REVIEW

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Using Chemical Genetics and ATP Analogues To Dissect Protein Kinase Function

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ABSTRACT Protein kinases catalyze the transfer of the γ-phosphate of ATP to a protein substrate and thereby profoundly alter the properties of the phosphorylated protein. The identification of the substrates of protein kinases has proven to be a very difficult task because of the multitude of structurally related protein kinases present in cells, their apparent redundancy of function, and the lack of absolute specificity of small-molecule inhibitors. Here, we review approaches that utilize chemical genetics to determine the functions and substrates of protein kinases, focusing on the design of ATP analogues and protein kinase binding site mutants.

One of the major challenges in postgenomic biology is to characterize the proteome in terms of protein–protein interactions and post-translational modifications. Protein phosphorylation controls processes as diverse as cell division, metabolism, and differentiation. It is perhaps the most universal regulatory modification used by organisms. More than 30% of all eukaryotic proteins are phosphorylated, with 99% of those modifications occurring on serine or threonine residues (*1*). The importance of such modifications is underscored by the study of disease states that often exhibit aberrant expression of protein kinases and phosphatases responsible for regulating the levels of protein phosphorylation.

To understand phosphorylation-mediated regulation of the proteome, it is essential to determine the substrates of each individual protein kinase and hence to define how signaling pathways operate. However, given the abundance of phosphoproteins and protein kinases (the human genome encodes >500 distinct protein kinases (*2*)), the identification of substrates for a particular protein kinase is a daunting undertaking. Here, we review recent progress in the application of chemical biology approaches to resolve this problem.

Limitations of Current Methods for Substrate Identification. The classical methodology for identifying the involvement of protein phosphorylation in a pathway was to isolate the candidate substrate through biochemical fractionation of animal tissue stimulated to undergo the process being studied. Ideally, the tissue had been preloaded with radiolabeled phosphate to ease subsequent analysis. Purified proteins would then be analyzed by Edman degradation to identify both the protein and the site(s) of phosphorylation by determining the cycle of release of the radiolabel. Many modifications to this basic protocol have been developed over

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the years, but it remains a difficult, complex, and time-consuming process that requires optimization for every individual protein and considerable quantities of tissue in order to yield sufficient protein for analysis.

One very useful modification to this approach was through the utilization of phosphospecific antibodies. These phosphospecific antibodies allow rapid and specific purification of modified proteins and have been particularly useful in the context of phosphotyrosine modification of proteins, as reviewed by Wang (*3*). However, the use of phosphoserine- and phosphothreonine-specific antibodies has had very limited success because of poor signal-to-noise ratios, and they are not widely employed.

Many recent analyses of protein phosphorylation have utilized the ever-expanding range of small-molecule inhibitors that target particular protein kinases (*4, 5*). These are widely available and are simple and rapid to use both *in vivo* and *in vitro*. By the addition of a specific inhibitor to a cell (or cell lysate), the involvement of a particular protein kinase in a given process can be rapidly determined. One major drawback to this approach in general is that the majority of these inhibitors target the ATP binding site of the kinase. This site is highly conserved between different protein kinases, and consequently, small-molecule inhibitors often lack absolute specificity (*6–8*); this makes it difficult to dissect the function of a single enzyme. For example, paullone inhibitors were initially described as specifically targeting cyclin-dependent kinase 1 (cdk1), resulting in cell-cycle arrest and antitumor activity (*9*). However, subsequent work with these inhibitors showed that they also inhibited glycogen synthase kinase (GSK), evidenced by an inhibition of Tau protein phosphorylation (*10, 11*). Cohen and coworkers (*6, 7*) have rigorously tested the specificity of a large number of kinase inhibitors and demonstrated many nonspecific effects.

Genetic techniques are available to eliminate (or modify) a given gene from a target organism and thus eliminate (or modulate the behavior of) a specific kinase. This allows the dissection of the function of a particular protein kinase within the organism. However, such approaches are time-consuming in higher organisms, and the resulting animal may not be viable. In addition, the removal of one component from a complex and incompletely understood system often leads to multiple effects on many components so that phenotypes may not be directly linked to genotypes. Investigations into the kinases that regulate cell division illustrate this point clearly. The cell-cycle kinase cdk2 was thought to be essential for entry into the synthesis phase (S-phase) of the cell cycle, and knockout mice lacking the cdk2 gene were hypothesized to be unlikely to survive. Surprisingly, mice lacking cdk2 were viable and displayed largely normal development (*12, 13*). Later work showed that the related enzyme cdk1 was able to compensate for the lack of cdk2 activity, and therefore the resulting phenotype of cdk2 null cells was little altered from that of wild-type cells (*14, 15*).

More synthetic systems can also be used to study protein phosphorylation, for example, *via* purified kinases and selected candidate substrates (*e.g.*, see ref 16) or a library of human proteins expressed in yeast (*17*). The isolation of individual kinases remains a technically challenging undertaking, requiring optimization for each enzyme as well as a substantial amount of starting material. Although candidate substrate selection is clearly biased by current knowledge, this can be largely eliminated by the use of libraries of recombinant proteins. However, libraries are often presented in an artificial environment (*in vitro* or within a heterologous organism), thus limiting their overall utility. Recently, Cohen and Knebel described a more elegant variant of this technique, termed KESTREL, in which a kinase is briefly incubated with a cell lysate (partially purified to remove other kinases) and [γ-^{32}P]ATP. False-positive substrates, which are proteins that are phosphorylated in the *in vitro* assay but are not *bona fide in vivo* substrates, are at least partially eliminated through the comparison of a panel of structurally related kinases and the identification of substrates that are specific to one member of this kinase panel (*18*). Using this technique, the authors identified substrate kinase pairs, including N-Myc downstream-regulated gene products 1 and 2 as substrates for serum- and glucocorticoid-induced kinase 1 (SGK1) (*19*) and methyltransferase-like protein 1 as a

KEYWORDS
Chemical genetics: The combination of synthetic chemistry with molecular biology to produce non-natural ligand/biomolecule pairs for dissection of biological function.
Protein kinase: An enzyme that catalyzes reactions that involve the transfer of phosphates from a nucleoside triphosphate (*e.g.*, ATP) to a protein substrate.
Adenosine triphosphate (ATP): An organic compound composed of adenine, the sugar ribose, and a triphosphate group. It serves as the major energy source that drives a number of biological processes within the cell.
Gatekeeper residue: The residue within the ATP binding site of a kinase that controls the accessibility of the substrate to an enlarged hydrophobic pocket.
Phosphorylation: The addition of a phosphate group to a protein or a small molecule.
Protein kinase substrate: A protein that undergoes phosphorylation.

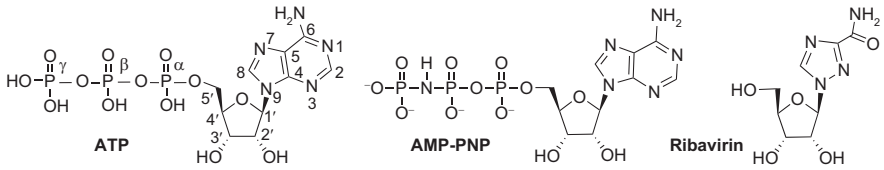


Figure 1. ATP; AMP-PNP, a commercially available ATP analogue; Ribavirin, a commercially available adenosine-derived antiviral drug.

substrate for protein kinase B (PKB)/Akt, among others (*20*). Such techniques are powerful, but they suffer the inherent drawbacks of *in vitro*/heterologous systems: kinases are supplied at nonphysiological levels with little or no regard for accessory subunits that may assist substrate selection. Temporal and spatial constraints are also largely ignored. For example, a kinase may only be expressed in a cell during one particular growth state and be confined to the nucleus; thus, it would not typically have access to many of the proteins in a whole-cell lysate.

Recently, there has been a widespread move toward using high-throughput mass spectrometry (MS) to survey the entire proteome for post-translational modifications. Such approaches are often coupled with the enrichment of phosphoproteins, for example, the use of affinity chromatography with immobilized metal ions that display some specificity for phosphoesters (*21–25*). These developments are very exciting in terms of cataloging the changes in proteins over time and in driving forward advances in technology to expand the sensitivity of MS. However, the application of such techniques is only as intelligible as the sample that is being analyzed, and the complexity of phosphorylation events *in vivo* makes interpretation extremely difficult.

Although the methods discussed above have drawbacks, it is important to note that they are powerful techniques and have been important in the identification of some kinase substrates. However, in order to build a complete picture of protein kinase pathways, a technique is greatly needed that combines the specificity of genetics approaches, the rapidity of the small-molecule approaches, and the sensitivity to encompass low-abundance targets. Chemical genetics has the potential to fulfill these criteria, and here we discuss the significant advances made so far and the developments required to drive this work forward.

The Application of Chemical Genetics to the Protein Kinase Substrate Identification Problem. This Review focuses on the application of chemical genetics to the identification of the substrates of protein kinases using analogues of the natural ligand ATP, the phosphate donor in the catalyzed reaction (see Figure 1).

Protein kinases are particularly well suited to analysis by chemical genetics: all protein kinases appear to use a common catalytic mechanism (*2*), their ATP binding domains are largely conserved in terms of primary amino acid sequence (*26*), and the structures of a number of distinct protein kinases have been solved to high resolution (*27–30*). These observations make chemical genetics approaches particularly powerful, because the establishment of a method of substrate identification for one kinase is likely to be applicable to many other distinct enzymes (*31–33*). In addition, altering the ATP binding site of the protein kinase to accept an unnatural ATP analogue would enable the products of phosphotransfer reactions (*i.e.*, the substrates of the kinase) to be specifically labeled, greatly improving the signal-to-noise ratio of these reactions.

ATP Analogues and Their Interactions with Protein Kinases. Various ATP analogues have been synthesized over the years, and they have provided a unique panel of reagents for dissecting protein kinase function. Modifications can generally be grouped according to the modified substructure of ATP: adenine base, ribose sugar, or the triphosphate moiety.

ATP Analogue Design: Base Modifications. Base-modified analogues incorporate an alteration to the adenine ring. Although it is synthetically possible to alter any of the atoms within or attached to the adenine ring, in this context most attention has been turned to the modification of the side chain at position 6 (see Figure 1 and Figure 2).

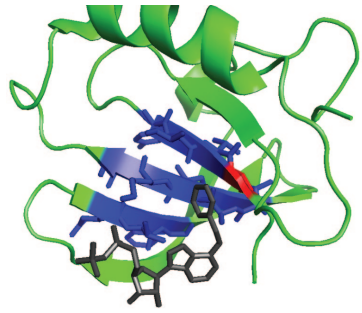
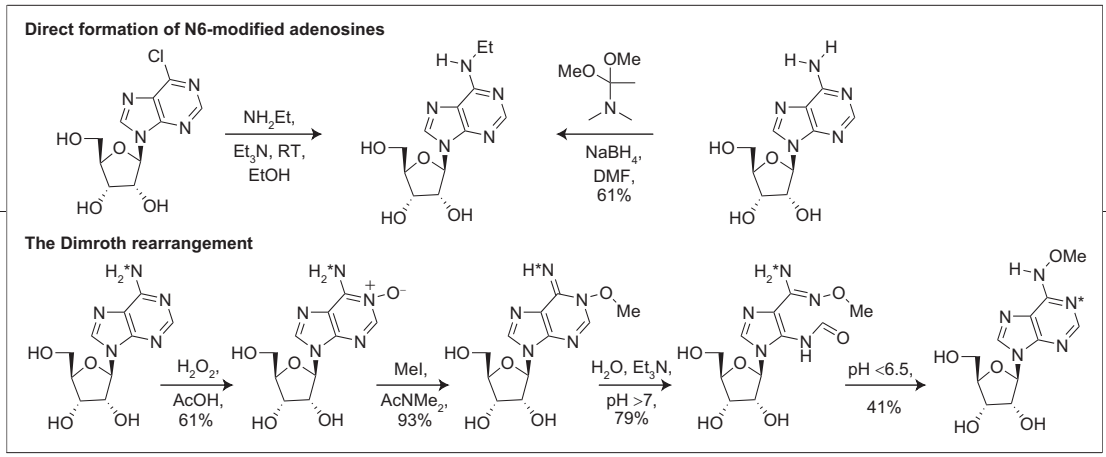


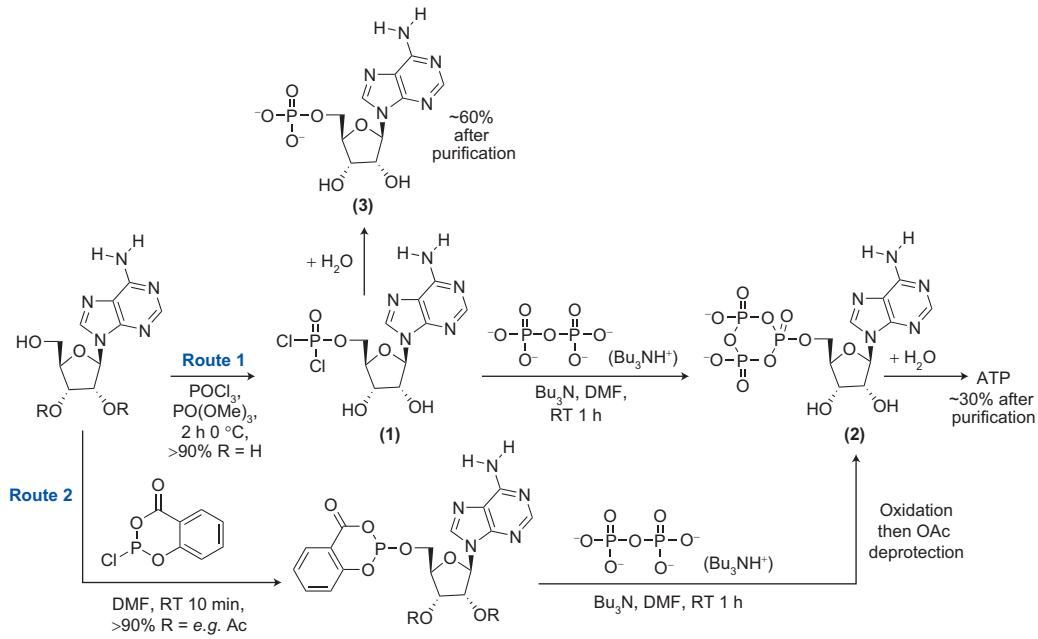
Figure 2. c-Src T338G mutant with N6-benzyl ATP (gatekeeper mutation in red) showing potential second site mutations in the β-sheet highlighted in blue. Molecular coordinates from Protein Data Bank 1KSW (*55*), generated using PyMOL software.



Scheme 1. Synthetic routes to N6-modified adenosines: direct formation and the Dimroth rearrangement. Amines considered in this Review: cyclopentylamine, phenylethylamine, NH_2OEt, NH_2OMe NH_2COMe, NH_2O*i*Pr, NH_2OBn, NH_2O-cyclohexane, cyclohexylamine, 1,3-dimethylbutylamine, 2-methylbutylamine, 3,3-dimethylbutylamine, *p*-tolylamine, *o*-tolylamine, *m*-tolylamine, piperidine, and pyrrolidine. Yields for direct formation by halide displacement are in the 80–95% range.

Position 6 can be modified by direct reaction with the exocyclic amine (*34*). Alternatively, adenosine is commercially available in the N6-chlorinated form, and this is easily modified by halide displacement reactions. If the chloride is displaced with an amine (other than ammonia), then the resulting adenosine analogue is sterically larger than natural adenosine, but an exocyclic NH group can be maintained (Scheme 1)(*35*). This reaction allows many groups to be incorporated at the N6 position. However, the incorporation of an alkoxyamino functionality by this method requires a large excess of the ancillary base, which may present purification problems for the product. An alternative strategy for the incorporation of an *N*-alkoxy functionality is the Dimroth rearrangement with adenosine (*36*). This involves oxidizing and then O-alkylating the N1 position, followed by a base-mediated ring-opening hydrolysis. Subsequent ring closure swaps the positions of the N6-exocyclic amine and the derivatized N1 nitrogen, thereby installing the *N*-alkoxy functionality at the N6 position (Scheme 1).

Nucleoside analogues can be converted to the nucleoside 5′-triphosphate by standard chemical means. The Ludwig modification of the Yoshikawa procedure is typically used to phosphorylate these compounds (*37*) (Route 1, Scheme 2) . The first step of this one-pot/three-step procedure involves the selective reaction of the primary 5′ OH with phosphorus oxychloride to give an unstable nucleoside 5′-dichlorophosphate intermediate (**1**). The selectivity for the 5′ OH is dependent on both the solvent and the exact reaction conditions. The best solvent has been found to be a trialkyl phosphate (*e.g.*, trimethyl phosphate), which gives a homogeneous reaction mixture. Additionally, it appears to accelerate the reaction and enhance chemoselectivity (*38, 39*). Intermediate **1** is then reacted with bis(tri-*n*-butylammonium) pyrophosphate in the presence of base to give a cyclic intermediate, **2**, which is then ring-opened by the addition of water to give the desired nucleoside 5′-triphosphate, usually in ~30% yield after purification. Purification of



Scheme 2. One-pot syntheses of nucleoside 5′-triphosphates. Route 1 is the Ludwig-modified Yoshikawa procedure; Route 2 is the Ludwig and Eckstein procedure.

nucleoside 5′-triphosphates is complicated by the multitude of charged and uncharged and organic and inorganic species in the crude reaction mixture. Generally, ion exchange chromatography is performed to isolate the tetraanionic species from the rest of the reaction mixture. This is followed by preparative reversed-phase HPLC (RP-HPLC) to yield the desired triphosphate. However, the hydrolytic instability of the phosphate linkages complicates this purification process. The reliable chemical synthesis of nucleoside triphosphates remains a significant challenge despite a wide range of available reagents and well-documented reactions (*40*). The problems are largely due to difficulties in coupling a charged, hydrophilic phosphate moiety with a highly polar, more hydrophobic nucleoside species that may bear more than one potentially reactive site. However several N6-modified adenosine 5′-triphosphates have been synthesized to probe kinase function.

The Ludwig-modified Yoshikawa procedure is one of the most commonly used chemical syntheses of nucleoside triphosphates (*41*). Being a one-pot procedure, it is relatively quick, and the yields, although low, are generally reproducible. Another commonly used one-pot nucleoside triphosphate synthesis is the Ludwig and Eckstein procedure (Route 2, Scheme 2) (*42*). This route has the advantage that the phosphorus III reagent is more reactive than phosphorus oxychloride and can be oxidized to give a P=O, P=S, or P → B at the α-position of the resulting triphosphate. The disadvantage is that the 2′ and 3′ OH groups must be protected, usually as the acetate.

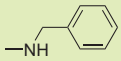
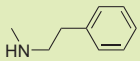
Alternatively, a nucleoside 5′-monophosphate (**3**) (synthesis shown in Scheme 2) can be converted to a diphosphate or a triphosphate. This chemistry is often used to incorporate modifications into the phosphate backbone and is discussed later in this Review.

In 2000, Burgess and Cook (*40*) published a comprehensive review of nucleoside triphosphate chemistries and the challenges associated with their synthesis. The field has not advanced significantly since then, and at this time the successful formation of the nucleoside 5′-triphosphate in acceptable yield remains the limiting factor.

N6-Modified adenosine 5′-triphosphates with enlarged bases are not able to interact productively with protein kinases because the ATP analogue is too bulky to correctly enter the binding site. Early studies with the virally encoded tyrosine kinase v-Src revealed that the ATP binding site could be altered to accommodate unnatural ATP analogues. There are major advantages to modifying the region of the kinase adjacent to the N6 of the adenine base. Firstly, it is buried deep in the ATP binding site, far away from regions of the kinase that are likely to be involved in substrate–regulatory interactions. Secondly, it is distant from the residues involved in catalysis. Therefore, an enlargement of the ATP binding pocket adjacent to the adenine N6 is unlikely to compromise kinase function, provided that the mutation does not interfere with the overall folding of the kinase. This approach to kinase chemical genetics through the use of N6-enlarged ATP analogues has been dominated by Shokat and coworkers (*43–54*). By aligning the sequences of v-Src with cAMP-dependent protein kinase and Cdk2, Shokat *et al.* (*43*) identified two residues (Val323 and Ile338) whose side chains were within 5 Å of the N6-amine of ATP. In a seminal work, Shokat *et al.* (*43*) generated a mutant v-Src in which both of these residues were replaced with alanine and expressed wild-type and mutant kinases in bacterial cells; they showed that N6-cyclohexyl ATP could inhibit ^{32}P transfer from [γ-^{32}P]ATP to a peptide substrate with the mutant kinase but not the wild-type enzyme, an indication of successful competition of the analogue with standard ATP for mutant kinase binding. This work was further developed to show that only an I338G mutation was required for v-Src to accept N6-modified ATP analogues (*44*). The crystal structure of N6-benzyl ADP bound in the c-Src (T338G) mutant (*45*) showed that the substrate binding site was unchanged by both the kinase mutation and analogue binding compared with wild-type c-Src and standard AMP-PNP. These data demonstrate that, although the kinase had been mutated, this mutation was unlikely to result in changes in substrate specificity. This point was substantiated through analysis of peptides targeted by the wild-type kinase/ATP and the mutated kinase/N6-benzyl ATP: both sets of phosphorylated peptides were largely indistinguishable (*45*). This is a finding of crucial importance and validates this chemical genetics approach to analyzing protein kinase function.

The conservation of the ATP binding site between different protein kinases has enabled the approach with v-Src to be more widely applicable. The ATP binding site of most kinases can be enlarged by mutation of a single amino acid equivalent to Ile338 of v-Src to reveal a hydrophobic pocket. Generally, the residue that is mutated

TABLE 1. Summary of the major results obtained with N6-modified ATPs and kinase mutants

ATP analogue	Enzyme	Substrate library	Context	Ref
N6-benzyl ATP	v-Src	NIH 3T3 cell lysates	Novel substrates identified.	(*55*)
		Peptide phosphorylation	A range of N6-modified analogues tested. N6-benzyl shown to be best.	(*44*)
		NIH 3T3 cell lysates	Mutating two conserved leucine residues on either side of the adenine ring to methionine improves specificity for the analogue.	(*56*)
		Peptide phosphorylation	Show that structure of enzyme complexed with ATP analogue and show that mutated enzyme retains the same specificities as wild-type kinase for peptide substrates screened.	(*45*)
	Cdk1	Yeast extracts	Novel substrates identified.	(*49*)
	Kin28 and Srb10	Yeast	Used in combination with selective inhibitor to dissect individual roles and identify new substrates.	(*57*)
	Pho85-Pd1 Cdk (plant)	Yeast	Screening method based on Tap-tagged yeast proteins. Novel substrates identified.	(*48*)
	Cdk7	Rabbit reticulocyte lysate	Investigation into the alternative substrate recognition systems of Cdk7. Known substrates found.	(*58*)
N6-phenylethyl ATP	v-Src	Peptide phosphorylation	N6-phenylethyl ATP shown to be better than N6-benzyl and N6-phenyl.	(*59*)
	Raf-1	HEK293 lysates	^{32}P radiolabeled analogue used to screen for novel substrates. Substrates not identified.	(*60*)
	JNK	HEK293 lysates	^{32}P radiolabeled analogue used to identify novel JNK substrates in HEK293 cell lysates.	(*61*)
	c-Src	Whole and digitonin-permeabilized cells	Expression of T338 Src in Src null cells. Lysates exposed to analogue ATPs to verify Fak as a target. Digitonin-permeabilized cells back up these data *in vivo*. Non-radiolabeled ATP analogue used and limitations discussed.	(*62*)
	YpkA	J774 macrophage cell lysate and bovine brain extract	A novel 36kDa band in lysates and extracts identified. Band was digested, and MALDI-TOF revealed protein to be otubain, involved in T cell anergy.	(*63*)
N6-cyclopentyl ATP	ERK2	Whole cells	ERK-QG overexpressed in cells, immunoprecipitated, resolved on a gel, and analyzed by MS. Novel substrates identified.	(*64*)
	v-Src	Mouse red blood cell lysate	A range of ATP derivatives tested; N6-cyclopentyl derivative shown to be best with this kinase.	(*43*)

has a bulky hydrophobic side chain and provides a natural barrier to N6-enlarged ATPs (Figure 2). This residue has been termed the "gatekeeper" residue (*46, 47*). The side chains of gatekeeper residues effectively "close the gate" so that the hydrophobic pocket is inaccessible to N6-substituted ATPs. However, it is a trivial exercise in molecular biology to mutate these restrictive amino acids to residues with smaller side chains. For example, mutation to glycine (which has only a hydrogen side chain) enlarges the region around position 6, "opening the gate" and enabling some of these substituted ATPs to gain sufficient binding affinity to function as a phosphate donor in kinase reactions. Such gatekeeper mutations have been introduced into a number of protein kinases (Table 1)(*31, 46, 47*).

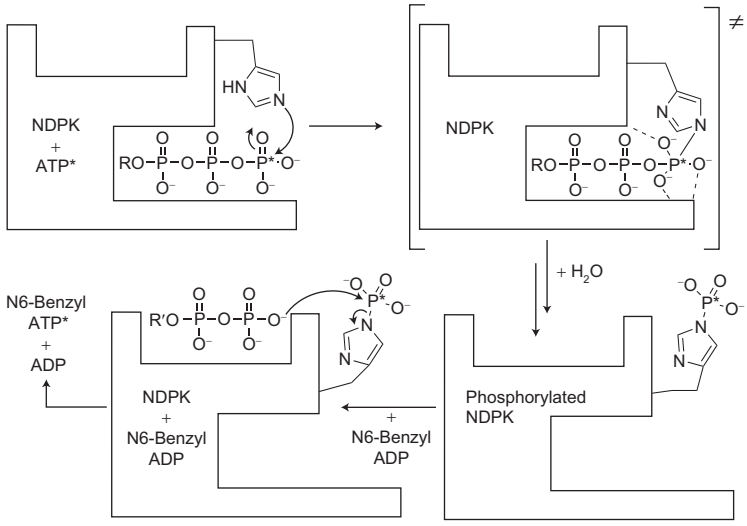
The use of ^{32}P γ-phosphate radiolabeled N6-modified ATP analogues has been successfully applied to many systems *in vitro*, including mammalian cell lysates (*43, 48, 50, 52, 55, 58–61*) and yeast (*46, 48, 49*). Mutant kinase-catalyzed ^{32}P phosphate transfer to substrates allowed the detection, separation, and identification of radiolabeled substrates (Table 1). For the phosphate transfer reaction to be selectively accomplished with the mutant over wild-type, it appears that,

in general, the N6 modification needs to be bulky. Groups that lack an exocyclic NH moiety (*e.g.*, N6-piperidinyl ATP) also appear to be generally poorer substrates, as do *N*-alkoxy and amide derivatives (*43, 61*).

The incorporation of the radiolabel onto the diphosphate has been achieved both enzymatically and chemically. For the enzymatic synthesis, nucleoside diphosphate kinase (NDPK) has been used with [γ-^{32}P]ATP as the ^{32}P phosphate donor and with the analogue diphosphate (*e.g.*, N6-benzyl ADP) as the ^{32}P phosphate acceptor (*50*) (Scheme 3) . The NDPK is immobilized on a column and incubated with [γ-^{32}P]ATP. This allows preloading of the NPDK with ^{32}P phosphate. The immobilized NDPK is then extensively washed to remove the ADP byproduct and any residual [γ-^{32}P]ATP (to avoid contamination of the final product with [γ-^{32}P]ATP). Finally, the [γ-^{32}P]-loaded NDPK is exposed to the analogue diphosphate, promoting phosphorylation of the analogue to produce the [γ-^{32}P]-labeled analogue triphosphate.

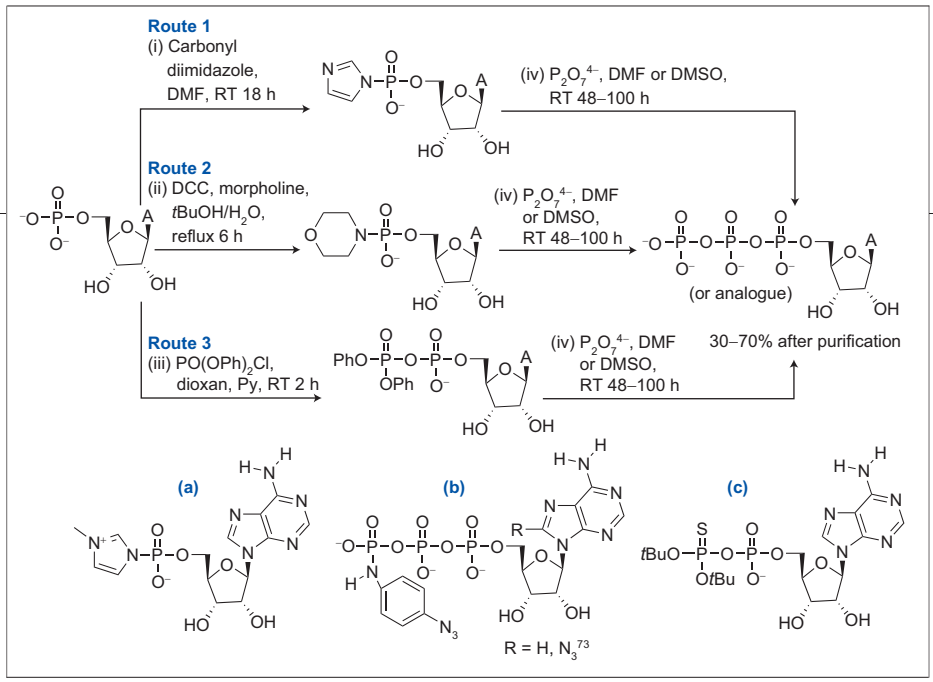
The chemical synthesis of [γ-^{32}P]ATP is based upon chemistry first reported by Hoard and Ott (*51*) and involves the synthesis of an intermediate nucleoside 5′-diphosphoroimidazolate. This intermediate is analogous to the histidine phosphate (Scheme 3) and is easily formed in good yield by the reaction of the nucleoside 5′-diphosphate with carbonyl diimidazole in a polar aprotic solvent (*e.g.*, DMF). Subsequent displacement of the imidazole with radiolabeled phosphoric acid, also in DMF, proceeds cleanly to give the desired [γ-^{32}P]ATP. No activation of the α-phosphate is seen. The product of these reactions is usually used crude; however, yields of ~60% can be obtained following preparative RP-HPLC.

Despite the large success with mutationally enlarged ATP binding sites, exceptions exist in which mutation of the gatekeeper site abolishes the enzymatic activity of the kinase, such as cell division cycle 5 (cdc5), MEK kinase 1, and G-protein-coupled receptor kinase 2 (GRK2) (*52*). Zhang *et al.*(*52*) showed that it is possible to regain kinase activity in the context of some mutant kinases by introducing second site mutations in amino acid residues surrounding the gatekeeper residue. For example, in GRK2, the gatekeeper mutation L271G caused an 80% drop in enzyme activity; however, the additional mutation S278V restored kinase activity to near-maximal levels. Examination of the structures of proteins engineered in this way indicates that the second site mutations were unlikely to directly result in enlargement of the ATP pocket but instead were all found in a β-sheet lying over the gatekeeper residue. These second site mutations generally introduced amino acid residues that are predicted to stabilize β-sheets (*53*) and hence are likely to promote the correct folding of the kinase into the active conformation. Using structure-based sequence alignments, the group identified other stabilizing mutations within this β-sheet. This re-established enzyme activity and thus further validated this hypothesis (*52, 54*).



Scheme 3. NDPK-mediated synthesis of N6-modified [γ-^{32}P]ATP. The radiolabel is denoted by an asterisk (*). Transition state as described in ref 120.

Although these approaches have been used to find some new substrates *in vitro* (see Table 1), the identification of substrates *in vivo* may prove more difficult because of the cell-impermeant nature of triphosphates and (should this problem be circumvented) because of the hydrolytic release of the γ-phosphate. Chaudhary *et al.* (*62*) reported that N6-phenylethyl[γ-^{32}P]ATP was hydrolyzed in <1 min of exposure to digitonin-perme-



Scheme 4. Phosphate activation/displacement chemistries for the synthesis of ATP (and ADP), A = adenine. Route 1 shows the synthesis of ATP *via* a 5′-phosphoroimidazolate. Also shown in (a) is the related *N*-methylimidazole intermediate. Route 2 shows the synthesis of ATP by the activation of AMP with morpholine. Analogous chemistry has also been used to incorporate further modifications to the γ-phosphate, as shown in (b) (73). Route 3 and (c) show typical intermediates used in the sacrificial phosphate approach to ATP synthesis.

abilized cells, and this resulted in free $^{32}PO_4^{3-}$. Previous studies have shown that such inorganic radiolabeled phosphate can be incorporated into the cellular ATP pool in both plants and animals over time scales of <0.1 s (*65, 66*). Clearly, this will substantially increase the level of nonspecific background labeling associated with this approach, and further research is required to reduce such side effects.

One of the major problems associated with the use of N6-enlarged ATP analogues is that the modified protein kinase can invariably utilize endogenous ATP as an alternative phosphate donor. This is a significant problem *in vivo* because the level of endogenous ATP is ~1 mM. Attempts have been made to circumvent this problem by creating ATP analogues with smaller bases and developing kinases with ATP pockets that are restricted in size to preclude natural ATP from binding. This size reduction in the adenine base was achieved either by effectively removing the pyrimidine C2 carbon atom from the adenine or by suitable derivatization of the closely related, commercially available analogue Ribavirin (Figure 1) (*67, 68*). However, these analogues appear to be of limited use because they were substantially less selective for the mutant kinases than the larger N6-modified analogues, probably because of a combination of a loss of rigidity in the purine ring system and the inherent flexibility of the kinase ATP binding region (*67*). Further work is required to develop this type of highly desirable reagent.

Other modifications of the adenine ring have received much less attention. Modifications at position 2 have not been studied in the context of kinase substrate identification. Modifications at position 8 raise the possibility of a steric clash with groups at the 5′ position on the sugar (*69–71*), which affects the conformation of the molecule and thus the relative orientation of the sugar and base. With the exception of the commercially available 8-azidoadenosine triphosphate (a photoreactive cross-linking reagent that is known to cross-link at other sites in addition to the ATP binding site), this modification is rarely used (*72–74*). Modification of the ring nitrogens would introduce a positive charge into the adenine ring and could lead to depurination (*75*). 7-Deazaadenosine is commercially available under the name Tubercidin and can be readily halogenated at position 7 prior to further modifications (*76*). This approach has not yet been exploited; however, 8-aza-7-deazapurine-like motifs occur in known kinase inhibitors, and they have been used to elucidate kinase function, as considered later in this Review.

ATP Analogue Design: Sugar Modification. The tolerance of kinases to modifications at the different sites in ATP varies with the kinase (*73*). In general, the modifications to the ribose that have been studied are well tolerated, although there are exceptions. In terms of probing kinase function, ribose-modified analogues are used less often than base- or phosphate-modified analogues. However, the 2′ and 3′ OH groups do not interact with the kinase and so are ideal sites at which to incorporate, for example, fluorescent tags (*77*).

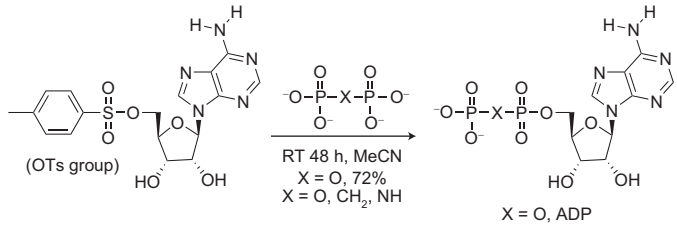
Adenosine and many of its derivatives are commercially available, whereas the synthesis of nucleosides is complicated by the need to control both stereochemistry (with respect to the sugar) and regiochemistry (with respect to the reactive nitrogen on the base) (*78*). The relative positions of the base and the phosphate backbone must also be maintained for the analogue to fit correctly into the ATP binding site. Taken together, these factors seem to have precluded significant investigation of ribose modifications.

ATP Analogue Design: Phosphate Backbone Modifications. One of the most common modifications to the phosphate backbone is to exchange the bridging oxygen between the β- and γ-phosphates for either an NR or a CH_2 group. This produces an analogue in which transfer of the γ-phosphate is prevented. Some of these analogues are commercially available (Figure 1) or can be made by appropriately modifying the pyrophosphate moiety in either the Eckstein- or Ludwig-modified Yoshikawa procedure (*79*). However, this type of modification is more typically introduced by an activation/displacement strategy with the nucleoside 5′-monophosphate (**3**, Scheme 1). Displacement of the activating group with a modified pyrophosphate gives the desired product. (Identical chemistry can usually also be applied to the synthesis of ADP analogues if phosphoric acid is used in place of pyrophosphate.) Different amine-based activating groups have been exploited. Perhaps morpholine (*80*) and carbonyl diimidazole are the most common (*81*) (Scheme 4) .

Although carbonyl diimidazole is an excellent reagent for activating nucleoside 5′-diphosphates (as in the ^{32}P labeling experiments described earlier), unwanted reaction at the 2′ and 3′ OH groups can be seen when trying to activate a nucleoside 5′-monophosphate (*82*). The alternative synthesis with the activating group on the non-nucleosidic phosphate has also been investigated but is much less widely used (*83*). The main problem with this class of reactions is to overcome the electrostatic repulsion between the two phosphate groups, which often leads to long reaction times and lower yields. The electrophilicity of the activated phosphate can be increased by the addition of metal ions (*84*) or by the use of a positively charged activating group (*e.g.*, *N*-methyl imidazole or 4-dimethylaminopyridine (*85*)) (Scheme 4).

Mixed anhydrides have been used less frequently to activate nucleoside 5′-monophosphates (Scheme 4) (*86*). This chemistry is based upon the different leaving group abilities of various phosphates. Generally, diphenylphosphate or di-*tert*-butylphosphorothioate acts as the sacrificial phosphate.

If the 5′ OH group is converted into a suitable leaving group (*e.g.*, OTs), then it is possible to form ADP or ATP by direct displacement with pyrophosphate or triphosphate, respectively, on a small scale (*87*) (Scheme 5) . On a larger scale, removal of the excess inorganic phosphate becomes a significant issue. The

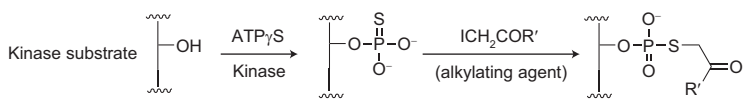


Scheme 5. Nucleoside activation/displacement chemistry for the synthesis of ADP (and ATP).

same chemistry, with noncleavable phosphate linkages (such as CH_2), has been validated and appears to be more general (*88*) (Scheme 5).

The majority of these analogues have been used in crystallization studies with kinases that can provide useful insights into binding states and reaction mechanisms; such information is also valuable in designing novel reagents to probe kinase function (*89*). One of the most widely used analogues is the nontransferable ligand AMP-PNP (Figure 1). AMP-PNP has been used in the structural determination of many protein kinases, including cyclin A2-Cdk2 (*90*), Pim1 kinase (*32*), and AKT/PKB (*30*). Historically, this application has been the largest to utilize ATP analogues to probe kinases, and many key results have been obtained. However, this will not be covered in more detail here.

One commonly employed analogue of ATP involves the substitution of the γ-phosphate with a thiophosphate group, resulting in ATPγS. This analogue can be used by protein kinases in place of ATP for substrate phosphorylation (*91*). However, this analogue also offers two distinct advantages: firstly, other cellular ATPases are unable to utilize the γ-phosphorothioate from ATPγS (meaning that the entire analogue is available exclusively to kinases), and secondly, the resulting thiophosphorylated protein is resistant to dephosphorylation by protein serine/threonine phosphatases, effectively stabilizing the modified substrate (*91, 92*). In addition, Facemyer and Cremo (*93*) showed that it was possible to alkylate the phosphorothioate moiety once



Scheme 6. Kinase-mediated protein thiophosphorylation and its subsequent alkylation.

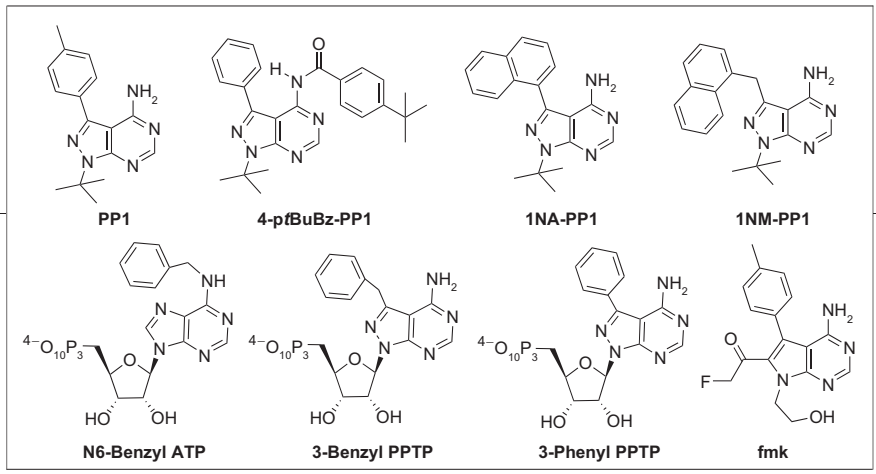


Figure 3. Structure of PP1 and related inhibitors designed to interact specifically with mutationally enlarged protein kinases (N4-(*p*-*tert*-butyl)benzoyl PP1 analogue, 1Na-PP1, and 1NM-PP1) structure of N6-benzyl ATP; structures of second-generation ATP analogues based on these PPI structures; structure of the rationally designed fmk-based covalent inhibitor 1-(4-amino-7-(2-hydroxyethyl)-5-*p*-tolyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)-2-fluoroethanone.

it had been transferred to the kinase substrate (Scheme 6) .

Such S-alkylation reactions have enabled the isolation of thiophosphorylated proteins, facilitating substrate identification (*93–96*). Facemyer and Cremo used a radioactive fluorescent conjugate to examine the phosphorylation of myosin with myosin light chain kinase. This work also resolved one of the issues surrounding the use of ATPγS (namely, that it is also possible to alkylate cysteine residues elsewhere in the substrate in the same manner) by suppressing the reactivity of the existing thiol groups before applying ATPγS (*93*). Early work focused on the reaction of phosphorothioate-tagged groups with fluorescent compounds. However, more recently, additions with biotin allow substrates to be affinity purified and subsequently identified *via* MS (*95, 96*). Other alkylating agents such as *p*-nitrobenzylmesylate (PNBmesylate) have also been reacted with the resulting thiol from ATPγS (*94*). In this example, the authors were able to purify the resulting substrates by immunoaffinity chromatography specific to the phosphorylated PNB group, removing the need to suppress the cysteine residues.

ATPγS is commercially available, and the enzymatic synthesis has been published (*97, 98*) using the same process as that detailed earlier (Scheme 3). The chemical synthesis of ATPγS analogues is more problematic. Only two chemical syntheses of ATPγS exist in the literature (*91, 99*), and neither of these approaches has been successfully applied to the synthesis of ATPγS analogues. The successful combination of substrate capture following thiophosphorylation with the N6-modified ATP analogues provides a powerful adjunct to existing chemical genetics approaches for the identification of kinase substrates (*95*).

An alternative modification of the phosphate backbone is exemplified in work from Philip Cole's lab. Parang *et al.*(*72*) used a morpholine-based activation strategy to incorporate a photoreactive azide group on the γ-phosphate (see Scheme 4). This modification did not prevent the kinase from binding to the substrate, and the modification could covalently cross-link the modified ATP to the substrate *in vitro* following irradiation. Petrousseva *et al.* (*100*) showed that, by varying the nature of the photoreactive substituent on the γ-phosphate, it is possible to change the rate of transfer of the γ-phosphate to the substrate, thus expanding the potential utility of this technique. Although it may be surprising that the kinase is tolerant to modifications at the γ-phosphate, analysis of crystal structures has shown that this site is partially exposed to solvent (*101*).

A second photoreactive group was incorporated at position 8 of the adenine to give the analogue (c) shown in Scheme 4. Parang *et al.* (*72*) showed that it is possible to induce *in vitro* kinase–substrate cross-linking, bridged by the ATP analogue (*72, 100*). If such a bifunctional ATP analogue could be introduced *in vivo*, it would provide an attractive route to substrate purification, simply by purifying the kinase; this would be especially facile if the kinase had been genetically engineered to contain an affinity tag. Alternatively, an affinity tag could be incorporated directly onto the γ-phosphate. ATP-biotin (ATP with a biotin attached to the γ-phosphate through a linker) is commercially available and has been shown to be a substrate for kinases (*101–103*). This leads to the covalent modification of the substrate with a biotin affinity tag, facilitating subsequent substrate isolation prior to identification. Although this approach worked well with artificial peptides, it was less satisfactory with genuine protein substrates. Nevertheless, this represents an exciting area for further development.

In a companion approach, Patricelli *et al.* (*104*) synthesized acyl-linked ATP/biotin and ADP/biotin conjugates. These bind in the kinase ATP binding site in the normal way, but the biotin portion is then covalently transferred to the kinase through reaction with a lysine residue (bioinformatic analysis indicates that most kinases have a suitably placed lysine residue). Although this system does not directly lead to substrate identification *per se*, it does provide a simple way to either analyze which kinases are present in a sample or assess both inhibitor potency and selectivity.

Inhibitors. The field of kinase inhibitors is one of the largest in medicinal and pharmaceutical chemistry. However, because the purpose of this Review is to illustrate how the use of ATP analogues in chemical genetics has enhanced our understanding of protein kinase biology, we have limited our scope to those inhibitors that have been designed specifically to interact in a defined way with mutationally enlarged ATP binding sites. Several more general reviews of kinase inhibitors have recently been published elsewhere (*105*–*107*).

The 8-aza-7-deazapurine-based inhibitor 4-amino-1-*tert*-butyl-3-phenylpyrazolo[3,4-*d*]pyrimidine (PP1) (see Figure 3) inhibits the activity of Src family kinases by occupying a large hydrophobic pocket in the native ATP binding site (*108, 109*). The major advantage of PP1-based inhibitors is that they are cell-permeable, unlike ATP analogues. This makes PP1-based reagents a more advantageous choice in the study of the inhibition of kinases *in vivo* and is reflected by the increasing diversity of studies taking this approach; representative examples of these studies are summarized (Table 2). The use of cell-permeable inhibitors designed to specifically target mutationally altered ATP binding sites neatly gets around the specificity problems associated with small-molecule inhibitors (as discussed earlier) and substantially enhances the power of this technique.

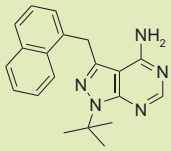
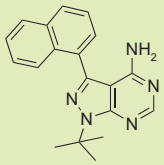
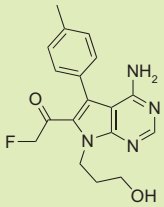
The selectivity of PP1 toward the Src kinases was found to be due to the Ile338 residue (the gatekeeper residue). When Ile338 was mutated to an amino acid with a more bulky side chain, the inhibitor's potency was reduced, whereas a smaller side chain substitution increased potency (*108*). Bishop and coworkers (*109*) were the first to investigate the potential of PP1 as a selective inhibitor by modifying the exocyclic amine group; this is an analogous modification to the N6 modification of ATP. Bishop *et al.* screened PP1 analogues against gatekeeper mutants of the Src-family kinases v-Src and Fyn. The most potent inhibitor identified was the N4-(*p-tert*-butyl)benzoyl analogue (4-p^{t}BuBz-PP1). A second set of analogues modified at the phenyl ring was then synthesized (*110*), and those bearing a naphthyl group were shown to be excellent and specific inhibitors for the mutant kinase (Table 2).

In 2001, Carroll and coworkers used 4-amino-1-*tert*-butyl-3-(1-naphthyl)pyrazolo[3,4-*d*]pyrimidine (1NA-PP1) to inhibit an analogue-sensitive mutant of Pho85. It is interesting that expression in Pho85 analogue-sensitive strains of the yeast *Saccharomyces cerevisiae* exposed to the selective inhibitor implicated a wider role in gene regulation for Pho85 than had been suggested from genetic studies (*118*). The same inhibitor has also been applied to v-erb and showed that this kinase is involved in S-phase progression (*116*); revealed that the autophagy kinase APG1 is used in vacuole trafficking during autophagy (*117*); and an inhibition of Ime2 in yeast inhibits meiosis (*115*). Kenski and coworkers (*54*) used 1NA-PP1 to investigate the requirements of a second site mutation to stabilize GRK2 activity when the gatekeeper mutation inhibited kinase activity.

An additional methylene unit joining the pyrimidine to the naphthyl ring structure gives the PP1-based inhibitor 4-amino-1-*tert*-butyl-3-(1-naphthylmethyl) pyrazolo[3,4-*d*]pyrimidine (1NM-PP1). This PP1-based inhibitor is also cell-permeable and has been applied to a number of cell systems (mainly yeast). In 2000, Bishop *et al.* (*46*) used 1NM-PP1 to selectively inhibit Cdc28 and revealed some discrepancies with previous studies, whether this is due to altered function of the kinase arising from the mutation of the gatekeeper site is not currently known. Several other experimental systems have revealed discrepancies between phenotypes uncovered by chemical genetics and other more traditional techniques; several of these discrepancies could be explained by nonenzymatic functions of the targeted kinase (such as regulatory interactions with other proteins) (*105*), although further work is required to eliminate the possibility that these differences are caused by the gatekeeper mutation itself.

Further additions have been made to PP1-based structures (Figure 3). The replacement of the *tert*-butyl group with the ribose and phosphate groups allows the PP1 derivative to be used as an 8-aza-7-deaza ATP analogue in phosphate transfer reactions. When 3-benzyl PPTP (8-aza-7-benzyl-7-deaza-ATP) was used by Kraybill and coworkers (*50*) as a phosphate donor, it was shown to be 4-fold more effective with the T338G c-Src mutant than N6-benzyl ATP, possibly because of the positioning of the hydrophobic pocket within Src. Inhibitors tend to bind more strongly in the ATP binding site than native ATP, so the key problem to overcome with this class of ATP phosphor-donor analogues was to find the balance between good selectivity for the analogue *versus* efficient release of the ADP analogue following substrate phosphorylation. In fact, 3-phenyl PPTP (8-aza-7-deaza-7-phenyl ATP) proved to be a poor phosphate donor,

TABLE 2. Modified kinase inhibitors and their use in chemical genetics studies with kinase mutants

Inhibitor	Enzyme	Substrate library	Result of kinase inhibition	Ref
1NM-PP1	Cdc28 (CDK1)	Yeast	Premitotic arrest contradicting previous temperature-sensitive work that showed a G1-phase arrest.	(*46*)
	MPS1	Yeast	Dependence of MPS1 activity for kinetochore attachment shown.	(*111*)
	Ire1	Yeast	Mutation of enzyme to accept inhibitor (yeast) resulted in reduction of kinase activity. However, 1NM-PP1 increased downstream endoribonuclease activity by substituting for the autophosphorylation.	(*112*)
	Cla4p	Yeast	Requirement of Cla4p for budding.	(*113*)
	CaMKII	Transgenic mice	Mice overexpressing mutant CaMKII were treated with NM-PPI and brain slices treated for analysis. *In vivo* treatments were successful *via* intraperitoneal (i.p.) injection and uptake through drinking water.	(*114*)
	Cdc28	Yeast	Cdc28 is required for late Ime2 activity.	(*115*)
1NA-PP1	v-erbB	NIH 3T3 cells	V-erbB is required during S-phase progression. Furthermore, i.p. injection of 1Na-PP1 inhibited the growth of subcutaneous tumors made from transformed cells.	(*116*)
	GRK2	HEK293 cells	GRK2 required a second mutation to maintain stability. GRK2 shown to play a role in initial ligand-induced receptor internalization.	(*54*)
	APG1	Yeast	Kinase activity of APG1 is essential for vacuole trafficking, however, it is not essential during induction of autophagosomes.	(*117*)
	Pho85	Yeast	Inhibition revealed differences between knockout studies and showed 250 genes regulated by Pho85 that were not already known.	(*118*)
	Ime2	Yeast	Requirement in meiotic nuclear division.	(*115*)
	RSK2 and MSK1	Cos-7 cells and HEK293 lysates	Selective inhibitors compared to mutations in RSK.	(*119*)

possibly because it retained too many characteristics of the inhibitor.

More recently, Cohen *et al.* (*119*) used a bioinformatics approach to compare the active site sequences of the 491 kinases encoded by the human genome to discern subclasses with distinguishing primary amino acid features in the ATP binding site. This approach identified a small group of kinases, including the p90 ribosomal S6 kinases (RSKs) with threonine at the gatekeeper position and a cysteine adjacent to the glycine-rich motif involved in triphosphate interaction. Using a modified adenine moiety as a structural scaffold, the researchers applied rational design principles to predict an inhibitor that would covalently target the cysteine of these kinases *via* a fluoromethyl ketone (fmk) group. Experimentation validated this approach, demonstrating the specificity of the fmk derivative 1-(4-amino-7-(2-hydroxyethyl)-5-*p*-tolyl-*7H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)-2-fluoroethanone (Figure 3) for the RSK subgroup of kinases. Further work indicated that other kinases could be sensitized to this fmk-based inhibitor only if they were mutated to contain threonine and cysteine in the appropriate positions (*119*). Such examples illustrate an alternative means of manipulating inhibitor sensitivity on the basis of kinase mutagenesis.

The application of modified inhibitors provides the only example reported to date of the use of a mutant kinase with an enlarged ATP binding site in a higher eukaryote. In 2003, Wang *et al.* (*114*) created a mouse strain overexpressing a mutant calmodulin-dependent protein kinase II (CaMKII), which accepts the PP1-based kinase inhibitor 1NM-PP1. The kinase inhibitor was administered to animals in their drinking water, and resulting behavioral and electrophysiological data showed a role for CaMKII in the formation of long-term memory. However, the authors were unable to specifically in-

hibit wild-type CaMKII, and therefore some residual activity remained that may have masked other functions of CaMKII. Such approaches do illustrate the promising future application of these chemical genetics tools to real biological systems in multicellular organisms. However, the power of this technique will be realized most when a modified kinase can directly replace that of the endogenous gene, thus eliminating any residual activity.

Summary. Engineering protein kinases and their ligands offers great potential to dissect the functions of these important biological regulators, perhaps in a manner that is inaccessible by other techniques. The establishment of a number of model systems that use ATP analogues or small-molecule inhibitors to target the ATP binding site of protein kinases, especially those with enlarged binding sites, has laid the foundations for substantive progress in the next few years. This may be particularly true when chemical genetics techniques are coupled with highly sensitive proteomics analyses, such as protein identification by MS; the chemical genetics will provide a specific substrate for enrichment and identification *via* proteomics techniques. We have reached a stage where many of the elements required for chemical genetics to achieve its full potential are in place. Specific substrates can be labeled *in vitro*, and small-molecule inhibitors directed toward the enlarged ATP binding site can be used to define kinases. However, some areas still require significant input, primarily those centered on engineering both kinase and ligand so that phosphate transfer to substrates is maintained but endogenous ATP is no longer a competitor of the ATP analogue. In addition, the development of ATP analogues that can be internalized will further enhance the *in vivo* application of these approaches, an essential goal if a full catalog is to be made of the specific substrates of a protein kinase under defined physiological conditions.

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