way and iSee datapacks are freely available from the SGC website (http://www.thesgc.com).

Conclusion

The availability of structures of protein kinases in multiple forms (apo- and co-factor complexes, as well as complexes of representative inhibitors from diverse chemotypes) released by structural genomics should greatly enhance the ability of drug discovery programs to successfully design and develop new, potent and selective inhibitors. In particular, the availability of protocols and reagents generated for typical structural biology bottlenecks, such as protein expression and crystallization, will greatly reduce the time required to establish these tools in drug development projects. We also envision that the large number of successfully expressed proteins will result in several co-crystal structures of signal transduction complexes that might also include full-length kinases and their regulatory domains. These larger structures should allow a more realistic view of signal transduction pathways and shed further light on the structural requirements for catalytic activity and its inhibition. Finally, more complete structural coverage of the kinome, including screening data should facilitate the design of inhibitors that target more than one protein kinase, thus, enhancing the probability of a successful outcome when put into clinical practice. In our view, the main challenge that remains is to successfully design compounds that are devoid of unwanted toxicity caused by interactions with anti-targets (protein molecules that are vital for healthy tissue). Here, the availability of structural information from a large number of protein kinases and other adenine-utilizing enzymes in multiple forms might have the largest impact.

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comparing the apo structure (red) with the inhibitor complex (green). **(c)** Binding of an organometallic half-sandwich inhibitor to PIM1 [44]. This inhibitor efficiently fills the available space in the binding pocket due to enhanced chemical possibilities of the introduced inert metal centre.

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