Challenges in design of biochemical assays for the identification of small molecules to target multiple conformations of protein kinases

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Protein kinases are under intense investigation because they play an important part in human diseases. Recent advances in structural biology and medicinal chemistry show that in addition to the adenosine triphosphate binding site, other 'druggable' pockets are formed at their surface when they adopt specific conformations. These findings open up the possibility of designing compounds with new modes of binding. The identification of these new inhibitors requires assays to be designed that allow the conformational flexibility of these enzymes to be better exploited. This review describes how biochemical assays can be adopted to explore these new drug design strategies.

A study of different marketed drugs shows that most of the effort expended in pharmaceutical companies is directed toward a limited number of protein families [1]. One of them is the protein kinase family. Protein kinases are very attractive drug targets because they play an important part in various human diseases and because a lot of experience in medicinal chemistry and biology has been gained in this area [2].

Over the past few years, detailed enzymatic studies have allowed identifying the kinetic mechanism of various protein kinases, providing a better understanding of kinase catalysis [3]. Furthermore, structural biology has shown that protein kinases are very flexible and can adopt a variety of conformations [4]. A very important finding for drug discovery is that, when these enzymes adopt specific conformations, 'druggable' pockets are created at their surface. This opens up the possibility of designing inhibitors that do not bind as 'classical' inhibitors at the adenosine triphosphate (ATP) binding site.

'Nonclassical' ATP-competitive inhibitors have been described (for example see [5]) but in many cases their mode of action was only discovered after structural biology analysis (e.g. Gleevec [6]). In other words, these compounds were only identified because the targeted protein kinase could adopt a specific conformation under defined assay conditions. As a consequence drug screening assays should be set up in a way that permits the exploitation of entire conformational diversity of kinase targets because protein kinases can adopt multiple conformations.

Cell-based assays are *a priori* most suited for such purposes, because they allow protein kinases to be targeted in their physiological environments, where they take on multiple conformations. Several cell-based assays have been developed and are routinely used to identify inhibitors. However, in the investigation of possible new drug design strategies, the initial lead compounds identified usually have low potency and may show a poor ability to penetrate cells and/or a low metabolic stability. Consequently, they may not be identified in cell-based assays. The establishment of clear structure–activity relationships is also important at the beginning of medicinal chemistry programs, because this enables the working hypotheses to be validated and the work on optimizing the compounds to be started. The complexity of protein kinase regulation in cells may lead to complex results, making it more difficult to establish structure–activity relationships. Biochemical assays are an attractive alternative because they are simpler and allow the detection of weak inhibitors. They are broadly used today as primary screens in many drug discovery programs. However, the conformational diversity targeted in a biochemical assay is probably limited when compared with the diversity that can be achieved in a cell-based assay. Biochemical assays therefore need to be designed in such a way that they permit a larger number of protein conformations to be probed. This means integrating the information obtained from cell biology, kinetic studies and structural biology into the design of the assays.

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GLOSSARY

AKT RAC-alpha serine/threonine-protein kinase.
AKT 1, 2, 3 RAC-alpha serine/threonine-protein kinases 1, 2 and 3.
CAMKII Ca^{2+}/calmodulin-dependent protein kinase II.
CDK2 Cyclin-dependent kinase 2.
CDK4 Cyclin-dependent kinase 4.
CDK5 Cyclin-dependent kinase 5.
c-MET Cellular mesenchymal epithelial transition factor.
CSK C-terminal Src kinase.
CycD1 Cycline D1.
CycE Cycline E.
EGF-R Epithelial growth factor receptor.
ERK2 Extracellular signal-regulated kinase 2.
IKK I kappa kinase.
INS-R Insulin receptor.
JNK2α2 Mitogen-activated protein kinase 9 isoform α2.
Kinetic mechanism Sequence in which substrates bind to and products are released from the enzyme.
MAPKAPK2 Mitogen-activated protein kinase activated protein kinase 2.
MEK Mitogen-activated protein kinase.
MEK1 Mitogen-activated protein kinase 1.
MLCK Myosin light chain kinase.
P38 MAP kinase α p38 mitogen-activated protein kinase alpha.
PAK2 p21-activated protein kinase.
PDK1 Serine/threonine-protein kinase PDK1.
PHK Phosphorylase kinase.
PKA Protein kinase A.
PKC θ Protein kinase C θ.
PKD 1, 2, 3 Protein kinases 1, 2, 3.
ROCK I Rho kinase I.
ROCK II Rho kinase II.
S6K1 S6 kinase 1.
SKY1P SR kinase in yeast.
c-SRC Cellular proto-oncogene tyrosine-protein kinase src.
v-SRC Viral proto-oncogene tyrosine-protein kinase src.
T2CDPK Type II calmodulin-dependent protein kinase.
TBK TANK-binding kinase 1.
TRKA High affinity nerve growth factor receptor.
TPX2 Targeting protein for Xklp2.
VEGF-R2 Vascular endothelial growth factor receptor 2.

The purpose of this review is to provide the reader with basic information on the kinetic properties of protein kinases and on the parameters that modulate their activity and to show that, by applying this knowledge, it is possible to design biochemical assays that allow a larger number of drug discovery approaches to be explored.

Kinetic properties of protein kinases and applications to drug discovery

Protein kinases catalyze the transfer of a phosphate group (γ-PO_4) from ATP to serine, threonine or tyrosine residues located on proteins (P). Because they interact with two substrates, they are bisubstrate enzymes, and their kinetic parameters should be determined using rate equations established for bisubstrate enzymes. It should be considered that, for such enzymes, the Michaelis constant (K_m), the maximal rate (V_{max}) and the inhibition constant (K_I) of an inhibitor measured for one substrate might be influenced by the concentration of the other substrate. They are therefore apparent constants. The rate equations used to determine the kinetic parameters of bisubstrate enzymes depend on their kinetic mechanism. The random Bi Bi and ordered Bi Bi mechanisms [7] are often used to describe the kinetics of a bisubstrate kinase system.

In a random mechanism, both substrates can bind first to the free enzyme (E) and the rate equation (1) (Fig. 1a) is used to determine K_P, K_{ATP} and V_{max}. The constant α defines the degree to which the binding of one substrate influences the binding of the other substrate. The affinity of a substrate for the free enzyme is higher than its affinity for the complex [enzyme:other substrate] when $\alpha > 1$ and lower when $\alpha < 1$. Substantially different α values have been reported for protein kinases. For example, $\alpha = 0.04$ and $\alpha = 130$ have been measured with p38 MAP kinase α [8] and CDK2 [9], respectively (see Glossary of terms for detailed definitions of protein names). It is important to note that α values can be affected by the enzyme form (e.g. full-length versus truncated protein kinases, activation status, etc.) and the substrate (e.g. synthetic peptide or physiological substrate) used in the assay.

In an ordered Bi Bi mechanism, there is a mandatory order for binding to the free enzyme, and only one of the two substrates can bind to the free enzyme. The other substrate only binds to the complex [enzyme:first substrate]. Two different mechanisms can be envisaged with protein kinases: the one where ATP binds first to the free enzyme and the one where P binds first. The rate equation corresponding to a mechanism where ATP binds first to the free enzyme is given in Fig. 1b (Eqn 2).

The kinetic parameters of protein kinases are determined by fitting Eqn 1 or 2 with data obtained from measuring initial rates under steady-state conditions at different [ATP] and [P]. Because these equations have the same form it is not possible to distinguish between both mechanisms solely from this analysis. It is therefore necessary to elucidate the kinetic mechanism to determine which rate equation to use. This is usually done through competition experiments using dead-end inhibitors (compounds that compete with the substrates) and product analogs (ADP and phosphorylated products). The study of the different inhibition profiles obtained with these inhibitors enables the kinetic mechanism to be elucidated (interested readers can obtain more information in [10]). In some cases, the experimentally determined inhibition profiles do not fit the theory, and additional experiments (e.g. biophysical studies) must be carried out.

The kinetic mechanism of several protein kinases has been elucidated (Table 1). These preferentially catalyze the phosphorylation of protein/peptide substrates by random Bi Bi or ordered Bi Bi mechanisms (in most cases with ATP binding first to the free enzyme). It should be noted that, depending on the experimental conditions (for example, peptide versus protein substrate), different kinetic mechanisms are sometimes determined for the same enzyme (for a discussion on this topic see [3]).

Once the kinetic mechanism is firmly established the kinetic parameters can then be determined experimentally, allowing balanced assay conditions – as described by Copeland [11] – to be established. Assays that are run at substrate concentrations below αK (random mechanism) or K_m (ordered mechanism) favor the identification of competitive inhibitors, while assays run at substrate concentrations above αK (or K_m) favor the identification of uncompetitive inhibitors. Therefore, working at αK (or K_m)

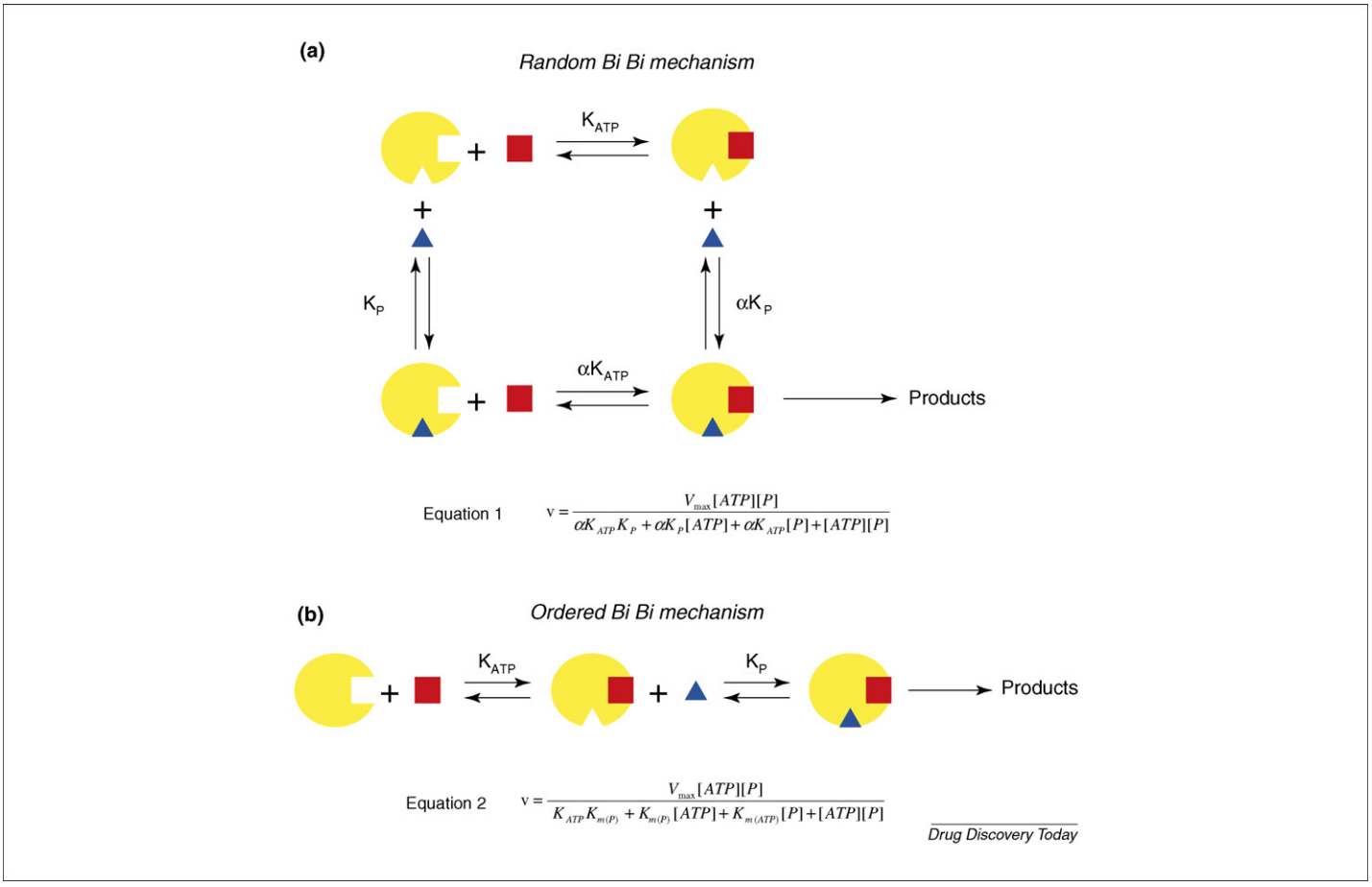

FIGURE 1

Kinetic mechanisms followed by protein kinases. The enzyme, ATP and protein substrate are represented in yellow, red and blue, respectively. **(a)** Random Bi Bi mechanism. K_{ATP} and K_P correspond to the dissociation constants for ATP and the protein/peptide substrate, respectively. For the significance of α, see the text. The rate equation (1) is used when it is assumed that all the association (substrates) and dissociation (products) steps are very rapid compared to the catalytic step. **(b)** Ordered Bi Bi mechanism with ATP binding to the free enzyme. K_{ATP}, $K_{m(ATP)}$ and $K_{m(P)}$ correspond to ATP dissociation constant and Michaelis constant for ATP and P, respectively. Eqn 2 is the rate equation for this mechanism (steady-sate assumption).

substrate concentrations enables different types of inhibitors to be identified more easily using the same assay conditions. The elucidation of the kinetic mechanism may also help in the design of binding assays that permit the direct interaction between the inhibitor and its targeted enzyme to be measured. For example, for an enzyme following an ordered Bi Bi mechanism where ATP interacts first with E, the binding of competitive inhibitors of P to the free enzyme may not occur in the absence of ATP (e.g. [12]). Determination of the kinetic mechanism also allows more detailed analysis of inhibitor properties. Figure 2 represents the inhibition of an enzyme following a random Bi Bi mechanism by an ATP-competitive inhibitor (I). Rearranging the rate equation (3) (for a fixed [P]) and expressing K_I as an apparent K_I (K_I^{app}) leads to Eqn 4 (Fig. 2), which shows that, unless β = 1, (K_I^{app}) depends on [P]. Very few reports describe β values in the literature. Nevertheless, Gao *et al.* have measured β = 49 when ADP was tested as inhibitor of PDK1 [13] showing that β values significantly different from 1 can be measured. For enzymes such as PDK1, therefore, the binding of ATP-competitive inhibitors might be significantly affected by the protein/peptide substrate. Several cavities exist at the ATP-binding site of protein kinases, and often compounds with different binding modes do not occupy the same cavities [14]. Little is known about the effect of protein/peptide substrates on these cavities but one can imagine that they may affect the geometry of some of them. Consequently, the determination of the β values may help to establish whether inhibitors with different binding modes are affected in the same way by protein/peptide substrates.

In recent years, significant effort in medicinal chemistry has been directed toward the discovery of small-molecule inhibitors that target multiple conformations of protein kinases. To assist the identification of protein kinase inhibitors in this effort, it is important to design biochemical assays that take into account the biological properties of these enzymes and their complexes with substrates and other cofactors. Biochemical assays using recombinant proteins cannot recapitulate the complexity of cellular systems, but using different experimental conditions it is possible to enlarge the diversity of the protein conformations so that compounds with different modes of action can be identified. The next section describes some of the parameters that can help in achieving this kind of conformational diversity.

Biological properties of protein kinases and applications to drug discovery

The activity of protein kinases in biochemical assays can be modulated by several parameters. In this section we shall focus on three of them: the protein; the protein/peptide substrate and

TABLE 1

Kinetic mechanisms found in protein kinases

Protein kinase	Proposed kinetic mechanism	Refs
AKT-1, AKT-2, AKT-3	Ordered – 1: ATP; 4: ADP	[25]
Aurora-A	Random	[15]
CAMKII	Random	[26]
CAMKII	Ordered – 1: ATP; 4: ADP	[27]
cAMP-independent kinase	Random	[28]
CDK2/CycE	Random	[9]
CDK4/CycD1	Ordered – 1: ATP; 4: PP	[29]
CDK5/p25	Random	[9]
CSK	Random	[30]
EGF-R	Ordered – 1: P; 4: ADP	[31]
	Random	[32]
ERK2	Random	[33]
	Random	[34]
	Random	[35]
IKK	Random	[36]
	Random	[37]
	Random	[38]
IKK-i	Random	[38]
INS-R	Random	[39]
	Random	[40]
JNK2α2	Random	[41]
MAPKAPK2	Ordered – 1: ATP; 4: ADP	[12]
MEK	Random	[42]
MLCK	Random	[43]
p38 MAP kinase α	Ordered – 1: P; 4: PP	[44]
	Ordered – 1: ATP; 4: ADP	[45]
	Random	[8]
PAK2	Random	[46]
PDK1	Random	[13]
PHK	Random	[47]
PKA	Random	[48]
	Random	[49]
	Ordered – 1: ATP; 4: ADP	[50]
	Random	[51]
PKC θ	Ordered – 1: ATP; 4: ADP	[52]
PKD1, PKD2, PKD3	Random	[53]
ROCK I	Random	[54]
ROCK II	Random	[55]
S6KK1	Ordered – 1: ATP; 4: ADP	[56]
SKY1p	Random	[57]
c-SRC	Random	[58]
v-SRC	Ordered – 1: ATP; 4: ADP	[59]
T2CDPK	Ordered – 1: ATP; 4: ADP	[27]
TBK-1	Random	[38]
TRKA	Ordered – 1: ATP; 4: ADP	[60]
VEGF-R2	Ordered – 1: ATP; 4: ADP	[61]

the metal-ion cofactor. By combining them it is possible to create various experimental conditions where the target proteins take on different conformations, enabling inhibitors with different modes of action to be identified (Fig. 3).

The protein

Although the activity of a protein kinase is regulated in many different ways, it is important in the design of biochemical assays to closely mimic the physiological conditions. In the following text, some examples will be presented to show that choosing the right protein construct and/or including the right regulating factors can have dramatic effects on the output of an assay.

The phosphorylation of the serine/threonine-protein kinase Aurora-A leads to a conformational change, triggering its activation. Furthermore, its activity is further enhanced upon its association with the microtubule-associated protein TPX2. It has been shown that the potency of some ATP-competitive inhibitors of Aurora-A is affected by the presence of TPX2 [15], which does not directly interact with the ATP-binding site. This suggests that the binding of the inhibitors to the enzyme is altered, because TPX2 affects the accessibility and/or size of a binding pocket. Therefore, screening for Aurora-A inhibitors should be carried out with the Aurora-A–TPX2 complexes and not with Aurora-A alone. These findings, which could be extended to other protein kinases, show the importance of including the appropriate regulating cofactors in assays.

At its N-terminus the AKT protein possesses a pleckstrin homology domain linked to the kinase domain by a hinge region. Barnett *et al*. conducted a screen against a compound library and identified inhibitors that inhibit full-length AKT but not its isolated kinase domain [16]. Inhibition studies show that they are mixed inhibitors of ATP and peptide substrate, and it is proposed that they bind to a site that is created only when the pleckstrin homology domain is present. This highlights the importance of using full-length proteins in biochemical assays because they can adopt a larger number of conformations and have potentially more binding pockets at their surface. In addition, it has been shown that regions outside the kinase domain are also important for stabilizing specific conformations of the kinase domain. For example, the juxtamembrane domain of different receptor tyrosine kinases plays an autoinhibition part and helps to maintain these enzymes in an inactive conformation [17].

Various protein kinases are active as oligomers suggesting that their kinetic parameters are affected by their oligomerization status. This is the case, for example, for c-MET. Hays *et al*. have shown that the cytoplasmic domain of c-MET rendered dimeric by the presence of a fragment of the coiled coil motif from the nucleolar pore complex has kinetic properties that are different from those of the corresponding monomeric protein [18]. A screen carried out with dimeric c-MET might then lead to the identification of compounds that would not be identified in a screen with monomeric c-MET because the dimeric protein may take different conformations. This strategy could also be extended to other oligomeric protein kinases.

Many protein kinases are not phosphorylated in their inactive state, but are phosphorylated in their active state. For some of these enzymes, it has been shown that the activation process is a sequential and ordered mechanism. The comparison of the structure of active and inactive protein kinases shows that the activation of some of these proteins leads to major conformational changes. Furthermore, protein kinases in one activation state can adopt different conformations. Altogether, this means that these enzymes progress from their inactive to their active state by

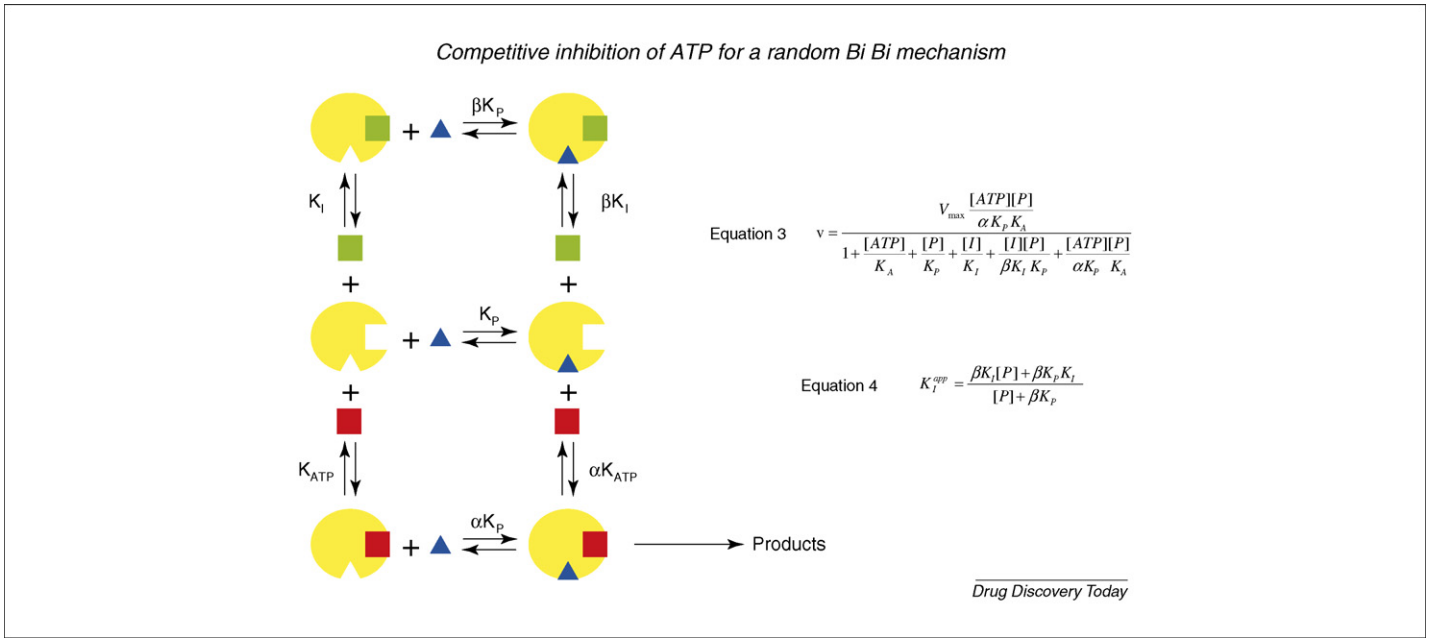

FIGURE 2

Competitive inhibition for a random Bi Bi mechanism. The enzyme, inhibitor, ATP and protein substrate are represented in yellow, green, red and blue, respectively. K_{ATP} and K_P are the dissociation constants for ATP and the protein substrate, respectively. K_I is the inhibition constant of the inhibitor I. For the significance of α, see the text. β is similar to α and represents the degree of interaction between P and I. Eqn 3 is the rate equation and Eqn 4 is the expression of the apparent inhibition constant (K_I^{app}).

multiple phosphorylations and that they can adopt different conformations in each activation state. This offers the unique possibility of targeting different conformations of the same enzyme [5]. To this end, biochemical assays can be set up with recombinant proteins that are in a specific activation state. Kinetic measurements with protein kinases in their active conformation (phosphorylated form) are rather straightforward, but this is not the case when they are in their inactive state (nonphosphorylated form). This is because of the nonlinearity of the reaction (presence of a lag phase at the early time points of the reaction) [19] and because of the complexity of the autophosphorylation process. Binding assays, which measure the direct interaction between a ligand and a protein, might then be more suited to evaluate the affinity of compounds for protein kinases in their inactive form. For example, Smith and Windsor have studied the binding of non-ATP-competitive inhibitors to phosphorylated (active)/nonphosphorylated (inactive) MEK1 by isothermal calorimetry and temperature-dependent circular dichroism in the presence and absence of nucleotide [20]. Their study illustrates perfectly how biophysical methods can be used as a complement to enzymatic assays for the characterization of the protein-inhibitor interaction. Many different biophysical methods – surface plasmon resonance-based analysis, isothermal titration calorimetry, differential scanning calorimetry, nuclear magnetic resonance, fluorescence-based thermal shift assays, among others – are available today and can be used to study the enzyme–inhibitor interaction.

It should be noted that knowing the activation status of protein kinases is very important when evaluating the specificity of protein kinase inhibitors. For example, an inhibitor designed to bind to the nonphosphorylated form of a protein kinase may not inhibit phosphorylated protein kinases and therefore may look specific. The 'specificity' of inhibitors determined with large panels of protein kinases might then be misleading if the activation state of each of these proteins is not precisely known and controlled.

The protein/peptide substrate

In most cases, peptides are used during drug discovery activities because they are easier to produce in larger amounts than the natural protein substrates. It has been shown that the K_m values for peptides are often higher than those for protein substrates and for some protein kinases; this difference is linked to a change in the rate of phosphoryl transfer at the active site of the enzyme [21]. Larger substrates may also do additional contacts with the enzyme. In cells the presence of these additional interactions increases the overall affinity of the physiological substrate, enhancing its effective concentration [22]. It is also known that the specificity of protein kinases can be modulated by regions that are outside the active site, as for example with cyclin-dependent protein kinases [23]. Altogether, this indicates that data generated with short synthetic peptides do not recapitulate the interaction between a protein kinase and its physiological substrate. Screens carried out with protein substrates may enable new inhibitor types to be identified, because the additional interactions formed between the protein substrate and the enzyme may create new structural features.

The metal-ion cofactor

The metal-ion cofactor is another element affecting the catalytic activity of protein kinases. Manganese (Mn^{2+}) and magnesium (Mg^{2+}) are used as cofactors in biochemical assays to stimulate the activity of protein kinases. However, Mn^{2+} exists only at trace levels in mammalian cells while Mg^{2+} is present at millimolar levels. It is therefore probably that protein kinases use only Mg^{2+} as

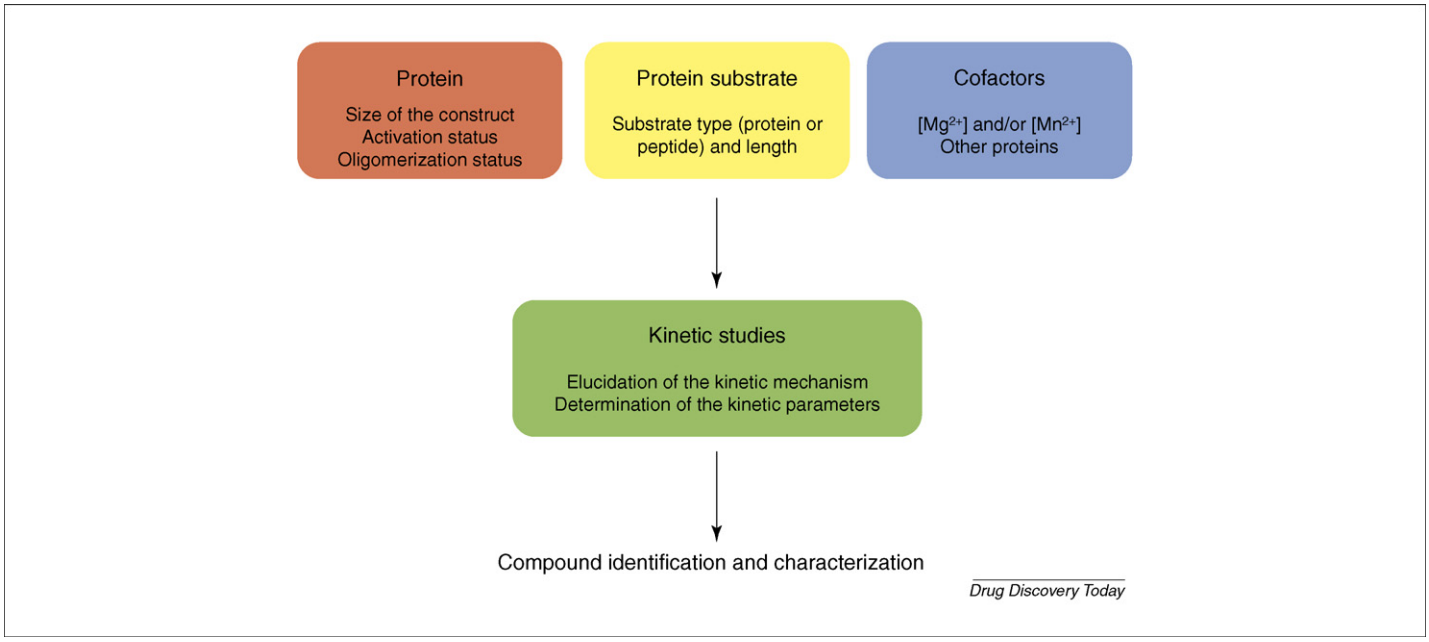

FIGURE 3

Important parameters for the design of protein kinase assays. Several parameters have to be considered when designing protein kinase assays. *Protein*: the size, the activation status (phosphorylated versus nonphosphorylated) and the oligomerization (when applicable) of the protein have to be considered. The stability and/or purification of the studied enzyme may prevent the use of some enzyme forms. Also important is the use of highly purified protein preparations (e.g. no contaminating protein kinase and/or phosphatases activities). *Substrate*: the protein substrate can be a protein or a synthetic peptide. Protein substrates are either the full-length natural substrates of the targeted protein kinase or part of it. Synthetic peptides can mimic short stretches of the natural substrate, peptides not related to the physiological substrate or random polymeric peptides. The former possibility is preferred but in some cases short peptidic fragments are poor substrates rendering assay development difficult. It is important to keep in mind that kinetic studies might be complicated if more than one phosphorylation site are present on the substrate. *Cofactors*: the optimal concentrations in Mg^{2+} and Mn^{2+} have to be determined for each enzyme and additional protein cofactor proteins (or short peptides) that help modulating and/or stabilizing the studied kinase can also be included in assays. *Kinetic studies*: the purpose of the kinetic studies is to determine the kinetic mechanism of the targeted enzyme and its kinetic parameters. These studies are carried out choosing the most appropriate protein construct, substrate and cofactors. The determination of the kinetic parameters permits to define which ATP and protein substrate concentration to use when testing compounds.

a cofactor in cells. In solution with ATP, Mg^{2+} forms the [MgATP] complex which binds to protein kinases. Biochemical studies have shown that an excess of free Mg^{2+} (Mg_{free}^{2+}) dose dependently affects the catalytic activity of protein kinases revealing that protein kinases possess two metal-binding sites [24]. The effect of the second metal ion on catalysis is variable and depends on the protein kinase [24]. Because the kinetic properties of protein kinases depend on [Mg^{2+}] and/or [Mn^{2+}], it is important to determine the effect of these two cations when designing biochemical assays. It might also be interesting to determine whether the properties of kinase inhibitors are also affected by $[Mg^{2+}]_{free}$.

Outlook and conclusions

In recent years, researchers working in the protein kinase field have made great progress in understanding the properties of these enzymes. These advances have shown that potential binding pockets (outside the active site) are present at their surface when they adopt specific conformations and compounds binding to some of these pockets have been identified. These initial breakthroughs show that it is possible to obtain inhibitors that do not interact with protein kinases in the same way as classical ATP-competitive inhibitors. This offers the possibility of developing protein kinase drugs with different pharmacological properties. For example, protein kinase inhibitors that bind to an allosteric pocket might be more specific than compounds that bind at the ATP-binding site. To explore these opportunities, it is important to utilize assays that allow the different conformations adopted by the target protein to be probed. Biochemical assays can be utilized for this purpose. However, the identification and profiling of conformation-specific inhibitors calls for a careful design of the biochemical assays. These assays were previously carried out with isolated kinase domains and synthetic peptides as substrates to identify classical ATP-competitive inhibitors and, in some cases, inhibitors with other modes of action. To identify inhibitors with new binding modes, these assays have to be modified in such a way that knowledge from the *in vivo* regulation of protein kinases can be helpful for the design of the biochemical assay. The protein status (length of the construct, phosphorylation levels, etc.), the nature of the substrate (synthetic peptide versus protein) and the presence of cofactors (additional proteins, etc.) are key parameters influencing the output of an assay and consequently the type of inhibitors identified. For example, an assay conducted with the phosphorylated form of a protein kinase will probably be unable to help in the identification of compounds that bind to the nonphosphorylated form of the same enzyme. Most probably, it will be necessary to develop more than one assay to target different conformational states of the same protein. Furthermore, the complete characterization of inhibitors may require the use of biophysical methods, especially if the compounds bind to a catalytically inactive conformation of the enzyme.

Acquired drug-resistant mutations have been frequently found in cancer patients in the clinic treatment with protein kinase drugs. Biochemical assays, together with structural biology studies, can be used to study the effects of mutations and to help in the synthesis of new drugs to overcome the drug-resistant mutations. It can be hypothesized that regions outside enzyme active sites are more prone to mutations because they contain fewer (if any) residues essential for their catalytic activity. Therefore, mutations inducing resistance might be more frequently found with inhibitors that bind to pockets outside the active site making in the future biochemical assays even more important in understanding how mutations affect drug binding.

In summary, the protein kinase field has made great progress with the result that many opportunities exist today for developing inhibitors with entirely new modes of action. Despite their apparent simplicity, biochemical assays will remain at the core of drug discovery activities to explore these new opportunities.

References

1 Hopkins, A.L. *et al.* (2002) The druggable genome. *Nat. Rev. Drug Discov.* 1, 727–730
2 Dancey, J. *et al.* (2003) Issues and progress with protein kinases inhibitors for cancer treatment. *Nat. Rev. Drug Discov.* 2, 296–313
3 Adams, J.A. (2001) Kinetic and catalytic mechanisms of protein kinases. *Chem. Rev.* 101, 2271–2290
4 Huse, M. *et al.* (2002) Conformational plasticity of protein kinases. *Cell* 109, 275–282
5 Liao, J.J. *et al.* (2007) Targeting protein multiple conformations: a structure-based strategy for kinase drug design. *Curr. Top. Med. Chem.* 7, 1394–1407
6 Schindler, T. *et al.* (2000) Structural mechanism for STI-571 inhibition of Abelson tyrosine kinase. *Science* 289, 1938–1942
7 Fromm, H.J. (1979) Summary of kinetic reaction mechanisms. *Methods Enzymol.* 63, 42–53
8 Szafranska, A.E. *et al.* (2005) Kinetic mechanism for p38 MAP kinase α. *FEBS J.* 272, 4631–4645
9 Clare, P.M. *et al.* (2001) The cyclin-dependent kinases cdk2 and cdk5 act by a random, anticooperative kinetic mechanism. *J. Biol. Chem.* 276, 48292–48299
10 Segel, I.H., ed. (1975) *Enzyme Kinetics*, Wiley
11 Copeland, R.A., ed. (2005) *Evaluation of Enzyme Inhibitors in Drug Discovery*, Wiley
12 Schindler, J.F. *et al.* (2002) Examination of the kinetic mechanism of mitogen-activated protein kinase activated protein kinase-2. *Biochim. Biophys. Acta* 1598, 88–97
13 Gao, X. *et al.* (2006) Steady-state kinetic mechanism of PDK1. *J. Biol. Chem.* 281, 21670–21681
14 Liao, J.J.L. (2007) Molecular recognition of protein kinase binding pockets for design of potent and selective kinase inhibitors. *J. Med. Chem.* 50, 410–424
15 Anderson, K. *et al.* (2007) Binding of TPX2 to aurora A alters substrate and inhibitor interactions. *Biochemistry* 46, 10287–10295
16 Barnett, S.F. *et al.* (2005) Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem. J.* 385, 399–408
17 Hubbard, S.R. (2004) Juxtamembrane autoinhibition in receptor tyrosine kinases. *Nat. Rev. Mol. Cell. Biol.* 5, 464–470
18 Hays, J.L. *et al.* (2003) Oligomerization-induced modulation of TPR-MET tyrosine kinase activity. *J. Biol. Chem.* 278, 27456–27463
19 Visco, C. *et al.* (2000) Activation of ZAP-70 tyrosine kinase due to a structural rearrangement induced by tyrosine phosphorylation and/or ITAM binding. *Biochemistry* 39, 2784–2791
20 Smith, C.K. *et al.* (2007) Thermodynamics of nucleotide and non-ATP-competitive inhibitor binding to MEK1 by circular dichroism and isothermal titration calorimetry. *Biochemistry* 46, 1358–1367
21 Lieser, S.A. *et al.* (2005) Coupling phosphoryl transfer and substrate interactions in protein kinases. *Biochim. Biophys. Acta* 1754, 191–199
22 Ubersax, J.A. *et al.* (2007) Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell. Biol.* 8, 530–541
23 Loog, M. *et al.* (2005) Cyclin specificity in phosphorylation of cyclin-dependent kinase substrates. *Nature* 434, 104–108
24 Sun, G. *et al.* (1997) Requirement for an additional divalent metal cation to activate protein tyrosine kinases. *Biochemistry* 36, 2139–2146
25 Zhang, X. *et al.* (2006) Kinetic mechanism of AKT/PKB enzyme family. *J. Biol. Chem.* 281, 13949–13956
26 Katoh, T. *et al.* (1991) Calmodulin protein kinase II. Kinetic studies on the interaction with substrates and calmodulin. *Biochim. Biophys. Acta* 1091, 205–212
27 Kwiatkowski, A.P. *et al.* (1990) Kinetic mechanism of type II calmodulin dependent protein kinase: studies of the forward and reverse reactions and observations of apparent rapid-equilibrium ordered mechanism. *Biochemistry* 29, 153–159
28 Gold, M.H. *et al.* (1974) Neurospora crassa protein kinase. *J. Biol. Chem.* 249, 2417–2423
29 Konstantinidis, A.K. *et al.* (1998) Purification, characterization, and kinetic mechanism of cyclin D1.CDK4, a major target for cell cycle regulation. *J. Biol. Chem.* 273, 26506–26515
30 Cole, P.A. *et al.* (1994) Evaluation of the catalytic mechanism of recombinant human Csk (C-terminal Src kinase) using nucleotide analogs and viscosity effects. *J. Biol. Chem.* 269, 30880–30887
31 Erneux, C. *et al.* (1983) The kinetics of tyrosine phosphorylation by purified epidermal growth factor receptor kinase of A-431-cells. *J. Biol. Chem.* 258, 4137–4142
32 Posner, I. *et al.* (1992) Kinetic model of the epidermal growth factor (EGF) receptor tyrosine kinase and a possible mechanism of its activation by EGF. *J. Biol. Chem.* 267, 20638–20647
33 Waas, W.F. *et al.* (2002) Transient protein–protein interactions and a random-ordered kinetic mechanism for the phosphorylation of a transcription factor by extracellular-regulated protein kinase 2. *J. Biol. Chem.* 277, 12532–12540
34 Wang, Z.X. *et al.* (2007) The complete pathway for ERK2-catalyzed reaction. *J. Biol. Chem.* 282, 27678–27684
35 Prowse, C.N. *et al.* (2000) Catalytic reaction pathway for the mitogen-activated protein kinase ERK2. *Biochemistry* 39, 6258–6266
36 Burke, J.R. *et al.* (1998) The multisubunit IκB kinase complex shows random sequential kinetics and is activated by the C-terminal domain of IκBα. *J. Biol. Chem.* 1998, 12041–12046
37 Peet, G.W. *et al.* (1999) IκB kinases α and β show a random sequential kinetic mechanism and are inhibited by staurosporine and quercitin. *J. Biol. Chem.* 274, 32655–32661
38 Huynh, Q.K. *et al.* (2002) Kinetic mechanisms of IkB-related kinases (IKK) inducible IKK and TBK-1 differ from IKK-1/IKK-2 heterodimer. *J. Biol. Chem.* 277, 12550–12558
39 Walker, D.H. *et al.* (1987) Substrate specificity and kinetic mechanism of human placental insulin receptor/kinase. *Biochemistry* 26, 1428–1433
40 Ablooglu, A. *et al.* (2001) Activation of the insulin receptor's kinase domain changes the rate-determining step of substrate phosphorylation. *Biochemistry* 40, 504–513
41 Niu, L. *et al.* (2007) Kinetic characterization of the human JNK2α2 reaction mechanism using substrate competitive inhibitors. *Biochemistry* 46, 4775–4784
42 Horiuchi, K.Y. *et al.* (1998) Competitive inhibition of MAP kinase activation by a peptide representing the αc helix of ERK. *Biochemistry* 37, 8879–8885
43 Geuss, U. *et al.* (1985) Steady-state kinetics of skeletal muscle myosin light chain kinase indicate a strong down regulation by products. *Eur. J. Biochem.* 153, 327–334
44 LoGrasso, P.V. *et al.* (1997) Kinetic mechanism of p38 MAP kinase. *Biochemistry* 36, 10422–10427
45 Chen, G. *et al.* (2000) Kinetic mechanism of the p38-α MAP kinase: phosphoryl transfer to synthetic peptides. *Biochemistry* 39, 2079–2087
46 Wu, H. *et al.* (2003) Evaluation of the catalytic mechanism of the p21-activated protein kinase PAK2. *Biochemistry* 42, 1129–1139
47 Tabatabai, L.B. *et al.* (1978) Kinetic mechanism and specificity of the phosphorylase kinase. *J. Biol. Chem.* 253, 2196–2202
48 Cook, P.F. *et al.* (1982) Adenosine cyclic 3′,5′-monophosphate dependent protein kinase: kinetic mechanism for the bovine skeletal muscle catalytic subunit. *Biochemistry* 21, 5794–5799
49 Kong, C.T. *et al.* (1988) Isotope partitioning in adenosine 3′,5′-monophosphate dependent protein kinase reaction indicates a steady-state random kinetic mechanism. *Biochemistry* 27, 4795–4799
50 Whitehouse, S. *et al.* (1983) Studies on the kinetic mechanism of the catalytical subunit of the cAMP-dependent protein kinase. *J. Biol. Chem.* 258, 3693–3701

51 Adams, J.A. *et al.* (1992) Energetic limits of phosphotransfer in the catalytic subunit of cAMP-dependent protein kinase as measured by viscosity experiments. *Biochemistry* 31, 8516–8522
52 Czerwinski, R. *et al.* (2005) Characterization of protein kinase C θ activation loop autophosphorylation and the kinase domain catalytic mechanism. *Biochemistry* 44, 9563–9573
53 Huynh, Q.K. *et al.* (2006) Protein kinase D directly phosphorylates histone deacetylase 5 via a random sequential kinetic mechanism. *Arch. Biochem. Biophys.* 450, 141–148
54 Futer, O. *et al.* (2006) Phosphoryl transfer is nit rate-limiting for the ROCK-I catalyzed kinase reaction. *Biochemistry* 45, 7913–7923
55 Trauger, J.W. *et al.* (2002) Kinetic mechanism for human rho-kinase II (ROCK-II). *Biochemistry* 41, 8948–8953
56 Keshwani *et al.* Kinetic mechanism of fully activated S6K1 protein kinase. *J. Biol. Chem.* (in press)
57 Aubol, B.E. *et al.* (2002) Mechanistic insights into Sky1p, a yeast homologue of the mammalian SR protein kinases. *Biochemistry* 41, 10002–10009
58 Boerner, R.J. *et al.* (1995) Kinetic mechanism of the forward and reverse pp60c-src tyrosine kinase reactions. *Biochemistry* 34, 16419–16423
59 Wong, T.W. *et al.* (1984) Kinetics and mechanism of angiotensin phosphorylation by the transforming gene product of Rous sarcoma virus. *J. Biol. Chem.* 259, 3127–3131
60 Angeles, T.S. *et al.* (1998) Kinetics of trkA tyrosine kinase activity and inhibition by K-252a. *Arch. Biochem. Biophys.* 349, 267–274
61 Parast, C.V. *et al.* (1998) Characterization and kinetic mechanism of catalytic domain of a human vascular endothelial growth factor receptor-2 tyrosine kinase (VEGFR2 TK), a key enzyme in angiogenesis. *Biochemistry* 37, 16788–16801