Pergamon

Tetrahedron 56 (2000) 9495–9502

TETRAHEDRON

Towards the Engineering of an Orthogonal Protein Kinase/Nucleotide Triphosphate Pair

Scott M. Ulrich,[a] Oleksandr Buzko,[a] Kavita Shah[b] and Kevan M. Shokat[a,*]

[a]Department of Cellular and Molecular Pharmacology, Box 0450, University of California at San Francisco, San Francisco, CA 94143, USA
[b]Novartis Institute for Functional Genomics, La Jolla, CA 92121, USA

Received 8 June 2000; accepted 3 August 2000

Abstract—Remolding protein/ligand interfaces has led to the development of new tools for the study of biological systems. Such methods allow one to engineer proteins with specificity for designed biological probes such as inhibitors, substrates or small molecule dimerizers. Previous work in our laboratory has resulted in engineered protein kinases with specificity for ATP analogs which are otherwise orthogonal ligands for wild type kinases. Kinase reactions of analog-sensitive mutant kinases in cell lysates using $\gamma^{32}P$ labeled ATP analogs allow identification of the direct substrates of the sensitized kinase. As an extension of this methodology, we have designed and evaluated an ATP analog, N^4 (benzyl) ribavirin triphosphate, which may be a suitable phosphodonor for a kinase that does not utilize ATP. Such a modification is likely to be necessary for in vivo kinase substrate labeling experiments where competitive phosphodonors are present in high concentrations. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Protein kinases are a family of enzymes that catalyze the transfer of γ-phosphate from ATP to tyrosine, serine or threonine amino acid residues of substrate proteins. Phosphorylation alters the enzymatic activity, binding capability or cellular localization of the substrate protein as a means to relay cellular signals from the environment such as the extracellular matrix, antigens and hormones such as insulin and growth factors.[1] Since the discovery of protein phosphorylation as a mechanism of signal transduction, the discovery of the v-Src and v-Abl oncogenes,[2,3] and the realization that protein kinases are an immense superfamily of proteins (2.1% of *C. Elegans* genes are protein kinases); protein kinases have moved to center stage in the field of signal transduction. Owing to their centrality in cell signaling, protein kinases have also become attractive therapeutic targets for such diverse diseases as diabetes and cancer.[23]

Protein kinases occur as cytosolic domains of receptor proteins, stimulated by extracellular ligand binding, or as non-receptor cytosolic enzymes. The molecular details of how protein kinases are regulated and activated have been elucidated by structural studies. Receptor protein kinases typically relay signals across the membrane by dimerization/oligimerization followed by *trans* autophosphorylation of the intracellular kinase domains, activating them to phosphorylate cellular protein substrates.[4] Cytosolic protein kinases invariably contain one or more binding domains that have regulatory and/or targeting functions. The earliest studied and most prevalent is the Src Homology 2 (SH2) domain, which binds specific phosphotyrosine sequences. Receptor kinase phosphorylations create SH2 binding sites, which modulate cytosolic protein kinases and other signaling enzymes. Src Homology 3 (SH3) domains are another module recognizing intra- or inter-molecular polyproline motifs for targeting and regulation.[5] It is the regulation and localization provided by these and other binding domains that direct kinase activity to the correct cellular location and ensure appropriate activation and correct signaling.[6]

Despite many years of intense study, the identity of *bona fide* substrates of most kinases remain unknown. Determining physiological kinase substrates is difficult for several reasons. First, kinases often have very little preference for a particular sequence flanking the phosphorylated residue.[7] While the targeting/binding domains serve to present meaningful physiological substrates to a particular kinase domain upon activation, the kinase domain itself is relatively promiscuous.[1,8–10] It is also well known that many important kinases are genetically redundant due to the presence of related family members. Thus, determining the targets of one kinase in a complex signaling network involving multiple kinases has proven intractable.[9]

To address this problem, we have previously reported a ‘chemical genetic’ approach to trace the substrates of a single kinase in the presence of all cellular kinases. This method involves engineering a kinase such that it is

Keywords: protein/ligand interfaces; dimerizers; phosphodonors.
* Corresponding author. Tel.: +1-415-514-0472, fax: +1-415-514-0822; e-mail: shokat@cmp.ucsf.edu

0040–4020/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0040-4020(00)00834-6

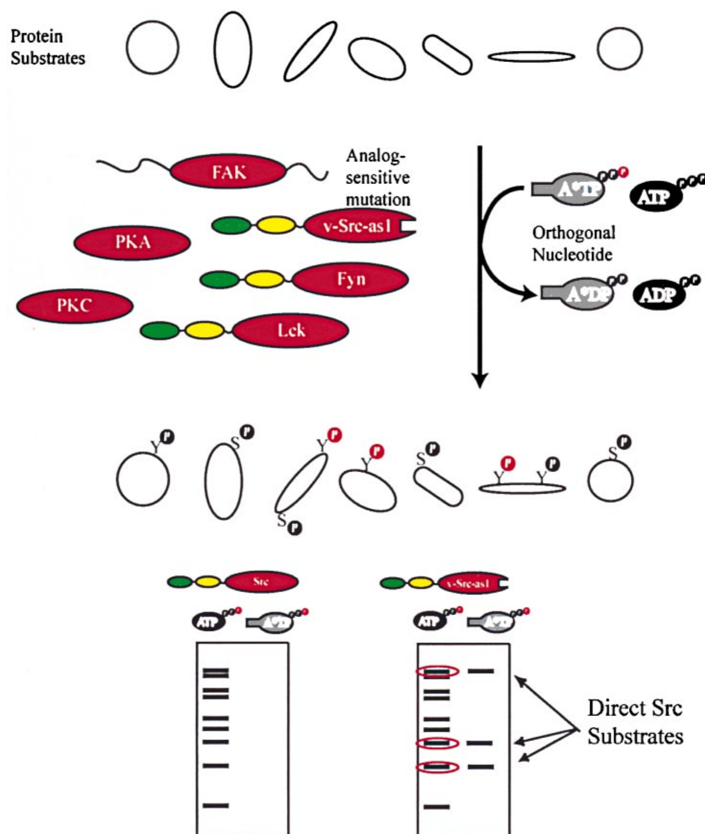


Figure 1. Schematic outlining the method of orthogonal A*TP analogs and the analog-sensitive allele v-Src-as1 to tag the direct v-Src substrates in the presence of multiple kinases.

uniquely able to recognize an unnatural ATP analog. The unnatural A*TP molecules are chemically modified with a benzyl 'bump' on N^6 of the adenine ring of ATP, rendering the nucleotide orthogonal to (a dead substrate for) all wild-type kinases. A single mutation (I338G in v-Src) sensitizes the mutant kinase to accept N^6 (benzyl) ATP (Fig. 1). The analog-sensitive allele is termed *V-SRC-AS1*. Addition of [γ^{32}P] N^6 (benzyl) ATP to cell lysates specifically radiolabels the direct substrates of v-Src-as1.[10,11] The mutation that renders v-Src-as1 sensitive to ATP analogs is readily generalizable to other Src family kinases and many other unrelated kinases (K. M. S., unpublished observations).

Here we describe our efforts to improve the sensitivity of the above method of tagging direct protein kinase substrates. Specifically, we aim to create a kinase which *only* utilizes the synthetic A*TP derivative. Engineering the *kinase* to be orthogonal to all other nucleotide triphosphates in the cell (ATP, GTP, CTP, TTP, etc.) will dramatically increase the efficiency of substrate labeling. 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, as the concentration of cellular ATP is quite high (1–5 mM).[24]

Design

The design goals (Fig. 2) for an ideal engineered kinase/synthetic nucleotide are:

1. Synthesize modified A*TP molecules orthogonal to wild type kinases.
2. Identify mutations that allow kinases to accept the orthogonal A*TP as an alternative phosphodonor.
3. Further modify A*TP→A**TP such that it can be accepted by a modified kinase that does not accept ATP.
4. Identify a mutant kinase that can accept this orthogonal A**TP while itself being orthogonal to ATP.

Design goals #1 and #2 are satisfied by the use of N^6 (benzyl) ATP as an alternate phosphodonor for v-Src-as1 previously reported by our laboratory.[11] Here we describe the design, synthesis, and characterization of an A**TP that may satisfy the third design goal. In order to design such an A**TP analog, we had to first design a kinase active site which does not bind ATP. In creating such an orthogonal kinase, we sought to use the same strategy that rendered N^6 (benzyl) ATP orthogonal to wild type kinases. Adding a steric bump to the N^6 position of adenine precludes N^6 (benzyl) ATP binding to the wild type active site. In an analogous fashion, an amino acid mutation from a small → large side chain in the active site of a kinase should block binding and phosphotransfer with ATP (Fig. 2). The conclusion from this exercise is that a kinase that does not recognize ATP for steric reasons would likely contain a *smaller* active site than wild type kinases.

Starting with the N^6 (benzyl) ATP scaffold, we sought to create a smaller nucleotide base that could be recognized by an active site that will exclude ATP as per design goal #4.

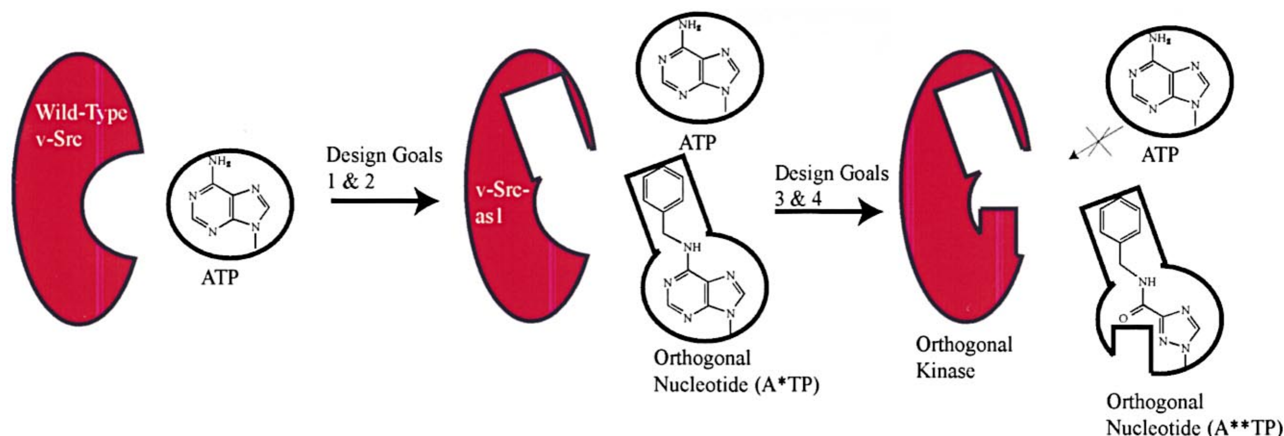


Figure 2. Second generation of A**TP and orthogonal kinase.

The C^2 and N^3 positions of ATP (blue in Fig. 3) do not make any specific hydrogen bonds with the kinase, so removing these atoms to create a smaller nucleotide would not disrupt any specific contacts present in the kinase–substrate complex. The resulting nucleotide (shown in Fig. 3b) is an amidine imidazole and has similar sterics and functionality to the triazole carboxamide (Fig. 3c) which is more stable and synthetically accessible. This nucleoside derivative (Fig. 3c) is N^4 (benzyl) ribavirin, the unsubstituted parent of which is an antiviral used clinically to treat Hepatitis C.[12] The amide moiety of ribavirin is capable of presenting a benzyl group to the I338G pocket similar to N^6 (benzyl) ATP. The ribavirin scaffold also satisfies hydrogen bond donor/acceptor interactions with the kinase seen in all ADP and inhibitor bound kinase crystal structures.[27] Previous work on carboxamide substituted 5-membered ring nucleotides has shown their capacity to retain adenine-like hydrogen bonding in DNA duplexes.[29]

This report describes the synthesis of N^4 (benzyl) ribavirin triphosphate (N^4 (benzyl) RTP) and its structure–activity relationship with both v-Src and v-Src-as1.

Results and Discussion

We first used computer aided modeling to determine whether N^4 (benzyl) RTP, (A**TP) could bind v-Src-as1 using a modified version of the molecular docking suite AutoDock.[17] To confirm that our docking algorithm could accurately predict small molecule-protein interactions, we tested it with the known A*TP analog, N^6 (benzyl) ATP and the c-Src-as1 kinase. The X-ray crystal structure of c-Src bound to ADP has been determined.[20] The structure of c-Src is likely to be identical to that of v-Src since the two proteins have almost identical active site residues, hence, we used it as a model in our docking studies. We used the molecular graphics package INSIGHTII[19] to modify the structure of the wild type enzyme and introduce the T338G change with the assumption that such a mutation would be unlikely to affect the position of the protein backbone.[25] N^6 (benzyl) ATP was docked to both c-Src and c-Src-as1. The top scoring orientations were compared to the structure of the c-Src/ADP complex.

This analysis showed that N^6 (benzyl) ATP binds to c-Src-as1 in a fashion similar to that of ADP bound to wild type c-Src (Fig. 4b). The binding orientation difference has a root mean square deviation (RMSD) of 1.7 ± 0.4 Å (calculated for a set of the five top scoring orientations). The adenine ring system of N^6 (benzyl) ATP occupies the same region in space within the binding site of the enzyme and forms essentially the same interactions with surrounding residues as ADP. These include the two essential hydrogen bonds between the N^6 amino group and NH group of E339 and N^1 nitrogen of ADP and carbonyl of M341. The ribose ring is positioned in the same location with a minor shift, which does not significantly affect orientation of the phosphate

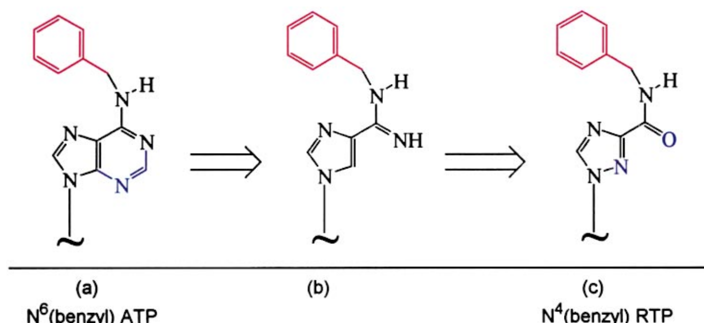


Figure 3. N^4 (benzyl) RTP as an A**TP. N^6 (benzyl) ATP (a) was modified to remove N^2 and C^3 in order to accommodate a smaller kinase active site which is orthogonal to ATP. (b) The structure was further modified for greater stability to give (c), N^4 (benzyl) ribavirin.

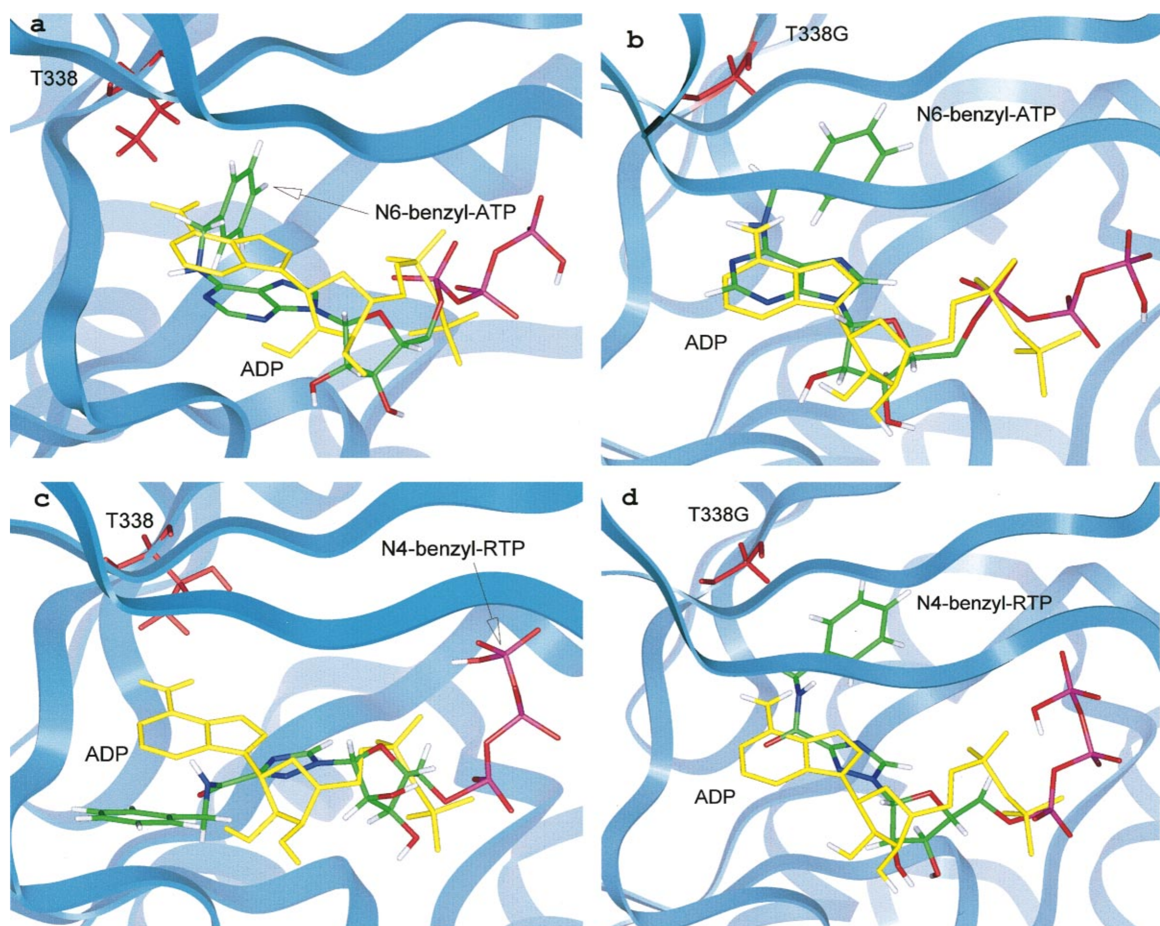


Figure 4. Graphic representation of docking results. N^6 (benzyl) ATP (a, b) and N^4 (benzyl) RTP (c, d) docked to wild type c-Src and v-Src-as1 overlaid with ADP from the experimental structure solved by X-ray crystallography.

moiety. The benzyl 'bump' of N^6 (benzyl) ATP fits into the engineered pocket as predicted by our general binding model.[26]

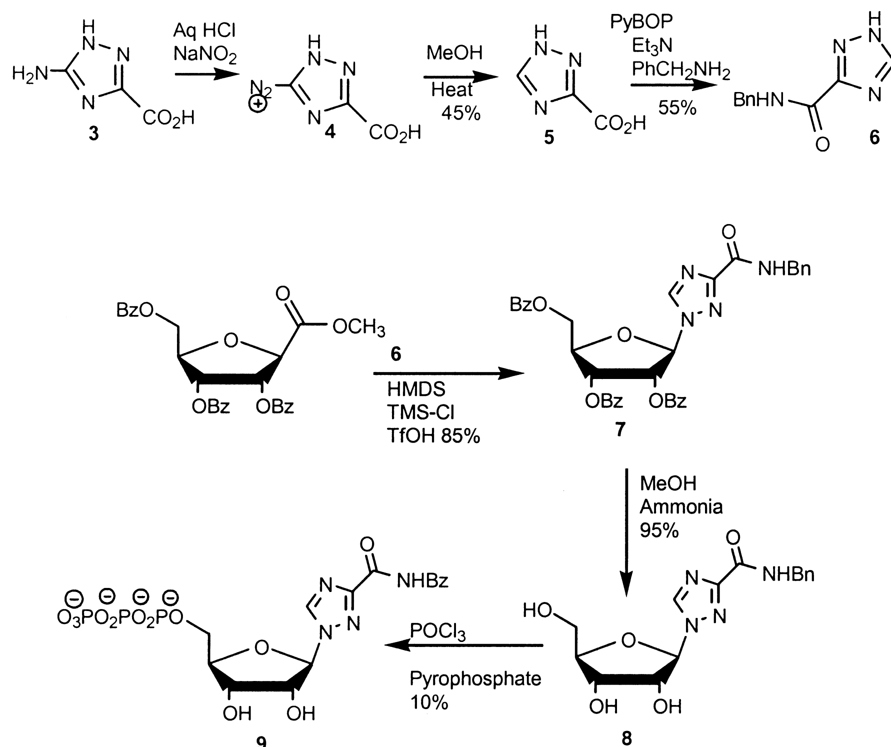
Furthermore, when N^6 (benzyl) ATP was docked to c-Src, which lacks the engineered pocket to accommodate the benzyl 'bump', no consensus binding orientation was found (one orientation is shown in Fig. 4a). This confirms the experimental observation that N^6 (benzyl) ATP cannot be accommodated by the wild type active site. These calculations are in agreement with the experimental observation that N^6 (benzyl) ATP is orthogonal to wild type v-Src and is an efficient substrate of v-Src-as1.[11] The ability of computer modeling to confirm N^6 (benzyl) ATP binding to c-Src-as1 validates the use of docking to guide our A^{**}TP design.

The A^{**}TP analog N^4 (benzyl) RTP was first docked to wild type c-Src to test orthogonality. Docking of N^4 (benzyl) RTP to wild type c-Src showed no distinct consensus binding orientation (one orientation is shown in Fig. 4c) suggesting it is poorly recognized by wild type active sites. The benzyl substituent, which confers orthogonality to N^6 (benzyl) ATP, is able to rotate out of the pocket into the solvent in the case of N^4 (benzyl) RTP because of the flexible linker (Fig. 4c). It appears that it could bind the kinase by virtue of extensive hydrophobic contacts, which compensate for the lack of specific hydrogen bonding.

Next, N^4 (benzyl) RTP was docked to c-Src-as1 to test if the new scaffold could recapitulate the appropriate interactions that make N^6 (benzyl) ATP a substrate for v-Src-as1. N^4 (benzyl) RTP appears to bind in a fashion comparable but not identical to the one observed for ADP (Fig. 4d). Root mean square deviation of common atoms is 2.2 ± 0.5 Å relative to ADP. The benzyl group of N^4 (benzyl) RTP is positioned similarly to N^6 (benzyl) ATP within the engineered pocket. However, the triazole and ribose rings do not overlay well with the imidazole and ribose rings of ADP. In addition, the N^4 of the analog is positioned in such a way that one of the hydrogen bonds (to E339) is not satisfied. These observations reflect the greater flexibility of N^4 (benzyl) RTP relative to the purine scaffold.

Overall, our docking results predict that N^4 (benzyl) RTP should behave like N^6 (benzyl) ATP, being orthogonal to wild type v-Src and accepted by v-Src-as1.

To experimentally test our modeling predictions, N^4 (benzyl) RTP was synthesized according to Scheme 1. Amino triazole carboxylic acid **3** was converted to the C^3 diazo compound and deaminated in situ by heating in methanol.[16] The resulting acid **5** was coupled to benzylamine with PyBOP.[13] Glycosylation of **6** was performed using the procedure of Vorbruggen[14] and the resulting product **7** deprotected by treatment with ammonia in

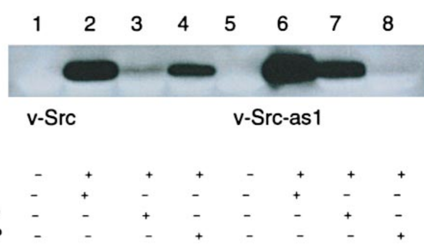


Scheme 1.

methanol to afford **8**. Addition of the triphosphate moiety to yield N^4 (benzyl) RTP **9** was carried out as described in Ludwig et al.[28]

The ability of the wild type v-Src and v-Src-as1 to utilize N^4 (benzyl) RTP as a phosphodonor was tested in a kinase-mediated reaction. Truncated forms of v-Src and v-Src-as1 Δ(77–225) were expressed as GST fusions from *E. coli* and purified by glutathione affinity chromatography. A green fluorescent protein modified with an optimized Src phosphorylation site (IYGEF addition at position 235, GFP_{235}IYGEF) served as an exogenous protein substrate.[15] Phosphorylated GFP_{235}IYGEF was detected with a monoclonal antiphosphotyrosine specific antibody, 4G10.

The results from this assay show that GFP_{235}IYGEF is an efficient kinase substrate (Fig. 5, lane 1 and 2). As a control, the I338G mutation sensitizes v-Src-as1 to the orthogonal analog, N^6 (benzyl) ATP (Fig. 5, lanes 3 and 7).



GFP-IYGEF	-	+	+	+	-	+	+	+
ATP	-	+	-	-	-	+	-	-
N(6) (benzyl) ATP	-	-	+	-	-	-	+	-
N(4) (benzyl) RTP	-	-	-	+	-	-	-	+

Figure 5. GFP_{235}IYGEF western blot assay of wild type v-Src (lanes 1–4) and v-Src-as1 (lanes 5–8) with various nucleotides. Lane **1**, **5**, no GFP control; lane **2**, **6**, 100 μM ATP; lane **3**, **7**, 100 μM N^6 (benzyl) ATP; lanes **4**, **8**, 100 μM N^4 (benzyl) RTP.

The data on the new A^{**}TP analog, N^4 (benzyl) RTP is surprising based on our modeling predictions. First, N^4 (benzyl) RTP is not orthogonal to wild type v-Src as indicated by the amount of phospho-GFP product (Fig. 5, lane 4). The structural modifications to N^6 (benzyl) ATP to give N^4 (benzyl) RTP make the nucleotide lose its orthogonality, presumably due to the flexible linker attaching the benzyl 'bump'. This flexible linker must give sufficient conformational freedom to allow the benzyl moiety of N^4 (benzyl) RTP to swing out of the wild type active site. Such a conformation can been seen in the docked structure of N^4 (benzyl) RTP to wild type c-Src (Fig. 4c). This alternate binding mode is not available to N^6 (benzyl) ATP (Fig. 4a).

An even more striking deviation from our design hypothesis is that adding the I338G 'hole' (v-Src-as1) abrogated the ability of N^4 (benzyl) RTP to be a phosphodonor (Fig. 5, lane 8), exactly the opposite of design goal #3. The additional conformational flexibility of N^4 (benzyl) RTP may again explain why v-Src-as1 is not catalytically competent with N^4 (benzyl) RTP but uses N^6 (benzyl) ATP with good efficiency. The hydrophobic benzyl substituent of N^4 (benzyl) RTP may be able to bind deeper and tighter to the active site, inhibiting catalytic turnover, as suggested by our modeling studies. The modeled structure of N^4 (benzyl) RTP docked to v-Src-as1 (Fig. 4d) shows that a key hydrogen bond from the amide N–H to the protein backbone carbonyl of glutamate 339 is not satisfied. It may also be that N^4 (benzyl) RTP binds the active site in a catalytically unproductive orientation. In any case, N^4 (benzyl) RTP does not serve as an alternate substrate for v-Src-as1 as we expected.

These results suggest that N^4 (benzyl) RTP is not a good

$A^{**}TP$ for an orthogonal protein kinase. However, the assay in Fig. 5 was performed in the absence of ATP. Our modeling predicts weak binding of N^4 (benzyl) RTP by wild type v-Src; a weak binder should compete poorly with the natural substrate (ATP) for wild type kinase active sites.

Since the impetus of the new design goals is to be able to trace kinase substrates in cells, we asked if N^4 (benzyl) RTP is conditionally orthogonal to wild type v-Src in the presence of the competitive phosphodonor, ATP. This was carried out by measuring N^4 (benzyl) RTP inhibition of a wild type v-Src/[γ^{32}P] ATP reaction. A trace amount (nM) of [γ^{32}P] ATP was added to wild type v-Src and the GFP_{235}IYGEF protein substrate in kinase buffer. N^4 (benzyl) RTP was added at varying concentrations. No inhibition was observed with up to 100 μM N^4 (benzyl) RTP (Fig. 6a). This result demonstrates that in the presence of even nanomolar amounts of ATP, N^4 (benzyl) RTP is a very poor alternate substrate for wild type v-Src ($IC_{50}>1$ mM). Therefore, N^4 (benzyl) RTP satisfies design goal #3 under cellular conditions where the ATP concentration is >1 mM and thus is *conditionally* orthogonal to wild type kinases, and is potentially useful as a substrate for an engineered kinase in a substrate labeling experiment.

The structure activity relationship of N^4 (benzyl) RTP and v-Src-as1 was also explored using the same competition assay. Again, v-Src-as1 and GFP_{235}IYGEF were incubated with a trace amount of [γ^{32}P] ATP and N^4 (benzyl) RTP was added at varying concentrations. The antiphosphotyrosine blot shows that N^4 (benzyl) RTP has remarkably high affinity for the v-Src-as1 active site ($IC_{50}<1$ μM), (Fig. 6b). This result, although disappointing, suggests that the structural modifications converting N^6 (benzyl) ATP to N^4 (benzyl) RTP do not abolish interaction between the nucleotide and kinase. However, we designed N^4 (benzyl) RTP to be phosphodonor, not an inhibitor. If N^4 (benzyl) ATP is not catalytically competent due to its high affinity, its orientation may be modulated by changing the nature of the chemical 'bump' or engineered pocket to achieve catalysis.

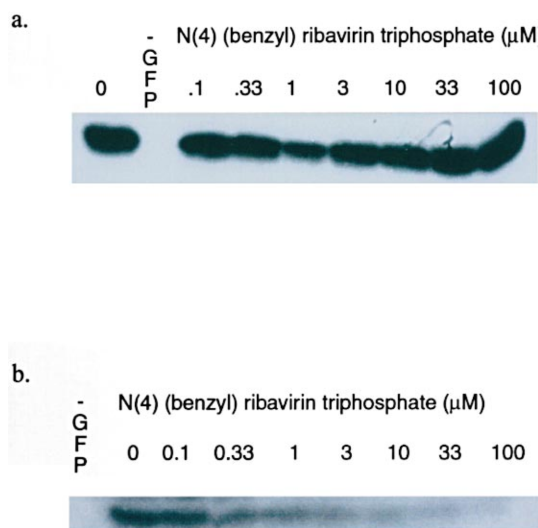


Figure 6. Inhibition of wild type v-Src (a) and v-Src-as1 (b) by N^4 (benzyl) RTP. A trace amount of [γ^{32}P] ATP was incubated with kinase, GFP_{235}IYGEF protein substrate and varying amounts of N^4 (benzyl) RTP in kinase buffer.

Conclusion

Reengineering small molecule/protein interfaces to achieve high specificity has been a powerful tool in the design of kinase inhibitors and small molecule dimerizers.[21] Rational remolding of binding surfaces requires sophisticated modeling of noncovalent interactions and chemical intuition. A significantly more difficult proposition, the engineering of a new catalytic binding site also requires engineering new binding interactions, but this alone does not guarantee catalysis. Enzyme catalysis requires subtleties of reactive group positioning and a fine balance of substrate binding, transition state stabilization and product release. Our initial efforts to design a second generation of unnatural nucleotide triphosphate ($A^{**}TP$)/engineered kinase underscore the difficulty of capturing these elements in a rationally designed system.

Although the N^4 (benzyl) RTP molecule is not strictly orthogonal to wild type v-Src as we hoped, the affinity of the active site for N^4 (benzyl) RTP is >100 fold lower than for ATP. In a substrate labeling experiment, cellular ATP will out compete N^4 (benzyl) RTP with wild type kinases. The nucleotide will behave as a dead substrate for wild type kinases under the high ATP concentrations found in cells.

Another difficulty encountered for this new $A^{**}TP$ analog is a result of removing the N^2 and C^3 atoms from the purine ring to generate a smaller base. The added flexibility yielded an $A^{**}TP$ that has remarkable affinity for the v-Src-as1 kinase containing the engineered pocket yet is not a competent substrate. Different I338G-like mutations in v-Src-as1 may modulate the affinity and orientation of N^4 (benzyl) RTP and allow catalysis. Similarly, other chemical 'bumps' may modulate the orientation and affinity of interaction, and such experiments on newer generations of $A^{**}TP$ analogs are underway. Potentially, alternatives to rational design such as error prone PCR, phage display or gene shuffling could be used to identify mutant kinases that are efficient catalysts with N^4 (benzyl) RTP.

Materials and Methods

Docking

We used a modified molecular docking suite, AutoDock, originally developed at the Scripps Research Institute.[17] The suite is based on the simulated annealing algorithm applying adiabatic cooling as the docking process progresses. It includes binding energy grid calculation program, evaluating interaction energies in every point of the scoring grid. The molecule of the ligand is processed to include electrostatic charges, as well as information about rotatable bonds. The docking routine introduces stepwise translational or rotational changes to the small molecule evaluating its binding parameters via the scoring grid, thus, moving it in the direction of descending energy gradient. Binding energy is evaluated as a sum of

non-bonded and electrostatic interactions, as well as hydrogen bonding. The ligand is flexible, whereas the protein target is kept rigid.

In order to add reliability to the performance of the program, we have introduced elements modeling hydrophobic interactions within the binding site. Among other improvements are introduction of additional atom types, including 'polar hydrogen' type and hydrogen bonding potential energy function with a higher attraction component to optimize modeling of hydrogen bonds.[22] The most apparent improvement has been observed for protein targets with relatively open binding sites lacking multiple Van der Waals contacts and/or hydrogen bonding. In such cases, as it could be seen from analysis of the binding site, hydrophobic effects played major role in positioning the small molecule.

Each experiment included 25 independent docking runs to ensure statistical significance of the results. Every run started with the small molecule placed outside of the binding site approximately 15 to 20 Å from the target. Resulting orientations have been stored as separate coordinate files and clustered according to their scores. We used SPARTAN[18] to minimize the structures of the ligands and to derive electrostatic charges using MNDO basis set. The protein atoms have been assigned charges with CVFF91 force field as a part of molecular modeling suite INSIGHTII.[19] All docking experiments were performed on a workstation Silicon Graphics O2.

v-Src mutagenesis, expression and purification was carried out as previously described.[10]

Kinase reactions were carried out in 20 mM Tris pH 8, with 100 mM KCl and 10 mM $MgCl_2$. 3 μg GFP-IYGEF and 1–10 ng kinase was added[15] and the reactions carried out at room temperature for 20 min. The reactions were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Phosphotyrosine was detected by 4G10, an antiphosphotyrosine specific monoclonal antibody followed by GαM HRP (Upstate Biotechnology) chemiluminescence.

IC_{50} determinations for N^4 (benzyl) RTP was carried out by incubating wild type and I338G v-Src kinase with 10 μCi [$\gamma^{32}P$] ATP and varying amounts of cold N^4 (benzyl) RTP in 20 mM Tris pH 8, 100 mM KCl, and 10 mM $MgCl_2$. These reactions were separated by SDS-PAGE electrophoresis, dried and exposed to X-ray film overnight.

Chemical Synthesis

1,2,4-Triazole-3-carboxylate (5) was prepared by the method of Grinshtein in Ref. 16. The carboxylate is thermally labile and used without further purification.

1,2,4-Triazole-3-benzamide (6). Compound **5**, (1.3 g, 11.5 mmol) was suspended in dry acetonitrile (60 mL). To this, triethylamine (2.5 mL, 34 mmol) was added, upon which the solution turned clear. To this solution, PyBOP (5.0 g, 11.3 mmol) was added and followed immediately by benzylamine (1.5 mL, 13 mmol). The reaction was stirred at rt under argon for 2 h. The insoluble tan product was collected by filtration and washed with saturated aqueous sodium bicarbonate and methanol to yield **6** (55%). ^1H NMR (DMSO-d_6, 400 MHz) δ 9.12 (s, 1H), 8.43 (s, 1H), 7.21 (m, 3H), 7.18 (m, 3H), 4.41 (d, J=4.5 Hz, 2H). HRMS (+EI) 202.0855; calcd 202.2057 for $C_{10}H_{10}N_4O$.

N^4 (Benzyl) ribavirin 2,3,5 tri-*O*-benzoate (8). To β-D-ribofuranose 1-acetate 2,3,5-tri-*O*-benzoate (0.74 g, 1.48 mmol) in dry acetonitrile, compound **6** (0.3 g, 1.48 mmol) was added and the suspension stirred at rt under argon. Hexamethyldisilazane (0.38 mL, 1.6 mmol) was added and the reaction stirred for 0.5 h. To this, chlorotrimethylsilane (0.29 mL, 1.6 mmol) was added, followed immediately by trifloromethanesulfonic acid (0.20 mL, 2.1 mmol), upon which the solution turned clear. The solution was stirred at rt under argon for 5 h. The reaction was quenched by the addition of saturated sodium bicarbonate (200 mL), and the product was extracted with EtOAc (3×200 mL). The organic layers were combined and dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on silica gel (2:1 CH_2Cl_2:EtOAc, R_f=0.5) to yield **8** (0.81 g, 85%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.40 (s, 1H), 8.03 (m, 2H), 7.91 (m, 4H), 7.48 (m, 5H), 7.32 (m, 10H), 6.32 (d, J=3.2 Hz, 1H), 6.17 (m, 1H), 6.07 (t, J=5.6 Hz, 1H), 4.75 (m, 2H), 4.62 (m, 3H).

N^4 (Benzyl) ribavirin (9). Compound **8**, (0.8 g, 1.25 mmol) was stirred in 2 M ammonia in methanol (25 mL) for 2 days at rt. The solvent was removed in vacuo and the residue chromatographed on silica gel (7:1 EtOAc/MeOH) to yield **9** (0.41 g, 96%, R_f=0.4). ^1H NMR (DMSO- d_6, 400 MHz) δ 9.04 (t, J=4.8 Hz, 1H), 8.85 (s, 1H), 7.27 (m, 5H), 5.79 (d, J=3 Hz, 1H), 5.54 (d, J=4.2 Hz, 1H), 5.16 (d, J=4.2 Hz, 1H), 4.87 (t, J=3.9 Hz, 1H), 4.40 (d, J=4.5 Hz, 2H), 4.31 (q, J=3.6 Hz, 1H), 4.10 (q, J=3.9 Hz, 1H), 3.91 (q, J=3.3 Hz, 1H), 3.57 (m, 1H), 3.46 (m, 1H). HRMS (+EI) 334.1263; calcd 334.3322 for $C_{15}H_{18}O_5N_4$.

N^4 (Benzyl) ribavirin triphosphate (10). Benzyl ribavirin **9** (0.06 g, 0.18 mmol) was dissolved in trimethyl phosphate (0.5 mL) and stirred under argon on ice. Phosphorous oxychloride (60 μL, 0.65 mmol) was added and the reaction stirred at 0° for 1.5 h. Bis (tributylammonium) pyrophosphate [prepared by dissolving 80 % pyrophosphoric acid (0.18 g, 0.81 mmol) in 1:1 water/ethanol (2 mL) followed by addition of tributylamine (0.5 mL, 2.1 mmol) and evaporated in vacuo to dryness] in dry DMF (1 mL) was added quickly and stirred for 1 min. The reaction was quenched by addition of 1 M TEA-B pH 7 (5 mL) and the solvent was removed in vacuo. The residue was dissolved in water (2 mL) and purified by HPLC (Rainin) over a Poros HQ/M column, 0–1 M gradient of TEA-B buffer pH7. MS. (-ESI), m/z[M−1]=573.

Acknowledgements

Thanks to the Balmain lab, P. J. Alaimo and other members of the Shokat lab for helpful comments on the text. This

work was supported by a grant from the National Institutes of Health (2R01CA70331) and the Sandler Foundation.

References

1. Hunter, T. *Cell* **2000**, *100*, 113–127.
2. Hunter, T.; Cooper, J. A. *Ann. Rev. Biochem.* **1985**, *54*, 897–930.
3. Bishop, J. *Cell* **1985**, *42*, 23–28.
4. Schlessinger, J. *Trends Biochem. Sci.* **1988**, *13*, 443–447.
5. Pawson, T. *Nature* **1995**, *373*, 573–580.
6. Pawson, T.; Scott, J. D. *Science* **1997**, *278*, 2075–2080.
7. Songyang, Z.; Carraway, K. L. I.; Eck, M. J.; Harrison, S. C.; Feldman, R. A.; Mohammadi, M.; Schlessinger, J.; Hubbard, S. R.; Smith, D. P.; Eng, C.; Lorenzo, M. J.; Ponder, B. A. J.; Mayer, B. J.; Cantley, L. C. *Nature* **1995**, *373*, 536–539.
8. Kamps, M. P.; Sefton, B. M. *Oncogene Res.* **1988**, *3*, 105–115.
9. Brown, M. T.; Cooper, J. A. *Biochim. Biophys. Acta* **1996**, *1287*, 121–149.
10. Shah, K.; Liu, Y.; Deimengian, C.; Shokat, K. M. *Proc. Nat. Acad. Sci. USA* **1997**, *94*, 3565–3570.
11. Liu, Y.; Shah, K.; Yang, F.; Witucki, L.; Shokat, K. M. *Chem. Biol.* **1998**, *5*, 91–101.
12. Witkowski, J. T.; Robins, R. K.; Sidwell, R. W.; Simon, L. N. *J. Med. Chem.* **1972**, *15*, 1150–1154.
13. Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205–208.
14. Vorbruggen, H.; Krolikiewicz, K.; Bennua, B. *Chem. Ber.* **1981**, *114*, 1234–1255.
15. Yang, F.; Liu, Y.; Bixby, S.; Friedman, J.; Shah, K.; Shokat, K. M. *Anal. Biochem.* **1999**, *266*, 167–173.
16. Chiipen, G. I.; Grinshtein, V. Y. *Chem. Heterocycl. Comp.* **1965**, *1*, 420–421.
17. Morris, G. M.; Goodsell, D. S.; Huey, R.; Olson, A. J. *J. Comput.-Aided Mol. Des.* **1996**, *10*, 293–304.
18. *SPARTAN 5.1*, Wavefunction, Inc. 18401 Von Karman Ave., Ste. 370, Irvine, CA 92612, USA.
19. *INSIGHTII 98.0* Molecular Simulations, Inc., 9685 Scranton Road, San Diego, CA 92121-3752 USA.
20. Xu, W.; Harrison, S.; Eck, M. J. *Nature* **1997**, *385*, 595–602.
21. Clackson, T. *Curr. Opin. Struct. Biol.* **1998**, *8*, 451–458.
22. Buzko, O.; Bishop, A.; Liu, Y.; Shokat, K. M., in preparation.
23. Bishop, A. C.; Shokat, K. M. *Pharmacol.Ther.* **1999**, *82*, 337–346.
24. Voet, D.; Voet, J. G. *Biochemistry*; Wiley: New York, 1995, p 430.
25. Position of the backbone of the glycine mutant of p38 MAP kinase is identical to that of the wild type protein, as derived from crystallographic studies (unpublished results).
26. Liu, Y.; Shah, K.; Yang, F.; Witucki, L.; Shokat, K. M. *Bioorg. Med. Chem.* **1998**, *8*, 1219–1226.
27. Sicheri, F.; Moarfi, I.; Kuriyan, J. *Nature* **1997**, *385*, 602–609.
28. Ludwig, J. *Acta. Biochim. Biophys. Acad. Sci. Hung.* **1981**, *16*, 131–133.
29. Johnson, W. T.; Zhang, P.; Bergstrom, D. E. *Nucleic Acids Res.* **1997**, *25*, 559–567.