New Lead Generation Strategies for Protein Kinase Inhibitors – Fragment Based Screening Approaches

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Abstract: The protein kinase superfamily represents both an enormous opportunity and a unique challenge for drug discovery. Protein kinases play central roles in the cellular economy and it is well known that a large number of diseases involve aberrant protein kinase activity. This review discusses how fragment based screening strategies, such as virtual screening, NMR and high-throughput X-ray crystallography are being employed to identify new chemo-types to produce the next generation of protein kinase inhibitors.

Key Words: Kinase inhibitors, X-ray crystallography, lead generation, structure-based drug design, NMR, virtual screening.

INTRODUCTION

Eukaryotic protein kinases constitute a large family of homologous proteins that catalyze the transfer of the terminal (or gamma) phosphate of ATP to the specific hydroxyl group serine, threonine or tyrosine in a protein substrate. The phosphorylation of proteins, triggered in response to extracellular signals, represents a fundamental mechanism for the cellular control of many different functions, including gene expression, metabolic pathways, cell growth and differentiation, cytoskeletal integrity, cell adhesion, membrane transport and apoptosis [1,2]. The protein kinase family comprises two major subfamilies: the protein tyrosine kinases and the protein serine-threonine kinases. More recently, the histidine kinases, which phosphorylate imidazole nitrogen on a histidine residue, have also emerged as a novel class of signaling enzymes [3, 4]. To date, over 500 kinase-related sequences have been identified in the human genome, representing approximately 1.7% of our genome [5]. A comprehensive list of protein kinases, including sequence alignments and three-dimensional structural information can be found in the Protein Kinase Resource website at http://pkr.sdsc.edu/html/classification.shtml.

Malfunctions of cellular signaling have been associated with many diseases including cancer and diabetes [6-9]. The level of involvement of protein kinases in proliferative diseases was unearthed by the discovery that a large number of viral oncogenes encode activated protein kinases. For example, over expression of members of the epidermal growth factor receptor (EGF-R) family is associated with carcinomas of breast, lung, brain, prostate, GI tract and ovarian tissues [10-12]. Cytokines, hormones and growth factors are well known to bind and activate specific receptors in cells. However, the molecular mechanisms of signal transduction pathways have been elucidated by identifying the specific protein kinase cascades along with their downstream targets, which include some specific transcription factors. Protein kinases act in concert with cytokines, cell-cycle regulatory molecules, proteins of the apoptotic machinery and transcription factors via pathways that regulate cell metabolism, differentiation, proliferation and death. Many therapeutic strategies are aimed at critical components in signal transduction pathways and as such, the development of selective protein kinase inhibitors is generating considerable interest in the drug discovery community [9, 13-16].

PROTEIN KINASE ARCHITECTURE

Protein kinases contain a structurally conserved catalytic domain, first elucidated for the cyclic AMP-dependant kinase, PKA [17]. Currently, over 160 crystal structures of 40 unique protein kinase catalytic domains have been deposited in the Protein Data Bank (PDB). All protein kinases contain the unifying, structurally conserved catalytic domain, which consists of N- and C-terminal domains (Fig. (**1a**)) that are further divided into 11 sub-domains. The two terminal domains are connected through a single peptide strand, which acts as a hinge about which the domains can rotate with respect to one another upon binding of ATP and/or substrate (without disruption of the protein kinases secondary structure). Fig. (**1b**) outlines the predicted binding of ATP to a representative serine/threonine kinase, CDK-2 (PDB code 1hck). The adenine moiety of ATP binds via two hydrogen bonds to the backbone carbonyl and N-H functions of two non-consecutive residues on the protein. The ribose group of ATP is anchored to the enzyme via hydrogen bonds with residues at the beginning of the C-terminal domain (ribose binding pocket). The triphosphate group is coordinated by a magnesium ion that is ligated by Aspartate 145 and Asparagine 132 residues located in the DFG motif and the catalytic loop, respectively. In addition, polar interactions with several residues from the glycine rich loop, the conserved DFG motif and the catalytic loop further stabilize the phosphates and the transition-state generated during the phosphotransfer reaction.

PROTEIN KINASE INHIBITION STRATEGIES

Although the catalytic core of protein kinases has been evolutionarily conserved [18], the mechanisms by which the

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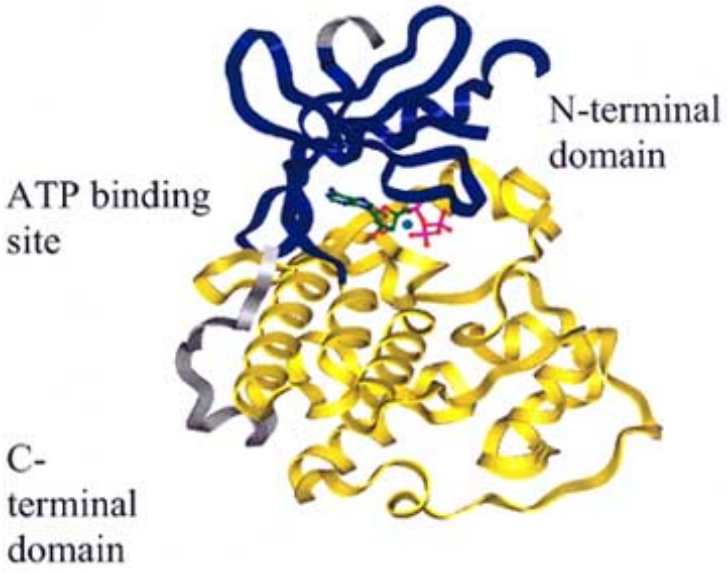

Fig (1a). Ribbon diagram of CDK-2 (PDB code 1hck), displaying N-terminal, C-terminal domains and ATP-binding cleft.

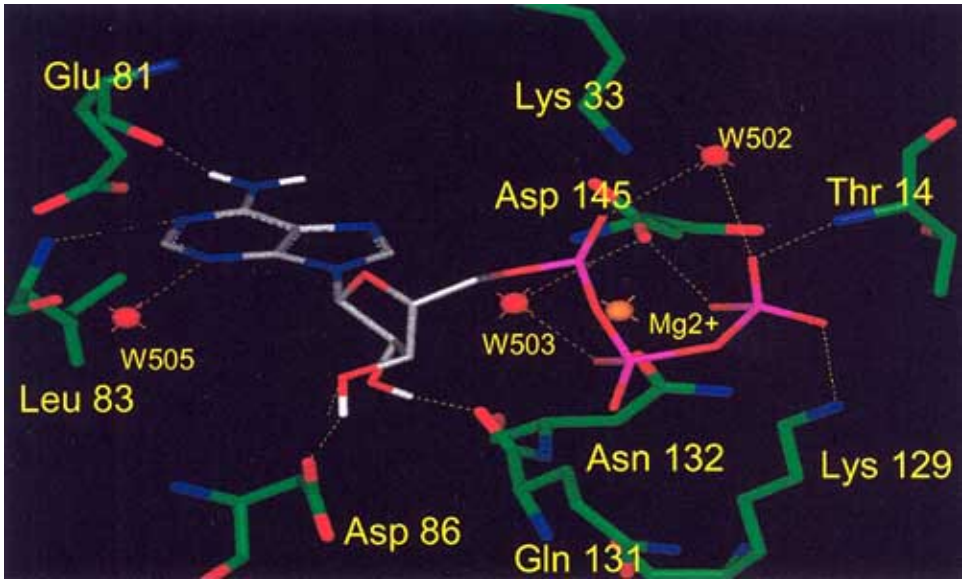

Fig. (1b). ATP binding to hinge region of CDK-2 (PDB code 1hck) catalytic cleft shown in AstexViewer^{TM}. The adenine ring makes key H-bond contacts to backbone carbonyl of Glu81 and backbone N-H of Leu 83.

inhibition of each kinase might be achieved may vary considerably. From an analysis of the enzymatic mechanism, three potential inhibitory mechanisms have been proposed: a nucleotide mimetic mechanism, a pseudo-substrate mechanism and a mechanism that involves locking the kinase into an inactive conformation by using surfaces other than the active site [19].

Most of the protein kinase inhibitors under development today are ATP-site directed inhibitors i.e. a nucleotide mimetic mechanism. In addition, several examples of inhibitors directed to non-catalytic domains of protein tyrosine kinases exist, locking the kinase into an inactive conformation [20-23]. To date the protein substrate binding site has not been successfully exploited for inhibitor design [24-26]. This is despite the fact that compounds, which block the enzyme function by substrate competition, might have an advantage over ATP mimetics, since the substrate-competitive inhibitors should be more specific and selective, in addition to the fact that they would not have to compete with high intracellular concentrations of ATP (typically around 1-5mM) [27].

There has been persistent concern in this field over the last 20 years that the high degree of structural conservation in the ATP binding cleft and the high intracellular concentrations of ATP with which an inhibitor must compete to generate sufficient cell activity, render the notion

of the ATP binding site as a drug target highly unlikely. Within the last several years, however, a large number of low molecular weight, potent ATP-competitive inhibitors, many of which show a high degree of selectivity against small panels of kinases (20-30 different kinases), have been identified [24, 28-34]. Selectivity is an important issue in the development of safe drugs, particularly with kinase inhibitors, because protein kinases share common sequences and structural homology in their ATP-binding sites, and are involved in key physiological processes. Only a fraction of all known kinases are currently available to assay, so for this reason it will be extremely challenging to design a completely selective ATP mimetic. In addition, there are many other proteins encoded by the human genome that rely on purine-based co-factors e.g. ATP synthases, phosphodiesterases (PDE's), etc.

Many recent studies, reporting the structures of kinases complexed with selective small molecule ATP site-directed inhibitors or AMP.PNP (a non hydrolyzable ATP analog), have provided a clear description of the ATP binding site, revealing the many conserved and non-conserved residues. Non-conserved residues are located in pockets that are not utilized for ATP binding and are likely to be unique to subsets of kinases. In several cases, structural biology efforts clearly show that compounds already known to be selective for a specific kinase (e.g. p38 [35]) or kinase class (e.g. the Src family kinases [36-38]), target the poorly conserved regions of the ATP binding site, thus providing a structural basis for the observed selectivity. Together, these results have increased confidence that developing inhibitors, directed at the ATP binding cleft, is a viable approach to selectively inhibiting protein kinases.

CURRENT LEAD GENERATION STRATEGIES – TO BE OR NOT TO BE "LEAD-LIKE" OR "DRUG-LIKE"?

During the 90's there was an enormous drive for increased productivity in the pharmaceutical industry. The industry became committed to massive increases in lead generation throughput and invested heavily in a range of technologies, such as high-throughput screening (HTS) and combinatorial chemistry (combi-chem), enabling the industrialization of lead generation *via* HTS of large numbers of drug-like compounds. However, there is now a growing doubt that these techniques, in isolation, will significantly increase the pharmaceutical industrys' productivity. Recently, several researchers have begun to advocate that compounds with good drug-like properties may not necessarily make the best leads for further optimization, because lead-like properties and drug-like properties, although not mutually exclusive, maybe significantly different [39-41].

Over the last 10-15 years, the industry has been active in defining drug-like properties. Lipinski's much cited "Rule of Five" [42] derives empirically from the vast amount of data that the industry has gathered on properties that maximize an oral drug candidate's probability of surviving development (molecular weight (MW) < 500, number of hydrogen bond donors < 5, number of hydrogen bond acceptors < 10, and clogP < 5). Other physicochemical properties, such as the number of rings, heavy atoms and rotatable bonds have also been used to predict drug-likeness [43, 44]. Although these rules are useful for assessing the risk profile of an oral drug candidate entering development, they do not necessarily define the properties of a good lead.

Rather than studying only drug-like properties, Hann *et al.* also studied a set of more than 450 pairs of commercial drugs and their corresponding leads [39]. Upon analysis, historical leads appeared to have lower MW, lower lipophilicity (clogP), fewer aromatic rings, fewer hydrogen bond acceptors, and lower Andrew's binding energy functions than the corresponding final drug. Teague and co-workers describe how many drug-like leads pass Lipinski's "Rule of Five", but have molecular weights (350-500 Da) and clogP values (3-5) at the high end of the preferred range [40]. Such leads are not the best starting points for optimization because addition of lipophilic groups to increase potency can adversely affect their pharmacokinetic properties. Lead-like fragments are therefore, smaller (MW = 100-350 Da) and more polar (clogP 1-3), so addition of lipophilic groups often improves both their potency and PK properties. Indeed, the average MW of successful drugs in the World Drug Index is in the low 300 Dalton's, which is similar to the average MW in current corporate collections [40]. This implies that corporate compound collections have evolved to be broadly drug-like with respect to MW and other features.

The MW and lipophilicity of initial leads typically increase during the lead optimization process. Thus, if the initial lead is already too drug-like, then the optimization process that is likely to be needed to tailor the molecule to the new receptor or enzyme will likely result in a higher MW and more lipophilic drug candidate [40]. The candidate may thus no longer possess drug-like properties. This suggests that when looking for leads, the guidelines suggested by Lipinski should be lowered so that leads that are found by HTS give more scope for further property optimization. Such drug-like property rules, however, have been applied almost universally to the design and selection of orally bioavailable compounds for lead discovery and not just for protein kinase inhibitors.

NOVEL LEADS FOR PROTEIN KINASE INHIBITORS – COMPLEX PROBLEM OR SIMPLE SOLUTION?

In considering whether leads should be less complex than drugs, another theoretical factor has been highlighted by Hann's group: the effect of increasing the complexity of the compounds screened on the hit rate [39]. As shown in Fig. (**2**), the statistical probability of finding a match between ligand and target protein decays exponentially as the size of the ligand increases, because as ligand complexity grows there are far more ways of obtaining a mismatch than a match. This is in conflict with the fact that any observed affinity would be high if a complex ligand does match. One interpretation of this model is that the industry, in trying to quickly identify high-affinity matches, has focused on screening complex drug-like compounds, but that in so doing it has unwittingly screened compounds whose complexity leads, on statistical grounds, to low hit rates. A

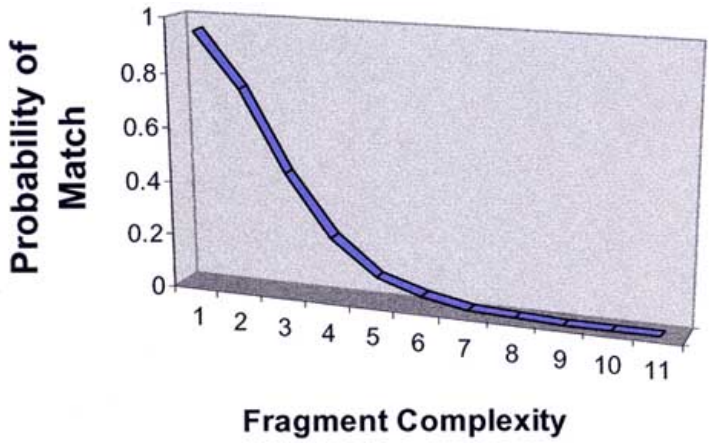

Fig. (2). Probability of ligand/protein 'match' decreases exponentially with increasing fragment complexity.

rational way forward is to initially screen simpler and more lead-like compounds that have a higher probability of efficiently binding (matching), even if they deliver less active starting points.

Outlined in Fig. (**3**) is a simple triazine-based scaffold with three growth vectors. If one considers that each of the growth vectors could be one of 100 different constituents, testing every possible drug-like molecule would require the production and screening of 10^6 compounds. However, by adopting a fragment-based approach, potentially only 300 would need to be made and screened to explore the same chemical space. Small, simple molecules are more desirable because they can penetrate deep into active sites without steric hindrance. The proposed sampling efficiency is based on the additive nature of the fragments, as compared to larger drug-like compounds needing a multiplicative procedure.

New methodologies to identify low MW fragments (MW 100-250 Da) that could be efficiently optimized into novel protein kinase inhibitors possessing good drug-like properties are a worthy cause. By definition these molecular fragments would have limited functionality and would therefore, exhibit weaker affinity (typically in the 50μm-mM range). This affinity range is outside of the normal HTS sensitivity range and as such cannot routinely be identified in standard bioassays due to the high concentration of compound that would be required, interfering with the assay and leading to significant false positives. As an alternative to pushing bio-assays into this affinity range, efforts are turning increasingly to biophysical methods such as NMR and X-ray crystallography for fragment-based screening approaches to find protein kinase inhibitor scaffolds and transforming them into novel, potent leads using structure-guided chemistry.

NMR SCREENING APPROACHES TOWARDS PROTEIN KINASE INHIBITOR LEAD GENERATION

Nuclear Magnetic Resonance (NMR) spectroscopy has long been used to detect the binding of small molecules and peptides to biomolecular targets, but has only recently been employed to screen fragment libraries for drug discovery. Wealth of NMR experiments have been developed to measure changes in NMR parameters (chemical shift, relaxation rates and intensity), which can be interpreted to provide information about the effects of protein-ligand binding on molecular rotation, translation and local nuclear environments. This alternative direct binding assay technique can be used to detect binders with affinities ranging from nanomolar to millimolar.

Pioneering methods have recently been developed, in which the detection of NMR signals in 2D Heteronuclear Single Quantum Coherence (HSQC) spectra of a ^{15}N- or ^{13}C-labelled protein is used to screen libraries of molecular fragments [47-51]. Perturbations to the NMR spectra of a

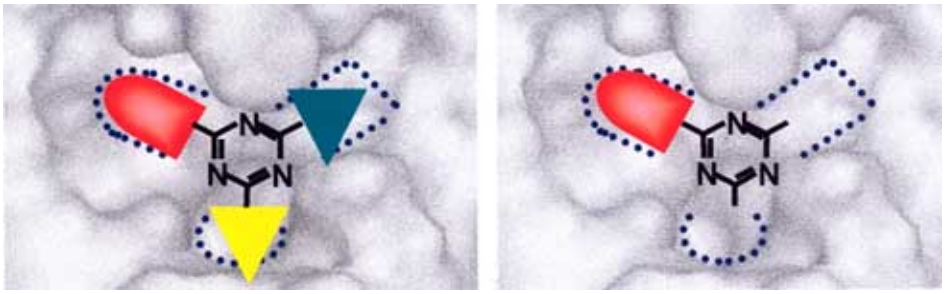

Fig. (3). A triazine-based scaffold with three variable points quickly becomes a relatively complex molecule able to enjoy a plethora of interactions with a protein target. The majority of these interactions, however, will not be conducive to efficient binding and only the net result of all three pockets interactions will be observed in a bioassay. A more elegant fragment-based approach allows efficient sampling of the same chemical space, producing a weaker binding but better quality 'hit'.

protein are used to indicate that ligand binding is taking place and to give some indication of the location of the binding site. Ligand titrations can then be used to estimate binding affinities, thus determining structure-activity relationships (SAR) by NMR. Once several molecular fragments that bind to the target protein have been identified they can then be linked together or "grown" using structure-guided chemistry to improve the affinity for the target protein.

Nevertheless, the use of NMR as a primary screen has some significant hurdles that can limit its use in a high throughput format. These are mainly: restrictions on the size of the protein target: amounts of isotope labeled protein required: protein purity: affinity of the ligands for the protein target: and the relatively low sensitivity of NMR compared to other spectroscopic techniques. This latter point dictates that NMR can require concentrations in excess of 10μM for the observation of small molecule NMR spectra and in excess of 100μM for the detection of small (<35kDa) proteins, even with state-of-the-art equipment. The rapid increase in NMR line-width with increasing molecular weight can be partially overcome using recent techniques at very high magnetic field strengths such as Transverse Relaxation-Optimized SpectroscopY (TROSY), thus extending the molecular weight limit to over 100kDa [52]. Currently, in order to complete an HSQC experiment in 30 minutes or less, the lower limit of the protein concentration is imposed by the sensitivity of the NMR spectrometer; around 50μM using high-sensitivity "cryoprobes" and 200μM for conventional probes.

Sample volume requirements are also high, typically 500μl for routine experiments, thus significant quantities of isotope-enriched protein (typically >0.5mg/sample, >90% pure) are sought, which impacts significantly on the number of compounds that can be screened [53, 54]. However, this requirement for comparatively high protein and ligand concentrations makes NMR well suited for detecting weak binding interactions.

In order to detect changes in the protein HSQC spectrum, a ligand must be present in approximately stoichiometric amounts and at concentrations around or above its K_d. For high affinity binding ligands, this imposes a lower limit that is set by the protein concentration. For weak binding ligands, the upper limit of the ligand concentration and hence the detectable K_d, is often set by the ligand solubility. In order to optimize use of protein and experiment time, ligands are usually screened as mixtures, at concentrations around the highest K_d that is to be detected. However, deconvolution of the positive mixtures is then required incurring a further commitment to sample supply and instrument resources. In addition, the utilization of mixtures may reduce a compound's solubility below the concentration required by the K_d, while further complicating the necessity of maintaining consistent buffer conditions (pH, ionic strength, % DMSO) between samples. Because of the inherently low sensitivity of NMR experiments, NMR screening requires compounds with much higher aqueous solubility than do conventional screening methods. Additionally, the need to optimize the NMR data collection throughput usually results in a compromise between data quality and acquisition time [55].

Other attempts in the NMR field to minimize resource and sample requirements when studying protein –ligand binding have focussed on the observation of the ligand using 1D-NMR techniques, particularly diffusion-edited measurements and transferred nuclear overhauser effects (TrNOE) and the utilization of a SHAPES compound library [56]. These 1D-NMR experiments eliminate the need for labeled protein while minimizing sample quantities and data acquisition time. The SHAPES compound library uses a very small set of molecular scaffolds (132) to represent a larger library (with common chemical features that make them more drug-like) where hits are used for virtual screening of the compound corporate collection. Again, the end result is to minimize both the sample requirement and experiment time. Larger libraries (2,000-200,000 molecules) with appropriate physicochemical properties have also been advocated for NMR screening [49, 50].

Ligand-detected NMR experiments do not impose an upper molecular weight limit on the target protein. They also do not require that the protein is isotopically labeled or that the protein is pure, so they are more generally useful with a wider range of protein targets. Typically ligands are screened as mixtures at concentrations around 100μM and with protein concentrations of about 10μM. Comparison of the ligand NMR spectrum in the absence and presence of protein is used to infer protein binding and, since less than five percent of ligand needs to be bound to protein to produce an observable change, these conditions are still capable of detecting interactions with K_d's as high as 1mM. The protein requirements are considerably reduced in comparison with protein-detected NMR experiments (approximately 10 fold). However, the detection of very weak binding (K_d>1mM) requires an increase in both protein and ligand concentrations whereas, for protein-detected experiments, only the ligand concentration needs to be increased, so this advantage maybe eroded.

Since the ligand mixture is directly observed, deconvolution of the mixture is not required in order to identify hits, unless there is severe overlap of signals or competition of ligands for the binding site is suspected. However, no direct information is obtained on the location or the number of ligand binding sites on the protein surface from such ligand-detected experiments. This information can only be inferred by further experiments where the weakly bound ligand is displaced by a high concentration of a higher affinity bound substrate or inhibitor whose binding has been previously characterized. Furthermore, the determination of ligand K_d's, or even the ranking of hits, involves assumptions about the nature of the bound state and may be problematical.

Lepre observes that for several targets, HTS screens of follow-up libraries based on NMR screening hit templates produce subsequent hit rates ten-fold higher than do general HTS screens, thereby demonstrating convergence towards more potent compounds [55]. This strategy has been applied to the design of DNA gyrase inhibitors starting from millimolar hits [57]. In this example, the lead optimization process employed SAR information and structure-based drug design. The group at Vertex have also rapidly differentiated between binding and non-binding compounds in small non-interacting mixtures, demonstrating the utility of the NMR

SHAPES method with several enzyme targets that include the 42 kDa protein p38 MAP kinase [56]. Recently, Hajduk *et al.* have demonstrated the ability of NMR-based screening to efficiently identify potent low-molecular-weight adenosine kinase inhibitors [58]. The subsequent molecules were novel, and thus unlikely to have been exactly represented in screening collections for HTS work.

The design of fragment libraries for NMR screening remains a highly competitive area and is strongly influenced by other technologies, such as virtual screening (VS), which are available to select the library members and by the chemical techniques that would be required to develop any hits. However, a few general comments can be made. Small molecular size (<250 Da) ensures that any hits, even with K_d's around 1mM, make few, comparatively strong interactions with the protein. These interactions are usually conserved as the hits are optimized and as additional interactions are added to improve potency. Good (1mM) solubility in aqueous buffers and high solubility in DMSO (10-100mM) is required for the preparation of NMR solutions and these restrictions are particularly acute for protein-detected experiments, where the ligand concentrations are higher. The optimum number of components in a mixture will depend on the observed hit rate for a given protein target and ligand concentration: low hit-rates permit the use of larger mixtures. Thus, an initial pre-screen maybe useful in order to estimate the hit rate that would be expected from a particular compound library [55].

Both protein-detected and ligand-detected NMR methods have been applied to kinases and kinase domains [56, 58, 59]. Generally the catalytic domains are at the upper size limit for protein-detected experiments and may be difficult to obtain in a highly purified form i.e. in a unique phosphorylation state. Thus, it is to be expected that the ligand-detected experiments would be the first choice for NMR-based screening, with positive hits being followed up by further NMR experiments using isotopically labeled protein or by other structural methods, such as X-ray crystallography [55, 56].

VIRTUAL SCREENING APPROACHES TOWARDS PROTEIN KINASE INHIBITOR LEAD GENERATION

Recent advances in parallel synthesiz, combinatorial chemistry and HTS have made it possible for chemists to synthesize large numbers of compounds. However, this still represents a tiny fraction of the total number of potential molecules that could be synthesized. Virtual screening (VS) encompasses a variety of computational techniques that allow chemists to reduce an enormous virtual library to more manageable proportions [60, 61]. Although still an evolving method, it can be viewed as a complimentary approach to experimental bio-assay screening and when coupled with structural biology, VS promises to increase the number and enhance the success of projects in the lead identification stage of the drug discovery process.

Structure-based virtual screening encompasses a variety of sequential computational phases, including target and database preparation, docking and post-docking analysis and prioritization of compounds for bioassay [see 60 & 61 for more detailed discussions]. One of the most important advantages of VS is the speed at which large computer processors can perform complex mathematical calculations in relatively short periods of time. Crystal structures of molecules complexed to protein kinases, for example, can be easily manipulated and modified in a simulated environment in order to assess the importance of different functional groups and residues for structure-based drug design (SBDD) efforts. However, with these advantages come limitations. Protein kinase-ligand complexes, for example, are assumed to have rigid conformations, although in reality they would be quite flexible, therefore preventing an assessment of alternative conformations. A method using computational sensitivity analysis is described by Wong to explain the interaction between PKA and Balanol [62]. This approach readily identified functional groups on the ligand that are significant in contributing to binding affinity and selectivity. The "rigid conformation" assumption was overcome by using molecular dynamics-based methods previously developed by Wong [63].

A VS campaign of a large compound database against a target protein can yield a vast amount of data that comprises the predicted binding conformation for each compound and predicted binding affinity (scoring function). Testing the compounds in simple rank order in the appropriate bioassay would then be the next step, but this is often misleading. Scoring functions in VS campaigns are often inadequate at predicting the true affinity of a ligand for a receptor. A recent study of the performance of several scoring functions of varying complexity, for predicting the binding affinities of several ligands to p38 MAP kinase, highlighted the inadequacies of some common scoring functions (PLP, Chemscore, Dock®), with little or no correlation found between the predicted and experimental binding affinities [64].

In recent years there have been several published successes for structure-based virtual screening, but so far few for protein kinases [60, 65, 66]. However, the technology is maturing into a viable method for the identification of hits and is being considered an essential tool for enriching the lead identification phase of the drug discovery process. Although there has been undoubted success with virtual screening, the false-positive rate remains high. Therefore, efforts to improve the quality of the scoring functions and the techniques used in the post-screening analysis are needed to progress this technology further.

At Astex, we have placed a great emphasis on constructing a web-based interface for analyzing the results from virtual screens. The interface allows the modeller to interactively filter hundreds of thousands of compounds on the basis of different scoring functions (e.g. Chemscore, Drugscore, Goldscore), simple molecular properties (e.g. cLogP Molecular weight), substructure queries, or 3D contacts made by the docked molecule. Compounds that pass the filters can then be visualized in the active site for final selection using AstexViewer™ [67]. A database of over 2 million commercially available compounds has been constructed for our virtual screening applications. The docking is performed using a proprietary version of GOLD [68] that has been substantially modified to allow docking with alternative scoring functions and pharmacophores.

GOLD is sometimes thought to be too slow for applications in virtual screening [60] but, in our hands, a million fragments (<300 molecular weight) can be screened per week on an 80 processor Linux cluster. Virtual screening has been used extensively to look for fragments that bind to kinases [Murray, C. W., Personal Communication]. We have found a significant number of hits that bind against four kinases, and for three of the kinases, we have obtained crystal structures of fragments derived from virtual screening.

STRUCTURE BASED DRUG DESIGN AND X-RAY CRYSTALLOGRAPHY APPROACHES TO PROTEIN KINASE INHIBITOR LEAD GENERATION

The use of structural information obtained by X-ray crystallography or computer assisted molecular modeling based on kinase domain homology has been a key factor in the design of selective protein kinase inhibitors [69]. As more structures are solved, the accuracy of the modeling for inhibitor design will ultimately improve. Pharmacophore models for ATP site-directed competitive inhibitors [70-73] have been obtained by combining three-dimensional structural information and structure-activity relationship data, providing directions for structure-based drug design approaches and interpretation of structure activity relationships (SAR) [70, 73-76].

Numerous technology advances have resulted in an exponential increase in the number of crystal structures being deposited into the Protein Data Bank (PDB) in recent years [77]. Currently, the PDB holds over 19,000 protein structures, most of which have been determined using X-ray crystallography. The three-dimensional structure of a therapeutic target of interest to drug discovery scientists is, therefore, likely to have been determined. Furthermore, it is expected that within the next five years, crystal structures of a large majority of the non-membrane protein targets of interest to the pharmaceutical industry will be available.

Non-liganded crystal structures of target proteins can help guide a lead discovery program, but the maximum value is derived from structures of the protein in complex with potential lead compounds. This is because many proteins can undergo some level of conformational movement upon ligand binding, which has proven very difficult to be predicted from the non-liganded structures alone. Water molecules can often play a key role in the interactions between ligands and proteins and their positions need to be established experimentally. Rapid determination of crystal structures of protein-ligand complexes is required to effectively guide the lead optimization phase of the drug discovery process, and also allows X-ray crystallography to be applied in a new way as a screening tool [78].

HIGH-THROUGHPUT X-RAY CRYSTALLOGRAPHY

Over the past two decades the potential rewards of structure-based drug design have continued to beguile the pharmaceutical industry. Unfortunately, the approach became overshadowed in the early nineties by other technologies such as combinatorial chemistry and high-throughput screening (HTS), which appeared at the time to offer a more effective approach for drug discovery. The goal of obtaining a crystal structure of the target protein, particularly in complex with lead compounds, was regarded as a resource-intensive, unpredictable and slow process. During that period it was clear that protein crystallography was unable to keep pace with the other drug discovery technologies being performed in a high-throughput mode and only found an outlet in the lead optimization of high-value compounds.

Significant technology developments in protein crystallography have resulted in many crystal structures for the majority of today's therapeutic targets, especially protein kinases [79]. Furthermore, the ability to rapidly obtain crystal structures of a target protein in complex with small molecules is driving a new wave of structure-based drug design [80]. New technologies and methods have been applied to X-ray crystallography to enable rapid high-throughput structure determination [78, 79, 81]. The process of producing crystal structures is multidisciplinary and advances in biochemistry, crystallization, molecular biology, X-ray data collection and computational analysis underpin high-throughput X-ray crystallography.

Production of novel proteins in a quantity and form that is suitable for crystallization and X-ray analysis occupies a significant amount of time in most structural biology groups and as such, there are numerous methods for the high-throughput parallel expression and purification of proteins [82, 83]. Typically, 10-50 mgs of protein is required to screen sufficient numbers of crystallization conditions to obtain initial crystals. Crystallization is often regarded as a slow, resource-intensive step with low success rates in obtaining good quality crystals. The use of biophysical methods to characterize protein samples, such as dynamic light scattering and the use of automation to improve the process by altering all the variables known to affect crystallization, has also greatly improved efficiency and success rates [84].

X-ray data collection has been transformed over the last 15 years by the advent of better X-ray sources and detectors [85]. Synchrotron radiation coupled with charged-coupled device (CCD) detectors have allowed complete X-ray datasets for a crystal to be collected and processed within hours instead of days. High-throughput X-ray data collection has also required the development of robotic systems that store and mount crystals sequentially, while maintaining the samples at liquid-nitrogen temperatures [86]. Concomitantly, new methods of electron density interpretation and model-building have allowed rapid and automated construction of protein models without the need for significant manual intervention [87].

HIGH-THROUGHPUT FRAGMENT-BASED SCREENING USING X-RAY CRYSTALLOGRAPHY

The application of X-ray crystallography as a high-throughput screening tool, requires the soaking of ligands into preformed protein crystals. Collection of the X-ray data from a protein crystal exposed to a ligand then needs appropriate analysis and interpretation of the resulting electron density. A crystallographer can spend many hours to several days assessing the data from a single protein/ligand experiment and this represents a key bottleneck for the use of X-ray crystallography as a method for screening compounds.

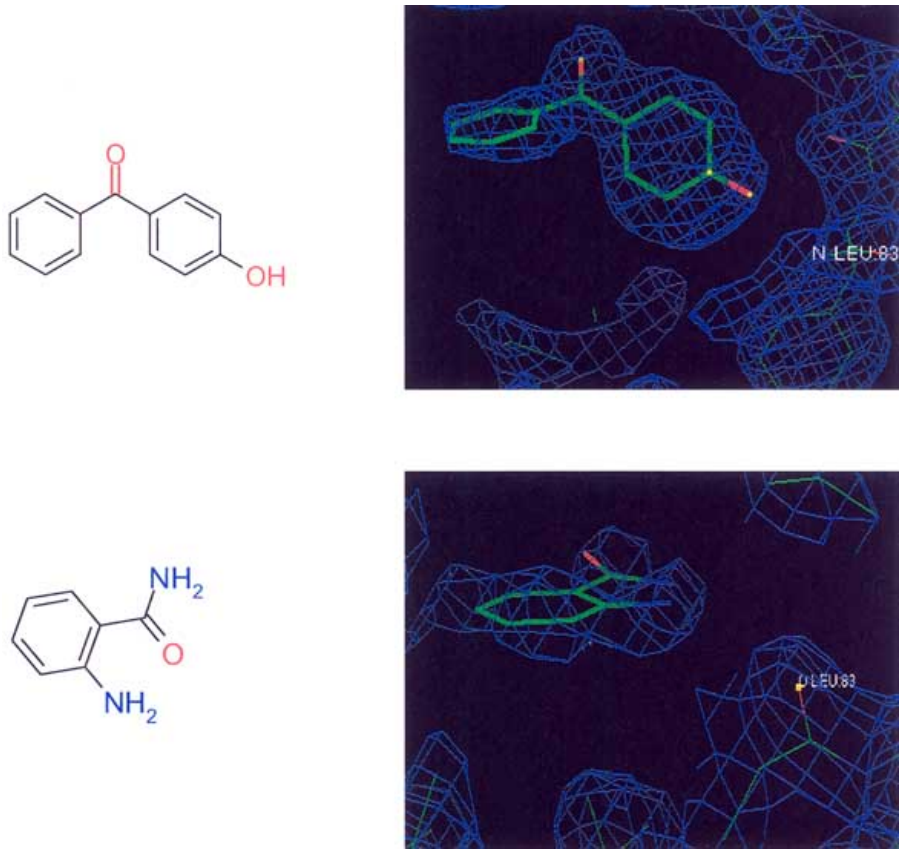

Fig. (4). Examples of small-molecules fragment bound to the hinge region of CDK-2. The electron density was interepreted and models of compounds were automatically fitted using AutoSolve® The electron density maps are contoured at 3s to ensure that the data are significant and density due to protein and solvent has been removed for clarity.

Software tools such as Quanta from Accelrys Inc. (San Diego, CA, USA) and AutoSolve® [80] from Astex (Cambridge, UK) enable a crystallographer to accelerate the time taken for data analysis and interpretation.

The electron density from soaking experiments using singlets and cocktails of fragments can be rapidly analyzed by AutoSolve®. The binding mode of the small-molecule fragment is defined by the electron density, so although the affinity may be in the millimolar range, the binding is ordered with key interactions being made between the compound and the protein. With high quality electron density, AutoSolve® can readily identify the correct compound bound at the active site from an experiment, where the crystal has been exposed to a cocktail of compounds. This provides a novel starting point for medicinal chemistry efforts.

In fragment based screening, X-ray crystallography has the advantage of defining the ligand-binding sites with more certainty than NMR spectroscopy. The binding orientations of the molecular fragments observed in X-ray crystallography then play a critical role in guiding efficient lead optimization programs. Different sets of molecular fragments can be used to target a particular protein. For example, at Astex, a screen of a "focussed set" of fragments against trypsin, based on known binders, such as benzamidine, 4-aminopyridine and cyclohexylamine has been performed [78, 80]. More recently, we used a proprietary "focused kinase set" against a key cancer target cyclin dependant kinase-2 (CDK-2), and demonstrated that a significant number of electron density maps were able to be unambiguously interpreted by AutoSolve®, revealing some novel structural motifs as shown in Fig. (**4**) [Wyatt, P., Unpublished Results]. Representatives of the "focussed kinase set" were dissolved in an organic solvent (such as dimethylsulphoxide (DMSO)) as singlets or cocktails, added to a single CDK-2 protein crystal and then left to soak for at least 1 hour to give the molecule(s) time to penetrate into the active site [78, 80]. The concentration of the molecular fragment is typically greater than 20 mM, reflecting the low-affinity that is expected.

Some of the first experiments, in which X-ray crystallography was used as a "screening tool" were reported by Verlinde [89]. More recently, Nienaber has described a method for screening using X-ray crystallography that focuses on soaking the target crystals with cocktails of compounds having differing shapes, so that they can easily be distinguished by visual inspection of electron density [90]. Furthermore, they have developed orally bioavailable inhibitors, containing a 2-aminoquinoline substituent, for urokinase using this approach.

Another key advantage of using molecular fragments for high-throughput X-ray crystallographic screening is the

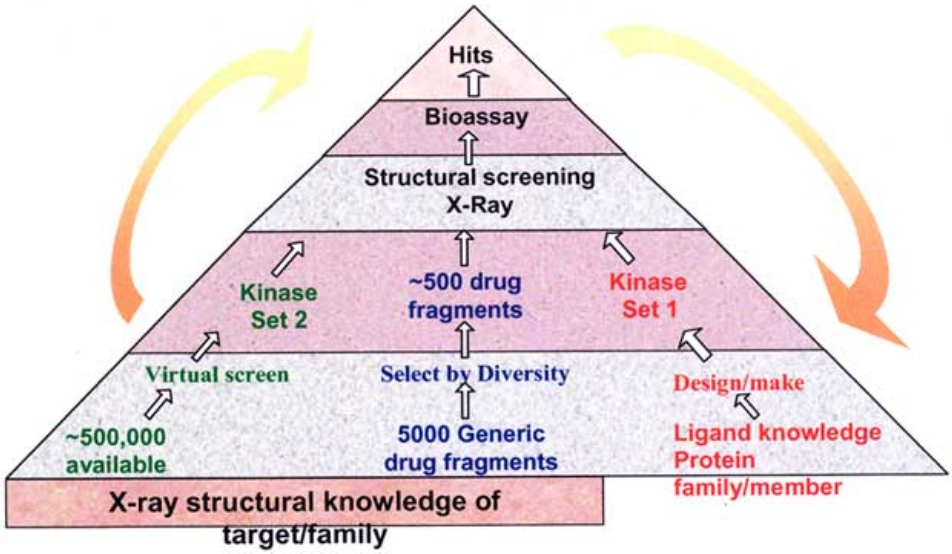

Fig. (5). Process for the selection of compounds. A variety of computational filters are applied to select and generate libraries of molecular fragments for screening using X-ray crystallography. Chemical databases containing millions of compounds are sampled to produce fragment libraries containing 500-1000 compounds.

significant amount of chemical space that is sampled using a relatively small library of compounds. For example, if the binding of several heterocycles is probed against specific binding pockets in a protein, the discrimination between a binding and non-binding event depends solely on the molecular complementarity and is not constrained or modulated by the heterocycle being part of a larger molecule. This becomes a far more comprehensive and elegant way to probe for new interactions than having the fragments attached to a rigid template, as might derive from a conventional combinatorial chemistry approach [91]. When all the above processes are coordinated they form a rational and powerful approach to lead discovery. A flow diagram in Fig. (**5**) outlines this Pyramid™ process Astex employs as part of its lead discovery platform.

STRUCTURE-BASED LEAD OPTIMIZATION OF MOLECULAR FRAGMENT "HITS"

Molecular fragments binding in the protein active site provides a valuable starting point for medicinal chemistry to optimize the interactions using a structure-based approach. The fragments can be fused onto a template or used as the starting point for "growing out" an inhibitor into other pockets of the protein, as shown in Fig. (**6**). The potency of a weakly-binding fragment can be rapidly improved using iterative structure-based chemical synthesis. For example, in one of our protein kinase lead discovery programs targeted against p38 MAP kinase, we identified a fragment, AT464 (MW = 180), which exhibited an IC_{50} of ~ 1mM in an enzyme assay. Using the crystal structure of AT464 bound to the protein kinase we were able to increase binding affinity by more than 20-fold, synthesizing just 20 analogs. The resulting compound, AT660, had an IC_{50} of 40μM [Murray, C. W.; Gill, A. L. Unpublished Results] and this novel lead series was further optimized to improve bioactivity using structure-based chemical synthesis. This resulted in the current lead series, which have IC_{50} values of less than 50nM against the enzyme and are active in inhibiting TNF-α release in LPS-stimulated cells. This improvement in affinity is produced by iteratively increasing the number of interactions between the protein and the compound (Fig. (**6**)). Using a structure-based chemistry strategy, progressing from millimolar hits to low nanomolar compounds for our first lead series required the synthesis of less than 150 compounds.

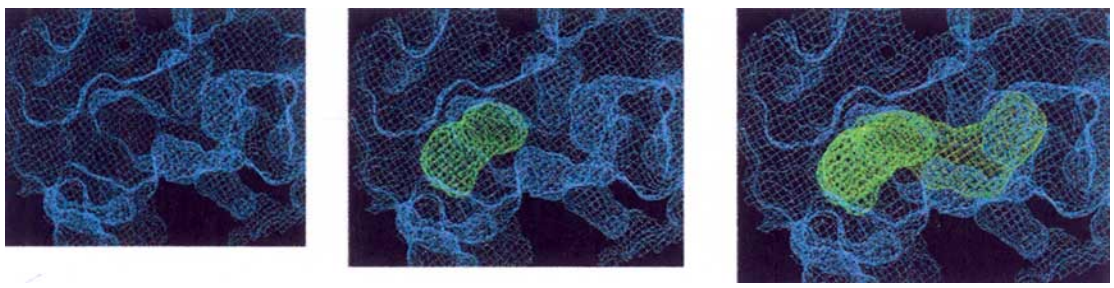

Fig. (6). Once a fragment has been bound into the active site, it can be used as a start point for iterative structure-driven chemistry by rational modification to occupy neighbouring pockets and pick-up key hydrogen bond contacts.

Another example, in our protein kinase lead discovery programs, of rapidly increasing potency of a weakly-binding fragment using iterative structure-based chemical synthesis is demonstrated in our CDK-2 program. The project team identified a number of chemically related inhibitors from crystallographically screening our proprietary 'focussed kinase set'. AT381 (MW = 118), which exhibited an IC_{50} of ~1mM in an enzyme assay, was chosen to perform rapid structure-guided chemistry upon. Using the crystal structure of AT381 bound to CDK-2 to guide synthesis of only 30 analogs, we were able to rapidly generate AT3409, with an IC_{50} of < 10nM [Wyatt, P., Unpublished Results]. Compounds from this and other series are also active in a range of normal and cancer cell lines including MRC5, HCT116 and HT29 cells.

SUMMARY

The key strengths of new lead generation strategies using fragment screening approaches are the potential to use less complex starting points and to work in areas of chemistry that have not previously been exploited. A strong intellectual property position continues to be a necessity for undertaking an expensive drug discovery campaign, and these methods present opportunities to explore chemical structures that are not already well represented in corporate collections or suppliers catalogues. This is particularly important in the protein kinase inhibitor arena, where currently many inhibitor scaffolds are based around a small number of well-documented heterocyclic templates [23, 26-32].

Although there are potentially significant benefits to fragment-based screening to generate novel protein kinase inhibitors, there are also some caveats. The hits identified are likely to have weak affinity (the smaller and less functionalized compounds screened are likely to yield only weak hits of tens to hundreds of micromolar). Weak leads may require more optimization iterations than typical drug-like leads, but in so doing they may also develop into new chemistries that are not yet represented in drug-like molecular screening collections.

The role of protein structure in developing novel protein kinase inhibitors will increase significantly over the coming years as more crystal structures become available for the protein kinase superfamily. Many recent technology advances in structure determination have allowed both NMR spectroscopy and X-ray crystallography to be used as a method for ligand screening. This has significantly increased the value for fragment-based lead discovery approaches where the initial molecular fragments are likely to have an affinity too weak to enable detection using traditional bioassay-based methods. Initial data generated using NMR and X-ray crystallographic screening of molecular fragment libraries indicates that novel kinase inhibitor scaffolds can be identified and subsequently optimized using rapid structure-based synthesis to generate useful lead compounds [56-58, 90, 91]. The potential of these new lead generation strategies in conjunction with the burgeoning power of VS to generate novel chemo-types as protein kinase inhibitors may be significant, particularly against those protein kinase targets which have remained intractable to date using conventional screening methods.

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