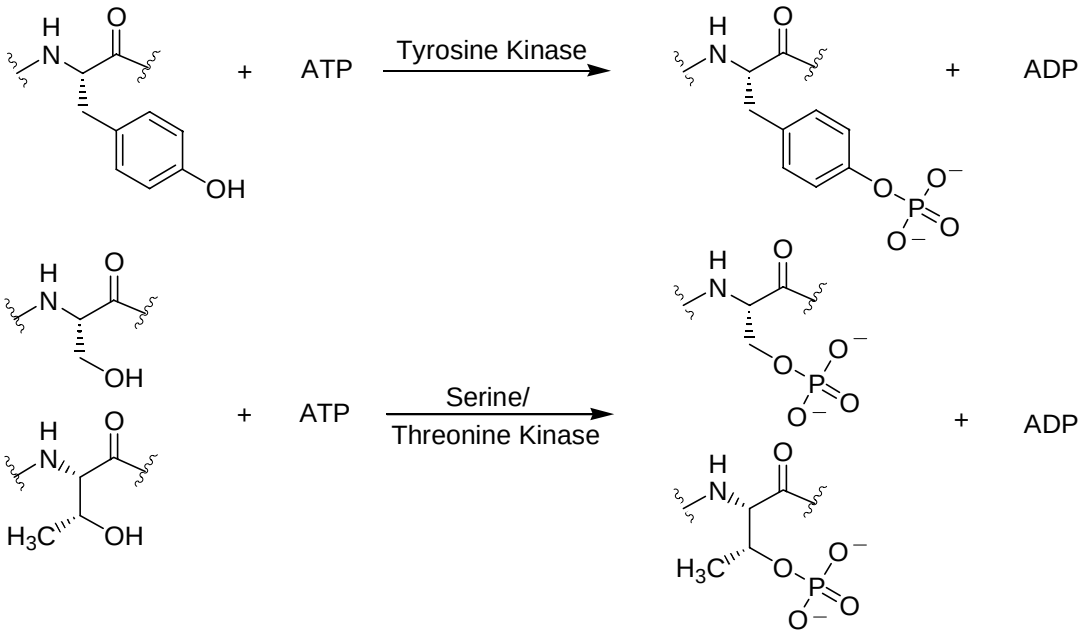
SEARCH FOR SPECIFIC KINASE INHIBITORS AND CELLULAR SUBSTRATES

Reported by Danica P. Galonic September 27, 2001

INTRODUCTION

Protein phosphorylation is the major post-translational modification used to control signal transduction.[1] In eukaryotic cells, protein kinases are the enzymes that catalyze the transfer of the γ-phosphoryl group from adenosine triphosphate (ATP) to the hydroxy groups in tyrosine, serine, and threonine in the protein substrates (Scheme 1).

Scheme 1. Kinase Catalyzed Phosphorylation of Tyrosine, Serine, and Threonine.



The protein kinase superfamily in humans contains approximately 2000 members, which are encoded by 2% of the genome.[2] Over 80% of the oncogenes and proto-oncogenes involved in human cancer encode for protein kinases.[3] Functional assignments in the protein kinase family would help deconvolute kinase-signaling pathways and uncover important targets for rational drug design. Systematic investigation of the role of each kinase requires design and synthesis of cell permeable inhibitors.[4] However, the enormous size of the kinase superfamily and the highly conserved nature of their active sites make the design of specific inhibitors very challenging.[5]

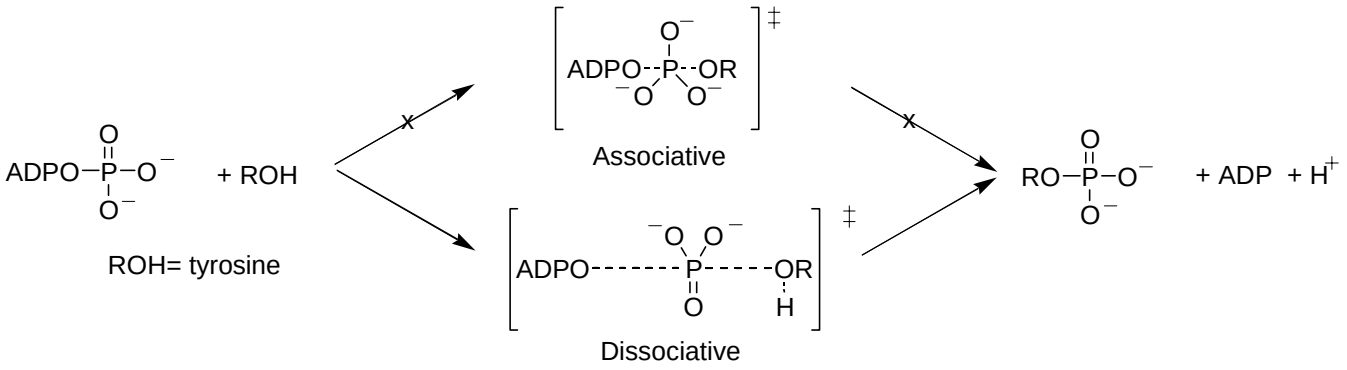
Tyrosine-phosphorylating Src kinases, and serine/ threonine phosphorylating cyclin-dependent kinases (CDKs) are among the most investigated kinase families. Src kinases are cytosolic signal transducers that participate in different cellular processes, including cell adhesion, growth, and

oncogenesis.[7] One of the members of this family, v-Src, is an important drug target because of its role in the development of breast, lung, and colon cancers. Cyclin-dependent kinases have important roles in the control of the eukaryotic cell cycle.[8] This report will focus on investigations directed toward the development of specific and potent competitive inhibitors of these enzymes, as well as on attempts to assign cellular functions to individual kinases belonging to these two classes.

KINASE MECHANISM

The catalytic mechanism of C-terminal Src kinase was investigated.[9] This enzyme catalyzes the transfer of the γ-phosphoryl group from ATP to a specific tyrosine in the protein substrate. The phosphoryl transfer is believed to proceeds through a dissociative (methaphosphate-like) transition state, as shown in Scheme 2.

Scheme 2. Associative and Dissociative Reaction Transition States for Protein Kinases.



This mechanism was supported by linear free-energy relationships studies. The Brønsted nucleophile coefficient for the forward reaction is small ($\beta_{nucleophile}$= 0- 0.1), indicating that there is little bond formation between the attacking nucleophile and the γ-phosphate prior to the departure of the leaving group.[10]

MEDICINAL CHEMISTRY APPROACH

Because of the important role kinases play in signal transduction pathways, these enzymes have become attractive therapeutic targets.[11] Medicinal chemistry has been the traditional way that enzyme inhibitors have been discovered. Although this approach has been successful in producing high potency kinase inhibitors, these compound are not sufficiently selective to be useful in the elucidation of signaling pathways; only with highly specific inhibitors can the role of individual kinases been investigated.

In the case of protein kinases, both rational drug design and combinatorial chemistry have been used to discover inhibitors. A potent inhibitor of Src family kinases discovered this way is pyrazolo[3,4-*d*]pyrimidine, PP1 (**1**), a novel heterocycle which inhibits two kinases from this family, c-Fyn and Lck, with an *in vitro* 50% inhibitory concentration (IC_{50}) of 5 nM.[12] However, this molecule also inhibits, though with decreased efficiency, some of the non-Src family kinases.

The purine ring system has often been employed as a scaffold for the design of kinase inhibitors.[8] A co-crystal structure of the CDKs family member CDK2 with purine olomoucine[13] (**2**, a compound that showed good selectivity for CDKs but modest potency: IC_{50} = 7μM), served as a guide for the development of 2,6,9-trisubstituted purine libraries of CDK inhibitors. These syntheses involved a combination of solid- and solution-phase chemistry.[14] The solid phase syntheses involved attachment of the purine ring to the solid support through the C2, N9, or C6 position (Figure 1).

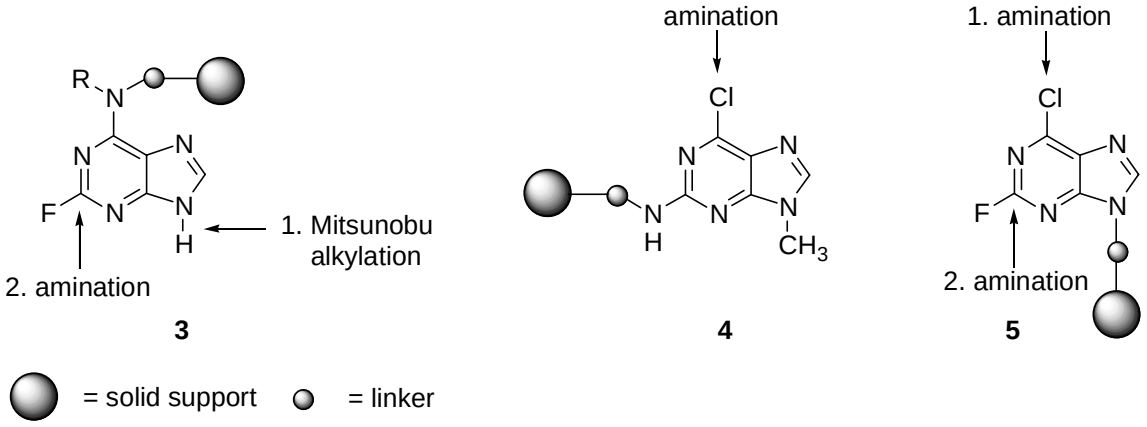


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Figure 1. Solid-Phase Synthesis Strategies leading to 2,6,9-Trisubstituted Purine Libraries.

Because solid phase synthesis required attachment of the linker to either C2, N9, or C6, this eliminates one potentially useful site for combinatorial modification. Therefore, to access derivatives in which diversity can be incorporated into all sites, two solution phase strategies were also developed (Figure 2). In strategy A, purine **6** was functionalized in the following sequence: (1) N9 was alkylated via a Mitsunobu reaction; (2) the amino group at C2 was activated by acylation with trifluoroacetic anhydride and then alkylated through a Mitsunobu reaction; (3) C6 position was aminated via nucleophilic aromatic substitution. In strategy B, the difference in reactivity between the halogen substituents at C6 and C2 in the compound **7** allowed for differential amination of these centers, after alkylation of N9.

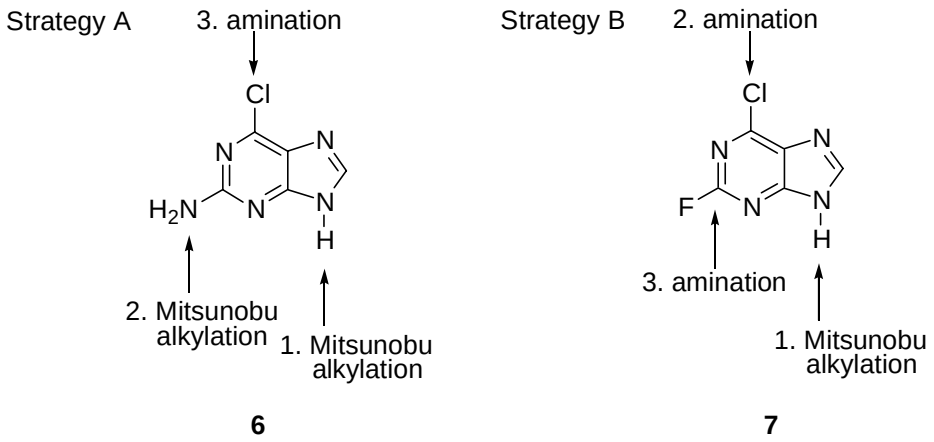
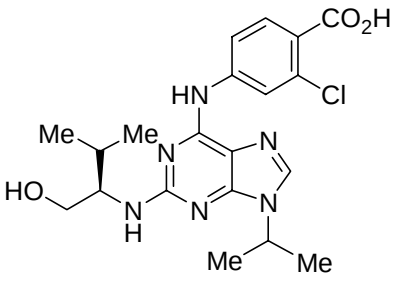


Figure 2. Solution-Phase Strategies for the Synthesis of 2,6,9-Trisubstituted Purine Libraries.

The compound libraries were screened for inhibition of CDK activity and human leukemic cell growth. Lead compounds were optimized by iterative synthesis based on structure-activity relationships. The most potent inhibitor found using this approach was purvalanol B (**8**), which has *in vitro* IC_{50} values lower than 10 nM toward numerous CDK-cyclin complexes.[15]



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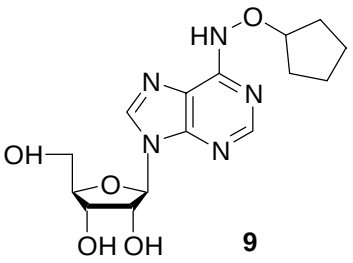
CHEMICAL GENETIC APPROACH

Although the medicinal chemistry approach has provided inhibitors of great potency, they show poor differentiation between specific kinases. In order to overcome the problems caused by genetic redundancy of protein kinase active sites, the Shokat group used site-directed mutagenesis in combination with structure-based design of small molecules to identify specific kinase inhibitors and direct cellular substrates. When a large amino acid residue in the enzyme active site is changed to a small one, a new binding pocket ('hole'), which is not present in the wild-type enzyme, is obtained. Ligands with incorporated large groups ('bumps') are then able to fit into the newly created hole present only in the engineered enzyme.[16] It was envisioned that the mutation of a large residue which is in a close proximity of nucleotide in the kinase active-site can create enlarged binding pocket. The ATP analog with incorporated large substituent would then be able to selectively bind to the engineered enzyme. At the same time, because of its size, the modified ATP analogue cannot interact with wild-type enzyme.

Inhibition of engineered kinases

Prototypical tyrosine kinase v-Src was chosen as a model kinase for this approach. By examining protein kinase crystal structures the researchers recognized that there is a structurally conserved bulky amino acid residue that is in close contact with the amino group attached to the C6 of ATP.[17] In the case of v-Src, this residue is isoleucine 338. Analysis of protein kinase sequence alignments confirmed that residue 338 (Src numbering) contains a bulky side chain in all known eukaryotic protein kinases. Mutation of the isoleucine 338 to either alanine (I338A) or glycine (I338G) created an enlarged binding pocket in the engineered enzymes. Substrate specificity of the v-Src mutants remained the same. I338G v-Src mutant was screened against various C(6)-*N*- substituted adenosine molecules. C(6)-*N*-(cyclopentyloxy) adenosine (**9**) showed the best potency (IC_{50} = 1 μM).[4] Surprisingly, this substituent introduces a much larger volume than was expected to be produced by the amino acid change that was made. By further analyzing available crystal structures of Src family kinases,[4] the investigators concluded that residue 338 acts as a 'molecular gate': once it is removed by mutation, it allows the C(6)-*N*- substituent to occupy another pocket that was already present in the enzyme active site.[18]



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Adenosine analogues, however, are not ideal inhibitors, because adenosine performs many cellular functions. Therefore, a second generation of inhibitors was made, based on the previously known tyrosine-kinase inhibitor PP1 (**1**).[19] The C(4)-*N*-(*p*-*tert*-butyl)benzoyl analog (**11**) was the most potent inhibitor of the I338G v-Src obtained by screening this new panel (IC_{50} = 430 nM). Furthermore, at nearly 1000-fold higher concentrations, these compounds did not inhibit wild-type Src. However, this compound could be disrupting potential hydrogen-bonding interactions with amino-acid residues in the kinase active site, as shown for *des*-methyl analog of PP1 (**10**, Figure 3).

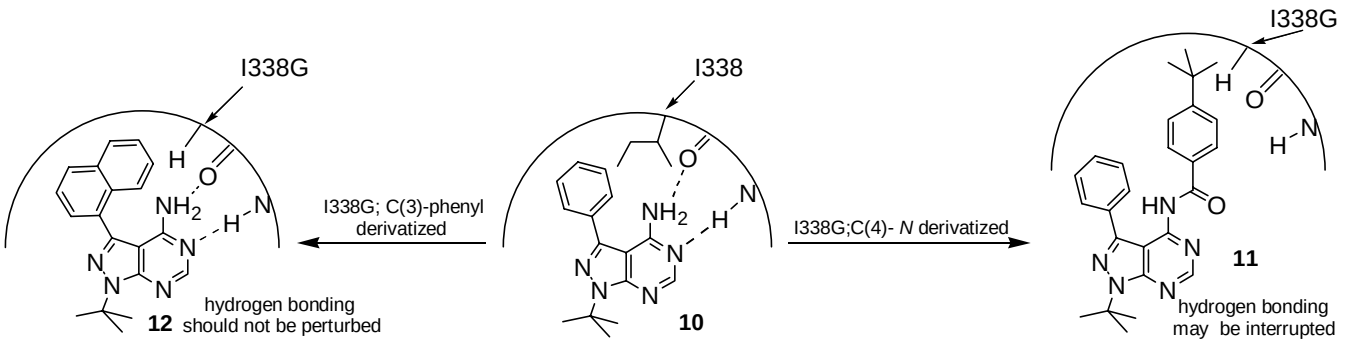


Figure 3. Predicted Binding Orientation of Two Classes of Derivatized Pyrazolo[3,4-*d*]pyrimidines.

It was envisioned that derivatization of the C3 phenyl ring with a bulky group could also afford compounds that complement I338G v-Src active site, but without disrupting any potential hydrogen-bonding interactions.[20] Indeed, naphthyl isomer **12** showed the excellent potency (IC_{50} = 1.5nM).

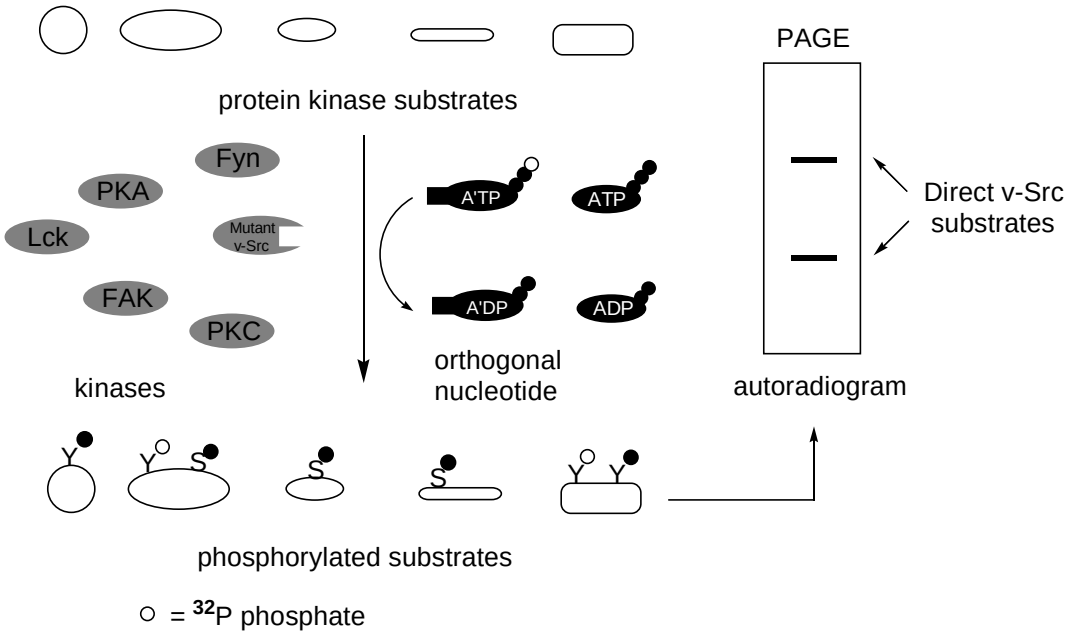
To investigate the utility of compound **12** as a specific kinase inhibitor in the context of a whole cell, NIH3T3 fibroplast cell lines that express either wild type or I338G v-Src were generated. Because v-Src is highly active, the majority of the tyrosine phosphorylation in these cells is a result of v-Src expression. To investigate the selective inhibition of the engineered v-Src, both wild-type and I338G v-Src expressing cells were incubated with varying concentrations of **12** in parallel experiments. It was shown that **12** strongly diminished tyrosine phosphorylation in cells containing I338G v-Src, within minutes, in a concentration-dependent manner. Wild-type v-Src-expressing cells showed no loss of phosphorylation signal in the presence of 500 nM **12**.

In order to test the engineering method in a more complex system, the budding yeast was chosen. The genome of this organism encodes over 120 protein kinases, including homologues of proteins from many mammalian kinase families. Mutation of the active-site residue in cyclin-dependent kinase Cdc28 of budding yeast, and the synthesis of a selective inhibitor, showed that this method can also be applied to serine/ threonine kinases.[21]

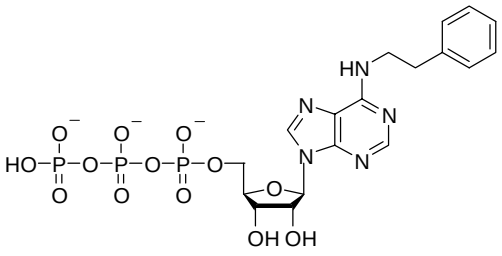
Determination of direct cellular substrates

In principle, the engineered kinase-modified nucleotide approach could be applied to the elucidation of the role of individual kinases in signal transduction pathways. In order to fulfill this goal, the following criteria should be satisfied: (1) the ATP analogue with a large substituent (A'TP) must be orthogonal to wild–type kinase, meaning that the unnatural ligand should not be utilized by the wild-type enzyme; (2) the mutant kinase should use A'TP with high catalytic efficiency; (3) the mutant kinase should exhibit reduced catalytic efficiency for ATP, so that in the presence of endogenous cellular ATP (1-5 mM), A'TP is the preferential phosphodonor; (4) mutations in engineered kinases should be functionally silent; a modified kinase should have the same biological function as the wild type kinase; and finally, (5) the engineering strategy should be broadly applicable to the other members of phosphokinase family.[22] The engineered kinase-modified nucleotide pair satisfying criteria 1-4 could be employed for labeling of cellular substrates of engineered kinases. If the γ-phosphate of a modified ATP analogue is radiolabeled and added into a cell containing the engineered kinase, the radioactive isotope would be transferred only to proteins that are direct substrates of the kinase of interest (Scheme 3); this would reveal the cellular role of the kinase of interest. Ideally, if the method were applicable to the whole kinase superfamily, elucidation of signal transduction pathways would be greatly simplified.

Scheme 3. Identification of Direct Cellular Substrates of Engineered V-Src.



Based on the previous findings for I338G v-Src mutant, the search for the optimal phosphodonor for this engineered enzyme started with C(6)-*N*-substituted nucleotides. A highly specific anti-phosphotyrosine antibody was used to detect phosphorylation of a specific tyrosine on the substrate. It was found that C(6)-*N*-(2-phenylethyl) ATP (**13**) is the best substrate for I338G v-Src mutant. The extra flexibility added into the phosphodonor by putting a two-methylene-unit spacer between the base and phenyl ring served to better orient the ATP analog **13** in the existing pocket in the nucleotide-binding site. As in the previous case, the modified ligand was not accepted by the wild-type kinase. Eliminating or drastically minimizing the ability of the engineered kinase to use ATP is likely to be necessary for *in vivo* protein kinase substrate labeling experiments, because the concentration of cellular ATP is high (1-5 mM).[21]



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CONCLUSION

Protein kinases play a central role in signal transduction. Systematic assignment of a role to each kinase that participates in signaling pathways requires the design and synthesis of specific inhibitors. Although potent inhibitors were synthesized via the medicinal chemistry approach, redundancy and overlapping substrate specificities made this protein family a difficult target for the design of highly specific inhibitors, even with the aid of combinatorial chemistry.[15] In a chemical genetics approach to kinase inhibition and signaling pathways elucidation, small-molecules syntheses and protein

mutagenesis were combined. In this way, highly potent and specific inhibitors of engineered kinases have been identified.[20] The molecular basis for inhibitor binding has been investigated.[18] This method is broadly applicable throughout the kinase family, and potentially as well, to other enzyme and ligand regulated systems.[23] The active sites of kinases have also been mutated in order to accept modified nucleotides.[17] The successful engineering of a kinase active site to accept a unique nucleotide analog would provide a handle by which the direct substrates of a given kinase could be traced in the presence of any number of cellular kinases.[21]

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