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Enzyme Activity Assays for Protein Kinases: Strategies to Identify Active Substrates

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Abstract

Protein kinases are an important class of enzymes and drug targets. New opportunities to discover medicines for neglected diseases can be leveraged by the extensive kinase tools and knowledge created in targeting human kinases. A valuable tool for kinase drug discovery is an enzyme assay that measures catalytic function. The functional assay can be used to identify inhibitors, estimate affinity, characterize molecular mechanisms of action (MMOAs) and evaluate selectivity. However, establishing an enzyme assay for a new kinases requires identification of a suitable substrate. Identification of a new kinase's endogenous physiologic substrate and function can be extremely costly and time consuming. Fortunately, most kinases are promiscuous and will catalyze the phosphotransfer from ATP to alternative substrates with differing degrees of catalytic efficiency. In this manuscript we review strategies and successes in the identification of alternative substrates for kinases from organisms responsible for many of the neglected tropical diseases (NTDs) towards the goal of informing strategies to identify substrates for new kinases. Approaches for establishing a functional kinase assay include measuring auto-activation and use of generic substrates and peptides. The most commonly used generic substrates are casein, myelin basic protein, and histone. Sequence homology modeling can provide insights into the potential substrates and the requirement for activation. Empirical approaches that can identify substrates include screening of lysates (which may also help identify native substrates) and use of peptide arrays. All of these approaches have been used with a varying degree of success to identify alternative substrates.

Keywords

Alternative substrate; antiparasitic; assay development; neglected tropical disease; orphan kinase; protein kinase

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

INTRODUCTION

Protein kinases (PKs) catalyze the phosphorylation of proteins at serine, threonine and tyrosine residues. This post-translation modification is important to most physiological processes; there are hundreds of different protein kinases that catalyze specific phosphorylation reactions. Kinases are important targets for drug discovery with now over 30 medicines approved that inhibit protein kinases, primarily for cancer [1]. There is interest to exploit the success of targeting kinases for non-infectious diseases [2–5], as well as neglected tropical diseases (NTDs). The druggability of the kinomes of *Plasmodia* [6, 7], Kinetoplastids [8], and bacteria [9] have been reviewed elsewhere, and there are opportunities for kinases as drug targets in other protozoa [10], helminths [11] and ectoparasites [12, 13].

PKs are a large and pervasive group of enzymes. Over 1 % of all genes encode for kinases, and they can be grouped into families and subfamilies by their deduced primary structure. PKs which do not fall into these major groups are called “other protein kinases” (OPK) [14]. OPKs can have orthologues and consequent predictive behavior based on homology to known kinases, or they can have no known orthologs (often called ‘orphan protein kinases’).

Many essential kinases have been identified in infectious organisms with genetic techniques (e.g. RNAi knockdown), of which some have been pursued as drug targets. For example, the sequenced genomes of three human-infective trypanosomatid protozoa, *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*, have allowed the kinome for each parasite to be defined as 179, 156 and 171 eukaryotic protein kinases respectively [15]. In *T. brucei*, the causative agent of human African trypanosomiasis (also known as African sleeping sickness), RNAi screens have identified over 100 of these kinases to be essential [16, 17], of which only a few have reported activity assays and screening for inhibitors. Deduced from the genome, *T. brucei* has over 30 orphan kinases displaying no homology to known kinases [16, 17]. The malarial parasite, *Plasmodium falciparum*, has over 80 kinases with many members lacking clear orthologues in the human kinome and many having been shown to be essential by reverse genetic techniques [18]. Further, many kinases from *P. falciparum* exhibit a high degree of structural divergence from their host counterparts. A number of *Plasmodium* kinases have recently been shown by reverse genetics to be essential for various parts of the complex parasitic life cycle, and are thus genetically validated as potential targets [6, 19, 20]. There are over 70 members in the *Cryptosporidium parvum* kinome of which only a few have been investigated; 35 % of these are classified as other protein kinases, and 25 % of these have no known orthologues outside of *Cryptosporidium* [21]. The Apicomplexans, including *Plasmodium*, *Toxoplasma* and *Cryptosporidium*, have two groups of kinases, the rhoptry kinases and FIKK kinases, unique to the clade [21]. Kinome of *Entamoeba histolytica* is predicted to have over 300 members, 112 of which are unclassified and 38 of which are classified as ‘other’ protein kinases [22].

A valuable tool for targeting kinases for drug discovery are enzyme assays. These can complement other approaches such as binding assays and cellular assays to provide new knowledge. They can be used for screening of compounds to identify new inhibitors and for evaluating specificity. Methodologies to determine physiological substrates have been

developed, but can be difficult and expensive [23–25]. Developing assays for parasite kinases can identify new selective inhibitors may be useful probes to help determine the physiological function of the kinase [23] as well as valuable starting points for drug discovery.

The chemistries for the phosphotransfer reactions catalyzed by protein kinases are similar, magnesium/ATP transfers the γ -phosphate group to an activated hydroxyl group (Ser, Thr or Tyr) on the acceptor protein substrate through an SN2 displacement reaction. Specificity for the reaction is provided by the interactions between the kinase and the protein substrate. The similarity of the catalytic chemistries for all kinases has provided an approach that uses non-specific substrates to mimic the activity of the physiologic substrates in the phosphotransfer reactions. While the use of non-specific substrates provides little information as to the biological function of a kinase, they enable enzyme assays to be developed that identify specific inhibitors of the kinases. Subsequently, the inhibitors can be used as chemical biology tools to help reveal the physiological function of the kinase. In this paper we review the approaches and methods used to identify non-specific substrates with a focus to identify inhibitors of non-human kinases in organisms that cause neglected tropical diseases.

AUTOPHOSPHORYLATION

Autophosphorylation is the kinase-catalyzed phosphotransfer to a Ser, Thr or Tyr residue of the kinase itself. Many kinases require autophosphorylation for full or increased catalytic activity for phosphotransfer to other proteins. Autophosphorylation occurs on a kinase's activation loop, in either a *trans* or *cis* (catalyzing phosphotransfer to another kinase molecule or to itself) fashion [26], and an autophosphorylated kinase may undergo a conformational change which better suits the kinase to accept exogenous substrates [27].

Several serine-threonine kinases have been screened using an autokinase assay. For example, a MAP kinase from *Toxoplasma gondii*, for which endogenous nor exogenous substrates have been identified, was recently shown to be inhibited SB505124, an inhibitor of transforming growth factor β type I receptors ALK4, ALK5 and ALK7 using the autokinase assay. Immunoprecipitated MAPK1 from *T. gondii* lysates was treated with SB505124. The inhibition of ^{32}P incorporation into TgMAPK1 in lysates (from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$) by SB505124 revealed an apparent IC_{50} of 125 nM [28].

Another example of autokinase assay is with the C2-domain-containing protein kinase (C2PK) immunoprecipitated from *E. histolytica*. Activity was measured *via* radiolabeled phosphotransfer ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$ [2.5 μM] for 1 h) to *trans*-autophosphorylate at Ser⁴²⁸. Biochemical analysis revealed a V_{max} of 66 nmol/min/mg. Autokinase inhibition assays identified staurosporine as an inhibitor of C2PK from *E. histolytica*, with an IC_{50} of 150 nM [29].

Autophosphorylation assays generally require more enzyme, as the enzyme itself is also its substrate. Consequently, these assays can be costly. Nevertheless, it is important to consider autophosphorylation when developing new assays, as many kinases require autophosphorylation for full catalytic activity with exogenous and endogenous protein

substrates. For instance, calcium-dependent protein kinase (CDPK) 3 from *T. gondii* required preincubation with ATP and CaCl_2 to auto-activate, prior to activity measurements with exogenous substrate Syntide-2 and $[\beta\text{-}^{32}\text{P}]$ ATP. Purfalcamine, a 2,6,9-trisubstituted purine with antiplasmodial activity and inhibitory activity on a CDPK from *T. gondii*, was shown to inhibit CDPK3 ($\text{IC}_{50} = 800 \text{ nM}$, $[\text{CDPK3}] = 100 \text{ nM}$) activity.

Other recent examples of Ser/Thr protein kinases in which autophosphorylation assays are where used to test inhibitors include PK7 from *P. falciparum* in testing pyrazolopyrimidine compounds [30].

Putative tyrosine kinases from parasites have been shown to exhibit autokinase activity. A Wee1-like kinase from *T. brucei* was recently expressed, but no protein substrate has been identified. Assays with generic protein and peptide substrates were not phosphorylated, however $[\text{P-}^{32}]$ was incorporated in the recombinant protein, and antiphosphotyrosine antibodies indicated that the phosphorylation site was a Tyr residue [31]. Tyrosine-kinase-like kinase from *P. falciparum* [32] and dual-specificity (Ser/Thr and Tyr) casein kinase from *P. falciparum* [33] have been shown to exhibit autophosphorylation, and it is believed that these activities regulate the kinases' activity to other protein substrates.

GENERIC SUBSTRATES

Generic alternative kinase substrates are used to test activity in many instances where endogenous substrates are not known [34]. Isoforms of casein (α and β) were used as substrates as early as 1954 [35]. Mixtures of up to four isoforms of casein have been used as kinase substrates. Dephosphorylated casein also can serve as a kinase substrate, particularly with new kinases which align with casein kinases (see next section for homology strategies in substrate selection). Dephosphorylated casein is commercially available, or it can be prepared by phosphatase treatment of casein prior to incubation [36]. Other successful generic substrates for kinase assays include myelin basic protein (MBP) and isoforms of histone, as well as synthetic peptides like kemptide (sequence =LRRASLG), Syntide-2 (sequence =PLARTLSVAGLPGKK) [34], Crosstide (sequence =GRPTSSFAEG) and CREBtide (sequence =KRREILSRPSYR) [37].

In the next section, we review in more detail kinase family-specific successes in substrate selection for new kinases which align with known kinases. Several kinase families accept generic protein or peptide substrates. For example, there has been success with MBP as a substrate for new kinases which align with mitogen-activated protein kinases (MAPKs). Recent successes include MAPKs from Apicomplexan *T. gondii* [38], Amoebae *E. histolytica* [39], Kinetoplastids of *Leishmania* spp. [40–44], *T. cruzi* [45] and *T. brucei* [Swinney DC, unpublished results], and helminths *B. malayi* [46] and *E. multilocularis* [47, 48]. Ashutosh and coworkers recently reported the specific activity of recombinant MAPK1 from *Leishmania donovani* to be 11.66 nmol ATP consumed/min/mg protein [43]. Likewise, new kinases aligning with kinases upstream in the MAPK signaling pathway from Apicomplexans (*T. annulata*) [49], Kinetoplastids (*T. brucei*) [Swinney DC, unpublished results] and *Leishmania mexicana* [50] and *Schistosoma mansoni* [51] can accept MBP as a protein substrate.

MBP has been used to show phosphotransfer activity in eukaryotic-like protein kinases (EPKs) from various bacteria [52–58]. Kimura and coworkers recently reported the cloning of 14 EPKs from *Myxococcus xanthus*. The selected EPKs had atypical motifs in their catalytic loops but contained all residues necessary for catalytic activity. Seven of the 14 showed activity in phosphorylating MBP, and four of those also showed autophosphorylation activity, with specific activities (200 μ M ATP) ranging 1–13100 and 1–10 pmol/min/mg, respectively [56].

Specificity has been demonstrated with generic substrates. For instance, FIKK kinase (named for its conserved FIKK-motif in the Apicomplexan-specific group of kinases) from *Plasmodium falciparum* showed activity with human histone H1 and *Xenopus laevis* histone H3, but was not active with *X. laevis* histone H2 [59]. Two recently expressed NIMA-related serine/threonine kinases (Nek) from *Giardia lamblia* have been shown to phosphorylate recombinant human histone H1, while only one of the two Neks phosphorylated human histone H3 [60]. There are also examples of Nek accepting MBP instead of histone [61, 62].

Similarly, cdc2-related kinases (CRKs), a group of cyclin-dependent kinases, from protozoan parasites have catalytic activity using histone H1 [63–67]. Histone was incubated at 0.167 – 0.333 mg/mL [64–67]. A second isoform of CRK from *E. tenella* was recently cloned, and the recombinant enzyme was incubated with a fluorescent-tagged histone H1-derived peptide. Engels *et al.* validated EtCRK2 as a drug target using the cyclin-dependent kinase-specific inhibitor flavopiridol, (IC₅₀ of 33 nM and K_i of 11 nM) which was similar to the human isoform (IC₅₀ 36 nM, K_i 19 nM). They also identified four chemically diverse compounds with K_i values in the low micromolar range [68].

Other generic peptide substrates were designed for specific kinase families. Kemptide (sequence = LRRASLG) is a synthetic oligomer derived from porcine liver pyruvate kinase which acts as a productive substrate for cyclic nucleoside mono- phosphate-dependent kinases (cyclic adenosine monophosphate (cAMP) -dependent kinases (PKAs) and cyclic guanosine monophosphate (cGMP) -dependent kinases (PKG)) [69]. Another common peptide substrate for PKAs and PKGs is GRTGRRNSI, derived from PKI, the heat stable inhibitor protein of PKA [70]. New kinases, from protozoan parasites, which align with PKAs or PKGs have shown activity with Kemptide [71–77]. PKGs from apicomplexans were recently reported to have an apparent K_m 28 μ M for Kemptide (*T. gondii* PKG) [71] and 19 μ M for biotinylated GRTGRRNSI (*E. tenella* PKG) [72]. Likewise, Crosstide [37] and Syntide-2 [78] were designed as generic substrates for protein kinase B (PKB) and calcium-dependent protein kinase (CDPK), respectively, and recently shown to be substrates for PKB [79] or CDPK [21, 80–83]. Typical peptide concentrations in kinase assays are about 200–400 μ M for Kemptide [71–75] and 100–150 μ M for Syntide-2 [82, 83]. Recently, four isoforms of CDPK from *C. parvum* were characterized (apparent K_m values for Syntide-2 ranging from 156 μ M to 426 μ M and k_{cat}s ranging from 12 min⁻¹ to 1370 min⁻¹). Syntide-2 (500 μ M) was used for an inhibitor screen [21]. For a recent inhibitor screen of CDPK3 from *T. gondii*, 1 mM Syntide-2 was chosen as a substrate concentration [81]. Biotinylated Syntide-2 was used in enzymatic assays of CDPK1 from *Neospora caninum* to discovery of seven inhibitors with IC₅₀s in the low nanomolar range and one inhibitor with an IC₅₀ of 513 nM [80].

Table 1 summarizes active generic substrates by kinase-type discussed in the text. Table 2 describes some recent successes with generic substrates in new kinases from protozoan parasites.

HOMOLOGY AND KNOWN FUNCTIONAL SUBSTRATES OF HOMOLOGUES

Kinase sequence alignments and homology have been used to map conserved catalytic domains and deduce phylogeny across classes of protein kinases [14, 27]. While all known typical and atypical protein kinases have structural similarities [100], primary sequences of catalytic domains in phylogenetic clusters correlate to the biochemical properties of the protein kinase, including substrate specificity [14, 27]. Feature of substrate specificity have been deduced from these alignments. For example, Li and coworkers have described putative specificity-determining residues and suggested a role for hydrophobicity in substrate specificity [101].

New kinases which align with the casein kinase (CK) family often are catalytically active, as mentioned above, with casein isoforms and dephosphorylated casein [36, 42, 102–112]. In addition CK-specific substrates have been reported. For instance, CK substrate CK-S (sequence = (RRKHAAIG(pS)AYSITA, where pS corresponds to phosphorylated serine) is a substrate for CK2 in *L. donovani*, [102]. Pep1 (sequence = RRKDLHDDEEDEAMSITA) and Pep2 (sequence = RRRADDSDDDDD) are selective substrates for CK1 and CK2, respectively, and not only have been used as substrates for new kinase activity, but have also been used to sub-classify CKs identified from parasites kinetoplastids [104–109, 111–113] and apicomplexans [33, 110, 114]. DDDEESITRR and KRRRAL(pS)VASLPGL [110] have also been used as CK1-specific peptide substrates, and RRREEE TEEE [110], RRREDEESDDEE, eIF2 β -derived peptide MSGDEMIFDPTMSKKKKKKKKP [114] and RRASADDSDDDEDL [115] have also been used as a CK2-specific peptide substrates. Typical Pep1 concentrations for activity assays are 100 – 800 μ M [104–108, 113], while Pep2 concentrations usually range 40 –200 μ M [33, 104–109, 112, 113]. Recombinant CK1.1 from *T. cruzi* had apparent K_m for β -casein of 5.7 mg/mL and for Pep1 of 128 μ M [108]. Recombinant CK1 β from *T. gondii* had an apparent K_m for partially dephosphorylated α -casein of 5 μ M, for β -casein of 12 μ M, for Pep1 of 15 μ M and for phosphopeptide substrate KRRRAL(pS)VASLPGL of 79 μ M [110]. CK2 α from *P. falciparum* has an apparent K_m for Pep2 of 137.5 μ M [114].

Sequence alignment has been successfully employed for functional substrate discovery with homologues of kinases which accept eukaryotic translation initiation factor 2 α and its orthologues as substrates. eIF2 α kinases from Apicomplexa have been shown to phosphorylate generic substrates such as α - and β -casein [85], but also putative eIF2 α isoforms from the parasite itself [85, 116] or from yeast [116, 117]. eIF2 α from Kinetoplastids are phosphorylated at Thr169, as opposed to other eukaryotic eIF2 α at Ser51; recombinant putative eIF2 α s have been successfully used as *in vitro* substrates for their kinases in *T. brucei* [118], *T. cruzi* [119], and *Leishmania infantum* [120].

Homology of glycogen synthase kinase-3 (GSK3), showcased in Ref. [121], has informed assay development in homologues from parasites. GSK3 has several synthetic exogenous

peptides which served as functional substrates, including CREBtide [122], GS-1 (sequence = YRRAAVPPS PSLSRHSSPHQ(pS)EDEEE) [123, 124], GSP-2 (sequence = YRRAAVPPSPSLSRHSSPHQ(pS) EDEEE) [124], and GSM (sequence = RRRPAS VPPSPSLSRHS(pS)HQRR) [125]. GS-1 has been used with recombinant homologues from *P. falciparum* [126] and *L. donovani* [127]. GSM was used as the substrate for the *M. tuberculosis* homologue [128]. In *T. brucei*, homologues of GSK3 β have been assayed with GSP-2 [129–131] and GSM [132]. Biotinylated GSP-2 and untagged GSP-2 had apparent K_m values of 2.4 μ M [129] and 8.4 μ M [131], respectively, and untagged GSM had an apparent K_m of 23 μ M [132].

LYSATES

In the absence of a known peptide or protein substrate, and when generic substrate-strategies fail, lysates of the kinase's organism can be used *in lieu* of substrate (Scheme 1). Cell lysates contain the native endogenous substrate, and have been used in kinase assays *in lieu* of purified active substrate [25]. It is worth noting that other kinases in the lysate must be inactivated. Treatments to inactivate the kinases from lysate include adding kinase inhibitors such as 5'-4-fluorosulphonylbenzoyladenosine [25], proteolytic digestion prior to kinase addition to the reaction [133, 134], and heat or acid denaturation. With some reaction mixtures, it may be necessary to treat tryptic digests with phosphatases to dephosphorylate potential reaction sites from peptides, followed by heat to inactivate the activity of the phosphatase [134]. The use of lysates also provide an opportunity to identify physiological relevant substrates [25]. This can be an added value to justify pursuing this approach.

ROP18, a rhoptry kinase of *T. gondii*, has shown phosphorylation activity to both parasite cultures and human foreskin fibroblasts that had been heated for 30 min at 56 °C in order to inactivate endogenous kinases [135].

PEPTIDE LIBRARIES AND MICROARRAYS

For new kinases where orthologues are not known, in the absence of autokinase activity and activity from generic substrates, libraries of proteins and peptides can be screened for substrate specificity (Scheme 1). Commercial kits of randomly generated protein and peptide libraries, as well as kinase family-specific libraries are available [136]. Further, commercial microarrays, or chips, have been developed for substrate screening of kinases, which contain libraries of proteins or peptides on solid support. These microarrays often have their own assay protocol and detection and analysis methods [137] can be used with kinase specificity results to determine amino acid motifs preferentially accepted by a new kinase [136].

Libraries and library arrays are available in many different types, and often they are clustered by knowledge-based or overall random library sets. The random sets consist of arbitrarily generated peptides based on combinatorial methods, while knowledge-based libraries consist of peptides derived from naturally occurring proteins [136]. Though costly, new methods of peptide synthesis and array technology [136, 138] make these libraries more comprehensive. This method is particularly convenient for orphan kinases which do not have known orthologues.

ROP18 phosphorylates immunity-related p47 GTPases from the mammalian host, but the full function and substrate specificity of ROP18 are not fully understood to date. Lim and coworkers reported the cloning of the catalytic domain of ROP18 and, the use of a positional scanning peptide library array. The investigators used 50 μ M cold ATP and approximately 1 μ Ci [γ - 32 P]-ATP in 8 h reactions and quantified the degree of phosphorylation in each library. They found TgROP18 to have a low peptide sequence selectivity, with a generalized motif of (X)-(X, not E)-(X)-(E)-(H)-(T)-(R/mixed, not P and not negatively charged)-(Ar)-(Ar)-(Ar), where X signifies any amino acid, the phosphoreceptor is underlined, and Ar denotes an aromatic amino acid, and they speculate the broad range of phosphoreceptor sequences suggests other specificity factors may exist to selectively allow TgROP18 to target its physiological substrates [139].

PfPK7, an atypical orphan plasmodial protein kinase, has been reported to autophosphorylate and phosphorylate the generic substrates MBP, histone H2A, and β -casein [84]. Peptide microarrays were used to further explore the substrate specificity of PfPK7 with peptide microarrays [30]. PfPK7 is one of many *P. falciparum* protein kinases which contains extensive insertions, reducing its identity to known kinases. Consequently, PfPK7 has no human orthologue, and is considered an orphan kinase. The two closest human homologues share 33 % and 26 % identity with PfPK7, and PfPK7 lacks activity related to these homologues. Using an arrayed peptide library, the sequence consensus for a phosphoreceptor of PfPK7 was found to be (r-R-R/K-K/R-S/T-P-K/R-K-R). The array-derived peptide specificity included a strong preference for positively charged amino acids lying 2, 3, and 4 residues on both sides of the Ser/Thr to be phosphorylated, in agreement with their structural observation of overall negative character in the active site cleft of PfPK7 [30].

Calcium-dependent kinase 1 (CDPK1) from *T. gondii* is a member of a calcium-dependent signaling pathway that is necessary to the survival of the parasite. This signaling pathway is poorly understood and its kinases are not fully characterized. The substrate specificity of TgCDPK1 was further tested by positional-scanning peptide array. Lourido and coworkers found a strong preference for Ser over Thr, for Arg at the -3 position to the residue of phosphotransfer, and hydrophobic residues in the -5 position. The peptide-array-generated consensus was in agreement with the peptide motifs derived from sulfur-covalent linkages to *T. gondii* proteins in incubations of the lysate with a thio-ATP analog [140].

CRKs typically phosphorylate histone H1 in the presence of cyclins. Walker and coworkers recently reported leishmanial CRK3 was tested with histone H1. A substrate finder assay which contained a library of 61 potential serine/threonine phosphoreceptors was used to find a fluorescently labeled peptide for an inhibitor screen. Of the 61 peptides, 5 were sufficiently phosphorylated by leishmanial CRK3, including two histone H1-derived peptides, and a motif of x-S/T-P-x-R/K was common to all of them. GGGRSPGRRRRK was used for inhibitor screens with the CRK3:CYC6 kinase complex. The assay with the library-derived peptide had a Z' score of 0.71, and potent inhibitors were identified for leishmanial CRK3 from two compound libraries [65].

FIKKs from *P. falciparum* and *C. parvum* were shown to have activity on canonical substrates (*cf.* Table 1), and their substrate specificities were further explored by means of a positional-screening peptide array. Osman and coworkers found a strong preference for basic residues, mainly arginine at positions three residues on either side of the peptide phosphorylation site, and a weaker preference for arginine at the -4 position. Knowing these preferences, an optimized substrate of sequence RRRAPSFYRK and three variants were tested, and the two isoforms had apparent k_{cat} s of about 20 min⁻¹ to 30 min⁻¹ (*P. falciparum*) or 70 min⁻¹ to 120 min⁻¹ (*C. parvum*) and K_m s ranging from 7 μ M to 150 μ M [87].

CONCLUSION

Kinase enzyme assays are a powerful tool to help identify new inhibitors. The inhibitors can be used as tools for chemical biology to learn about the physiological function of the kinases as well as starting points for drug discovery optimization. Enzyme assays measure function which can complement other types of assays such as binding assays. However, the challenge is to identify substrates for new kinases with unknown function. In this manuscript we review different approaches to identify substrates for kinases with respect to developing assays suitable to screen for inhibitors.

There is no consensus way to identify the substrate. Methods including auto-activation and use of generic substrates and peptides are the most straight-forward. However, these may not provide robust activity, and, when that is the case, more significant quantities of protein will be required for the assays and screens. A concern with any of the assays is the potential need for the kinases to be activated. Sequence homology modeling can provide insights into the potential substrates and the requirement for activation. More empirical approaches that can provide substrates include screening lysates (which may also help identify native substrates) and peptide arrays. These later approaches are less biased but also more costly.

Our recommendation is to begin with the simplest approaches (auto-activation and generic substrates) and then work down the flow-chart shown in Scheme 1. This strategy has been used by numerous investigators to identify substrates for new kinases.

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LIST OF ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CRK	Cyclin-dependent kinase-related kinase
eIF2α	Eukaryotic initiation factor 2 α
EPK	Eukaryotic-like protein kinase

MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
Nek	NIMA-related serine/threonine kinase
PKA	cAMP-dependent kinase
PKG	cGMP-dependent kinase

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Scheme 1.

Table 1

Canonical protein and peptide substrates for various types of kinases.

Kinase Type	Kinase Abbreviation	Canonical Substrate
mitogen-activated protein kinase	MAPK	MBP
mitogen-activated protein kinase kinase	MAPKK	MBP
FIKK kinase	FIKK	Histone
NIMA-related kinase	Nek	MBP, Histone
cyclin-dependent kinase-related kinase	CRK	Histone
cAMP-dependent kinase	PKA	Kemptide
cGMP-dependent kinase	PKG	Kemptide
protein kinase B	PKB/AKT	Crosstide
calcium-dependent protien kinase	CDPK	Syntide-2
glycogen synthase kinase	GSK	GSM, GSP-2, GS-1
casein kinase 1	CK1	Casein, CKPep1, see text for others
casein kinase 2	CK2	Casein, CKPep2, see text for others

Table 2

Recent applications of generic substrates for assays of new protein kinases from parasitic protozoa.

Organism	Kinase Assayed ^a	Successful Common Substrates ^{b,c}	Year	Reference
<i>Plasmodium falciparum</i>	CDPK4	MBP, Syntide-2	2009	[82]
<i>Plasmodium falciparum</i>	PK7	MBP	2005	[84]
<i>Plasmodium falciparum</i>	eIK1	α -cas., β -cas.	2009	[85]
<i>Plasmodium falciparum</i>	FIKK4.2	MBP	2014	[86]
<i>Plasmodium falciparum</i>	FIKK8	cas., MBP, Syntide-2	2015	[87]
<i>Plasmodium falciparum</i>	SRPK1	RSep	2010	[88]
<i>Plasmodium falciparum</i>	CLK-1, CLK-2	α/β cas., his. H1, MBP	2011	[89]
<i>Plasmodium falciparum</i>	CLK-1, CLK-2, CLK-3, CLK-4	α/β cas., his. H1, MBP	2014	[90]
<i>Plasmodium falciparum</i>	PKB	Crosstide, his. IIAS	2006	[79]
<i>Plasmodium falciparum</i>	PKG	GRTGRRNSI, Kemptide, MCPD-4 ^d , Glasstide	2006	[74]
<i>Plasmodium falciparum</i>	TKL2	α -cas., β -cas., his. H1, MBP	2013	[32]
<i>Plasmodium falciparum</i>	TKL3	α -cas., β -cas., his. H1, MBP	2010	[91]
<i>Plasmodium falciparum</i>	CDPK1	β -cas., dephosphorylated cas., his. H1, his. H2, his. H3	2009	[92]
<i>Toxoplasma gondii</i>	CDPKif3	his. IIAS	2009	[93]
<i>Toxoplasma gondii</i>	FIKK	cas., MBP, Syntide-2	2015	[87]
<i>Giardia lamblia</i>	Nek1, Nek2	BSA, his. H1, his. H3	2012	[60]
<i>Giardia duodenalis</i>	PKC	his. H1-IIIs	2007	[94]
<i>Leishmania major</i>	MPK7	MBP	2014	[95]
<i>Leishmania mexicana</i>	PK4	MBP	2005	[96]
<i>Leishmania</i> spp.	MPK10	dephosphorylated cas., his. H1, MBP, Ets1	2014	[42]
<i>Trypanosoma brucei</i>	TLK	cas.,	unpublished results	
<i>Trypanosoma brucei</i>	AUK1	MBP	2013	[97]
<i>Trypanosoma brucei</i>	PK50, PK53	α -cas., β -cas., his. H1, MBP	2010	[98]
<i>Entamoeba histolytica</i>	AK1	actin, his. IIIs	2014	[99]

^aKinase abbreviations are taken from respective references

^bBSA, bovine serum albumin; cas., casein; his., histone; MBP, myelin basic protein; RSep, Arginine-Serine repeat peptide (sequence = RSPSYGRSRSRSRSRSRSRSNSRSRSY)

^ccasein and histone nomenclature are taken from respective references

^dMCPD-4 sequence = GKRRKRSRKES