

Design and Use of Analog-Sensitive Protein Kinases

Many protein kinases can be engineered to accept analogs of ATP that are not efficiently used by wild-type kinases. These engineered kinases, which are referred to as “analog-sensitive” or “*-as*” alleles, are also often sensitive to protein kinase inhibitor variants that do not block the activity of nonmutant kinases. Selective *in vitro* use of radiolabeled ATP analogs by *-as* kinases can be exploited to identify the direct phosphorylation targets of individual kinases in complex extracts. In organisms in which it is practical to replace wild-type kinase genes with engineered alleles, the *in vivo* activity of an *-as* kinase can be reversibly blocked with an allele-specific inhibitor. Thus, analog-sensitive kinases can be effective tools for discovery of the cellular functions and phosphorylation targets of individual enzymes. A theoretical background for the design and use of these alleles is discussed in the Commentary section at the end of this unit, as are strategies for construction of candidate *-as* alleles of any kinase.

In vivo use of analog-sensitive kinase alleles requires access to kinase inhibitor analogs. This unit describes protocols for synthesis of 1NM-PP1 and 1NA-PP1 (see Basic Protocols 1 and 2), which are among the most effective allele-specific inhibitors of *-as* kinases. These protocols assume knowledge of organic synthesis, and it is advisable to work with a chemist that can provide assistance if one is not familiar with organic chemistry methods. Under no circumstances should one attempt these protocols if not comfortable with safety procedures fundamental to synthetic organic chemistry. Much of the equipment required for these procedures is uncommon in molecular biology groups but readily accessible in chemistry laboratories. The authors have provided information relevant to analysis of NMR spectra for researchers familiar with this technique. A detailed description of product analysis is, however, beyond the scope of this section.

The authors have also supplied a protocol for production of γ -³²P-labeled *N*⁶(benzyl)ATP, an ATP analog that is commonly selectively used by *-as* kinases, from the ADP analog (see Basic Protocol 3 and Support Protocols 1 and 2). This procedure employs enzymatic phosphorylation of *N*⁶(benzyl)ADP by immobilized nucleoside diphosphate kinase (NDPK), and can be used to produce other γ -labeled ATP analogs from ADP analogs (Mourad and Parks, 1966; Kraybill et al., 2002). Commercial availability of *N*⁶(benzyl)ADP, as well as other ADP analogs, is still somewhat limited. A method for synthesis of *N*⁶(benzyl)ADP has been published (Shah et al., 1997; Shah and Shokat, 2002). Labeling of ADP analogs by the procedure provided here requires moderately large quantities (~2.5 mCi) of [γ -³²P]ATP, and it is therefore advisable to ensure that this raises no issues with one's license to use radioactive materials.

A final protocol (see Basic Protocol 4) describes the assays for testing inhibition of analog-sensitive kinases in yeast.

NOTE: Although the protocols described in this unit are directly derived from published material, it is important to note that the use of analog-sensitive kinases, analog-sensitive kinase inhibitors, and ATP analogs is protected by U.S. patents 6,610,483; 6,521,417; 6,390,821 and 6,383,790, as well as patents in other countries, which are owned by Princeton University and exclusively licensed to Cellular Genomics Incorporated (CGI) of Branford, Connecticut. Information regarding accessing rights to make and use the technology can be obtained by contacting CGI.

SYNTHESIS OF 1NM-PP1

This protocol and the one that follows for 1NA-PP1 (see Basic Protocol 2) are based on previously published methods (Bishop et al., 1998; Bishop et al., 1999). The synthetic schemes are diagrammed in Figure 18.11.1. A representative NMR spectrum is presented in Figure 18.11.2.

Materials

1-naphthylacetic acid (Acros Organics)
Hexane
N,N-dimethylformamide (DMF; Aldrich)
Oxalyl chloride (Aldrich)
Tetrahydrofuran (THF; Aldrich)
Sodium hydride (Aldrich)
Malononitrile (Aldrich)
1 N H₂SO₄
Ethyl acetate
MgSO₄, anhydrous
1,4-dioxane (Aldrich)
Sodium bicarbonate (Aldrich)
Dimethyl sulfate (Aldrich)
Diethyl ether (Et₂O)
Silica Gel 60 (pore size 0.040 to 0.063 mm; Merck)
Ethanol (Aldrich)
Triethylamine (Aldrich)
tert-butylhydrazine hydrochloride (Aldrich)
Chloroform (CHCl₃)
1% (v/v) methanol in CHCl₃
Formamide (Aldrich)
Activated charcoal
Celite

Büchi Rotavapor Model R-200 or equivalent rotary evaporator
Ice bath
Separatory funnel
Oil baths, 80°, 100°C, 180°C
10-in. (25.4-cm) length × 2-in. (5.0-cm) i.d. chromatography column
TLC plates (Silica Gel F₂₅₄; EM Science) and tank
Reflux condenser
Filter paper
Buchner funnel

Synthesize 1-naphthylacetylmalononitrile

1. Dissolve 3.72 g 1-naphthylacetic acid (20.0 mmol) in 100 ml hexane.
2. Add 146 mg *N,N*-dimethylformamide (DMF; 2.0 mmol).
3. Add, dropwise, 12.7 g oxalyl chloride (100 mmol).
4. Wait for gas evolution to stop (~30 min), then remove solvent under reduced pressure in a Büchi Rotavapor or equivalent rotary evaporator.
5. Redissolve the residue (containing the acid chloride) in 30 ml tetrahydrofuran (THF).
6. Suspend 1.06 g sodium hydride (44.0 mmol) in 10 ml THF.

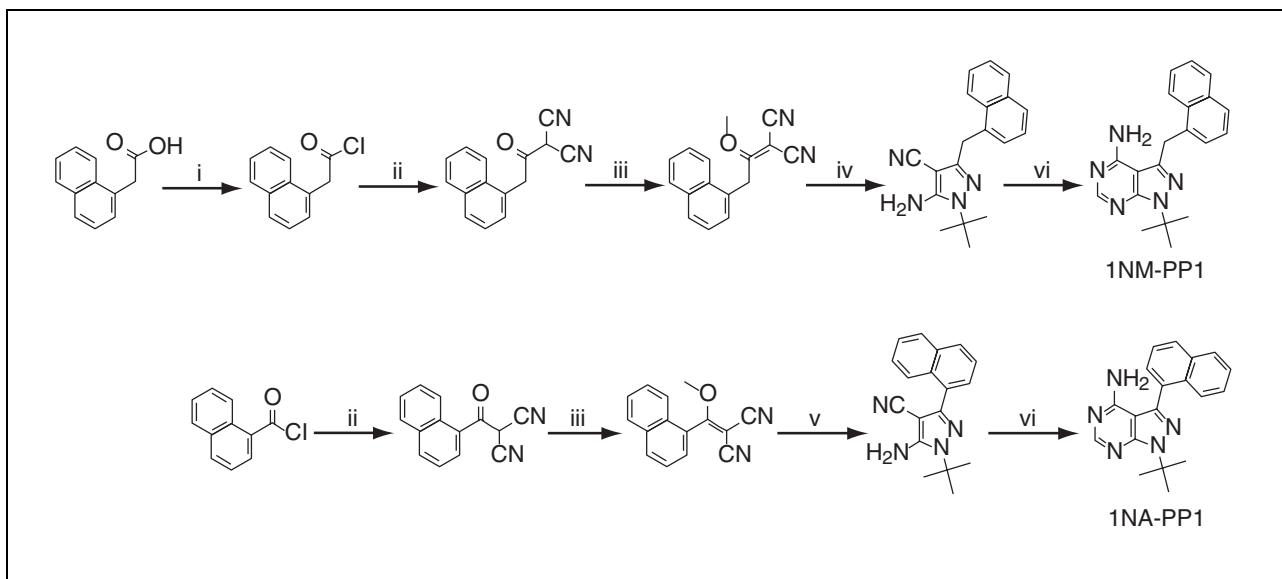


Figure 18.11.1 Synthetic schemes of 1NM-PP1 and 1NA-PP1. Conditions: (i) 5 eq oxalyl chloride, 0.1 eq DMF, room temperature, 1 hr; (ii) 2 eq NaH, 1 eq malononitrile, THF, 0°C to room temperature, 1 hr; (iii) 8 eq NaHCO₃, 5 eq dimethyl sulfate, dioxane/water (6:1), reflux, 1 hr; (iv) 2 eq triethylamine, 1 eq *tert*-butylhydrazine hydrochloride, EtOH, reflux, 1 hr; (v) 1 eq *tert*-butylhydrazine, DMF, room temperature, 1 hr; (vi) formamide, 180°C, 10 hr.

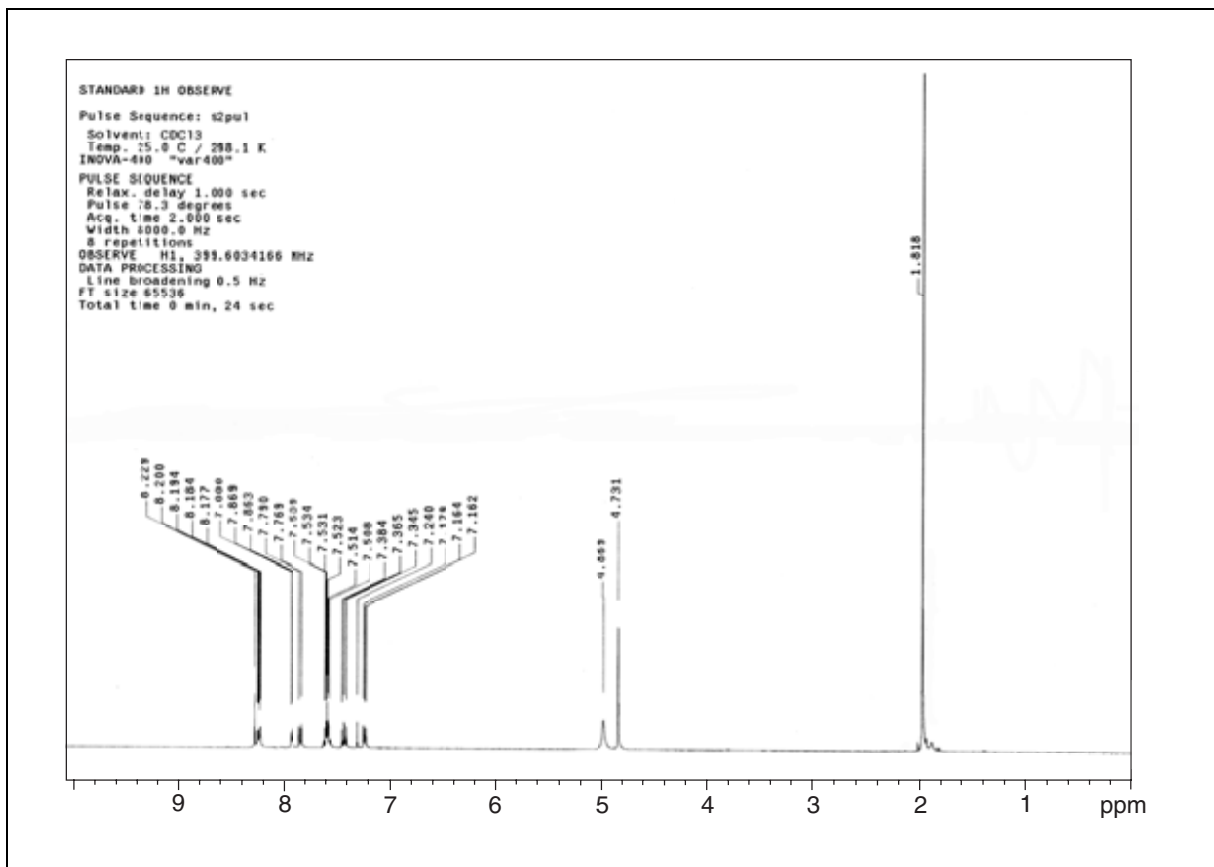


Figure 18.11.2 NMR data for 1-*tert*-butyl-3-naphthalen-1-ylmethyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine (1NM-PP1). Compound is white powder; ¹H NMR (CDCl₃, 400 MHz) δ 1.82 (s, 9H), 4.73 (s, 2H), 4.87 (br s, 2H), 7.17 (d, J = 7 Hz, 1H), 7.37 (t, J = 8 Hz, 1H), 7.52 (m, 2H), 7.78 (d, J = 8 Hz, 1H), 7.87 (m, 1H), 8.19 (m, 1H), 8.23 (s, 1H); ¹³C NMR (CDCl₃, 100 MHz) δ 29.2, 32.7, 60.0, 101.1, 123.5, 125.6, 125.8, 126.2, 126.6, 128.2, 128.9, 131.9, 133.9, 134.0, 140.5, 154.5, 154.7, 157.6, HRMS (EI) molecular ion calculated for C₂₀H₂₁N₅ 331.17993, found 331.17951.

7. Dissolve 1.45 g malononitrile (22.0 mmol) in 10 ml THF and add dropwise to the NaH/THF suspension (step 6) on an ice bath.
8. Add the THF solution of acid chloride from step 5 dropwise to the malononitrile solution with vigorous stirring. Stir for 1 hr.
9. Add ~15 ml of 1 N H₂SO₄ and 50 ml water.
10. Extract three times, each time with 100 ml ethyl acetate, in a separatory funnel.

The ethyl acetate phase is the top phase.

11. Combine ethyl acetate extracts and dry by addition of ~5 g anhydrous MgSO₄.
12. Evaporate the solvent under reduced pressure in a Büchi Rotavapor or equivalent rotary evaporator, to yield 1-naphthylacetylmalononitrile, a yellow solid.

Yield should be ~70%.

Synthesize 1-naphthylmethyl(methoxy)methylidenemalononitrile

13. Dissolve 2.23 g 1-naphthylacetylmalononitrile (from step 12; 10.0 mmol) in a mixture of 18 ml dioxane and 3 ml water.
14. Add 6.8 g sodium bicarbonate.
15. Add 4.9 ml of dimethyl sulfate with vigorous stirring. Heat the reaction mixture at 100°C on an oil bath for 2 hr.
16. Cool the reaction mixture to room temperature.
17. Dilute with 70 ml water and extract three times, each time with 100 ml Et₂O, in a separatory funnel.
18. Combine the Et₂O extracts and dry over anhydrous MgSO₄.
19. Evaporate the Et₂O under reduced pressure in a Büchi Rotavapor or equivalent rotary evaporator to give an oil.
20. Prepare a 10-in. (25.4-cm) × 2-in. (5.0-cm) column containing 200 g Silica Gel 60, saturated with 1:1 Et₂O/hexane (the mobile phase).
21. Dissolve the oil from step 19 in a small amount of mobile phase and apply to column. Elute with mobile phase. Sample the eluate as it emerges from the column and analyze by thin-layer chromatography (TLC) using 1:1 Et₂O/hexanes as the mobile phase. Pool fractions containing a species with R_f of ~0.24.
22. Evaporate solvent under reduced pressure in a Büchi Rotavapor or equivalent rotary evaporator to produce a white crystalline solid 1-naphthylmethyl(methoxy)methylidenemalononitrile.

Yield should be ~75%.

Synthesize 5-amino-1-tert-butyl-3-(1'-naphthylmethyl)-4-cyano-1H-pyrazole

23. Dissolve 1.74 g of 1-naphthylmethyl(methoxy)methylidenemalononitrile (from step 22; 7.0 mmol) in 50 ml ethanol.
24. Add 1.51 g triethylamine (15.0 mmol) and 0.92 g *tert*-butylhydrazine hydrochloride (7.4 mmol). Reflux at 80°C on an oil bath for 1 hr.
25. Cool the mixture and evaporate the solvent under reduced pressure.
26. Suspend the residue in water and extract three times, each time with 50 ml CHCl₃, in a separatory funnel.

The CHCl₃ phase is the top phase.

27. Combine the CHCl_3 extracts, dry over anhydrous MgSO_4 , and evaporate the solvent under reduced pressure in a Büchi Rotavapor or equivalent rotary evaporator, to give a yellow solid.
28. Prepare a 10-in. (25.4-cm) \times 2-in. (5.0-cm) column containing 100 g Silica Gel 60, saturated with CHCl_3 (the mobile phase).
29. Dissolve the yellow solid in a minimal volume of CHCl_3 and apply to the column. Elute with 1% methanol in CHCl_3 . Sample the eluate as it emerges from the column and analyze by thin-layer chromatography (TLC) using 1% methanol in CHCl_3 as the mobile phase. Pool fractions containing a species with R_f of ~ 0.1 .
30. Evaporate solvent to obtain a white solid, 5-amino-1-*tert*-butyl-3-(1'-naphthylmethyl)-4-cyano-1*H*-pyrazole.

Yield should be $\sim 75\%$.

Synthesize 4-amino-1-*tert*-butyl-3-(1'-naphthylmethyl)-1*H*-pyrazolo[3,4-*d*]-pyrimidine (1NM-PP1)

31. Suspend 0.9 g 5-amino-1-*tert*-butyl-3-(1'-naphthylmethyl)-4-cyano-1*H*-pyrazole (from step 30; 3.4 mmol) in 20 ml of formamide.
32. Heat this suspension at 180°C on an oil bath, stirring with a reflux condenser for 10 hr.
33. Cool reaction mixture and dilute with 80 ml water.
34. Collect precipitate by filtration on paper in a Buchner funnel.
35. Dissolve precipitate in 40 ml room temperature ethanol.
36. Add 1 g powdered activated charcoal. Boil at 80°C for 10 min.
37. Pour a celite pad on a Buchner funnel using a slurry of celite in ethanol. Filter the hot mixture through this celite pad. Collect the filtrate.
38. Evaporate the solvent to obtain 1NM-PP1 as a white powder.

Expect yields of $\sim 75\%$.

SYNTHESIS OF 1NA-PP1

1NA-PP1 can be synthesized in the same manner as 1NM-PP1, with two differences. First, the acid chloride (1-naphthoyl chloride) is directly used in the first step of the synthesis, since it is commercially available. Second, a different condition is used in the reaction that forms the pyrazole ring, giving much better yields than the original procedure. A representative NMR spectrum is presented in Figure 18.11.3.

Materials

1-naphthoyl chloride (Aldrich)
 Tetrahydrofuran (THF; Aldrich)
 Sodium hydride (Aldrich)
 Malononitrile (Aldrich)
 1 N H_2SO_4
 Ethyl acetate
 MgSO_4 , anhydrous
 1,4-dioxane (Aldrich)
 Sodium bicarbonate (Aldrich)
 Dimethyl sulfate (Aldrich)

**BASIC
 PROTOCOL 2**

**Analysis of
 Protein
 Phosphorylation**

18.11.5

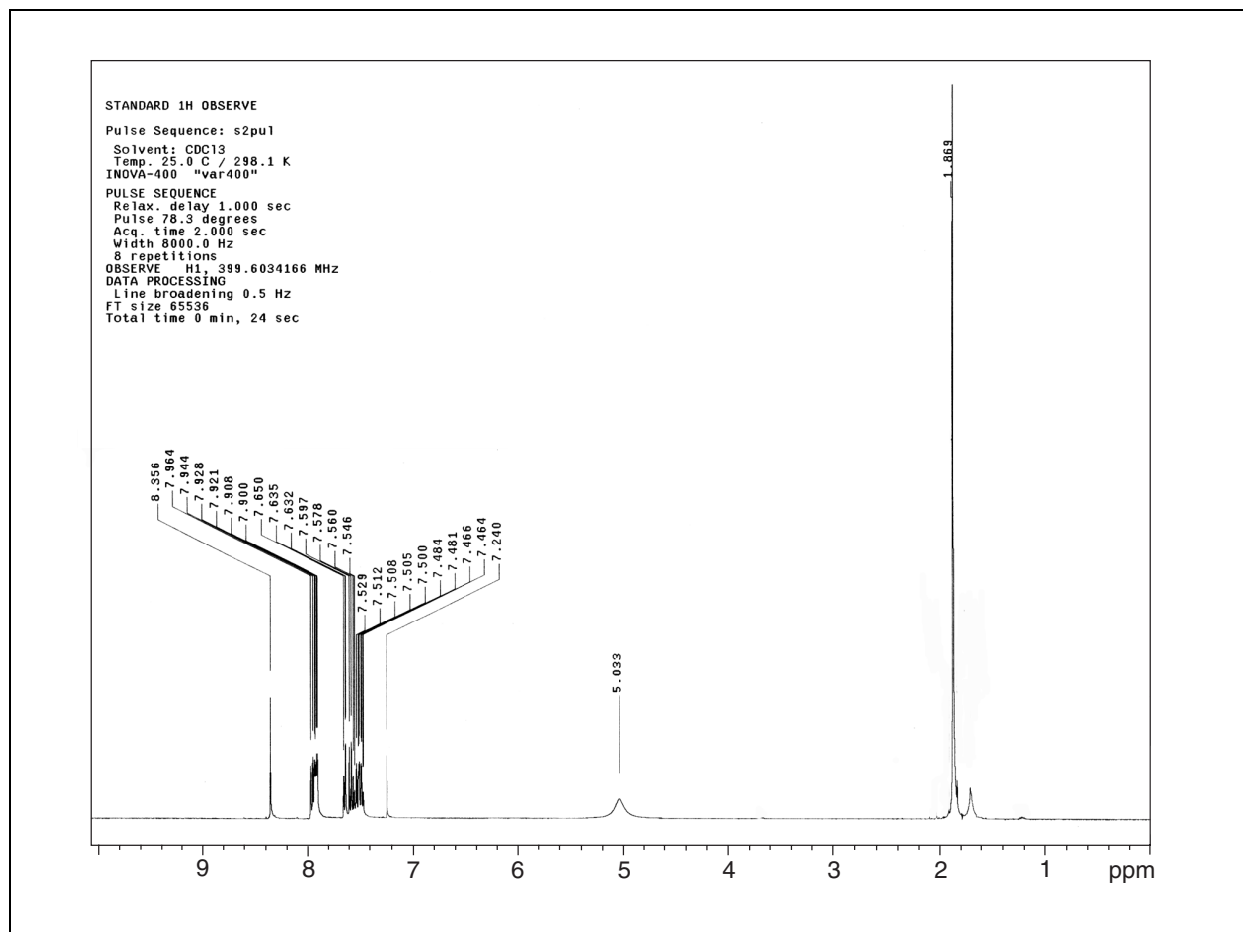


Figure 18.11.3 NMR data for 1-*tert*-butyl-3-naphthalen-1-yl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine (1NA-PP1). Compound is white powder; ^1H NMR (CDCl_3 , 400 Mhz) δ 1.87 (s, 9H), 5.04 (br s, 2H), 7.05 (m, 2H), 7.58 (t, $J = 8$ Hz, 1H), 7.64 (d, $J = 7$ Hz, 1H), 7.92 (m, 2H), 7.95 (d, $J = 8$ Hz, 1H), 8.36 (s, 1H); ^{13}C NMR (CDCl_3 , 100 Mhz) δ 29.3, 60.6, 101.5, 125.5, 125.6, 126.5, 127.1, 128.4, 128.4, 129.6, 130.6, 131.8, 134.0, 140.4, 153.9, 154.7, 157.7, HRMS (EI) molecular ion calculated for $\text{C}_{19}\text{H}_{19}\text{N}_5$ 317.16427, found 317.16247.

Diethyl ether (Et_2O)
 Silica Gel 60 (pore size 0.040 to 0.063 mm; Merck)
 Sodium ethoxide (Acros Organics)
tert-butylhydrazine hydrochloride (Aldrich)
N,N-dimethylformamide (DMF; Aldrich)
 Chloroform (CHCl_3)
 1% (v/v) methanol in CHCl_3
 Formamide (Aldrich)
 Ethanol (Aldrich)
 Activated charcoal
 Celite

Separatory funnel
 Oil baths, 80°, 100°, and 180°C
 10-in. (25.4-cm) length \times 2-in. (5.0-cm) i.d. and 8-in. (20.3-cm) length \times 1.5-in. (3.8-cm) i.d. chromatography columns
 TLC plates (Silica Gel 60 F₂₅₄; EM Science) and tank
 Reflux condenser
 Filter paper
 Buchner funnel

Synthesize 1-naphthoylmalononitrile

1. Dissolve 3.80 g (20 mmol) 1-naphthoyl chloride in 30 ml tetrahydrofuran (THF).
2. Dissolve 1.06 g sodium hydride (44.0 mmol) in 10 ml THF.
3. Dissolve 1.45 g malononitrile (22.0 mmol) in 10 ml THF and add dropwise to the NaH/THF suspension on an ice bath.
4. Add the THF solution of 1-naphthoyl chloride from step 1 dropwise to the malononitrile solution with vigorous stirring. Stir for 1 hr.
5. Add ~15 ml of 1 N H₂SO₄ and 50 ml water.
6. Extract three times, each time with 100 ml ethyl acetate in separatory funnel.

The ethyl acetate phase is the top phase.

7. Combine ethyl acetate extracts and dry by addition of anhydrous MgSO₄.
8. Evaporate the solvent to yield 1-naphthoylmalononitrile, a yellow solid.

Yield should be approximately 70%.

Synthesize 1-naphthyl(methoxy)methylidenemalononitrile

9. Dissolve 2.20 g 1-naphthoylmalononitrile (from step 8; 10.0 mmol) in a mixture of 18 ml dioxane and 3 ml water.
10. Add 6.8 g sodium bicarbonate.
11. Add 4.9 ml of dimethyl sulfate with vigorous stirring. Heat the reaction mixture at 100°C on an oil bath for 2 hr.
12. Cool the reaction mixture to room temperature.
13. Dilute with 70 ml water and extract three times, each time with 100 ml Et₂O.
14. Combine the Et₂O extracts and dry over anhydrous MgSO₄.
15. Evaporate the Et₂O under reduced pressure to give an oil.
16. Prepare a 10-in. (25.4-cm) × 2-in. (5.0-cm) column containing 200 g Silica Gel 60, saturated with 1:1 Et₂O/hexane (the mobile phase).
17. Dissolve the oil from step 15 in a small amount of mobile phase and apply to column. Elute with mobile phase. Sample the eluate as it emerges from the column and analyze by thin-layer chromatography (TLC) using 1:1 Et₂O/hexanes as the mobile phase. Pool fractions containing a species with R_f of ~0.22.
18. Evaporate solvent under reduced pressure in a Büchi Rotavapor or equivalent rotary evaporator, to produce a white crystalline solid, 1-naphthyl(methoxy)methylidenemalononitrile.

Yield should be ~75%.

Synthesize 5-amino-1-tert-butyl-3-(1'-naphthyl)-4-cyano-1H-pyrazole

19. Add 0.38 g sodium ethoxide (5.5 mmol) and 0.68 g *tert*-butylhydrazine hydrochloride (5.5 mmol) to 10 ml DMF. Stir mixture at room temperature for 0.5 hr.
20. Filter the mixture. Save the filtrate, which contains *tert*-butylhydrazine.
21. Dissolve 1.17 g 1-naphthyl(methoxy)methylidenemalononitrile (from step 18; 5.0 mmol) in 20 ml DMF. Add the *tert*-butylhydrazine-containing filtrate from step 20. Stir the reaction mixture at room temperature for 1 hr.
22. Evaporate the solvent under reduced pressure.

23. Suspend the residue in water and extract three times, each time with 50 ml CHCl_3 , in a separatory funnel.
24. Combine the CHCl_3 extracts, dry over anhydrous MgSO_4 , and evaporate the solvent to give a yellow solid.
25. Prepare an 80-in. (20.3-cm) \times 1.5-in. (3.8-cm) column containing 100 g Silica Gel 60, saturated with CHCl_3 (the mobile phase).
26. Dissolve the yellow solid in a minimal volume of CHCl_3 and apply to the column. Elute with 1% methanol in CHCl_3 . Sample the eluate as it emerges from the column and analyze by thin-layer chromatography (TLC) using 1% methanol in CHCl_3 as the mobile phase. Pool fractions containing a species with R_f of ~ 0.1 .
27. Evaporate solvent to produce a white solid, 5-amino-1-tert-butyl-3-(1'-naphthyl)-4-cyano-1H-pyrazole.

Yield should be $\sim 75\%$.

Synthesize 4-amino-1-tert-butyl-3-(1'-naphthyl)-1H-pyrazolo[3,4-d]pyrimidine (1NA-PP1)

28. Suspend 0.99 g 5-amino-1-tert-butyl-3-(1'-naphthyl)-4-cyano-1H-pyrazole (3.4 mmol) in 20 ml of formamide.
29. Heat this suspension at 180°C on an oil bath, stirring with a reflux condenser for 10 hr.
30. Cool the reaction mixture and dilute with 80 ml water.
31. Collect the precipitate by filtration on paper in a Buchner funnel.
32. Dissolve precipitate in 40 ml room temperature ethanol. Add 1 g powdered activated charcoal. Boil at 80°C for 10 min.
33. Pour a celite pad on a Buchner funnel using a slurry of celite in ethanol. Filter the hot mixture through this celite pad. Collect the filtrate.
34. Evaporate the solvent to obtain 1NA-PP1 as a white powder.

Expect yields of $\sim 75\%$.

PREPARATION OF γ - ^{32}P -LABELED N^6 (BENZYL)ATP

NDPK is a ubiquitous enzyme that catalyzes the exchange of γ phosphate between nucleoside triphosphate species and nucleoside diphosphate species by means of a bi-bi-ping-pong mechanism involving a phospho-enzyme intermediate (Weaver, 1962; Mourad and Parks, 1966). In this protocol, $6\times$ His-tagged NDPK is immobilized in the solid phase by metal affinity chromatography, and the column-bound NDPK is equilibrated with $[\gamma$ - $^{32}\text{P}]$ ATP in low magnesium to generate the labeled phospho-enzyme. The column is then washed to remove residual ATP, and a ten-fold molar excess of N^6 (benzyl)ADP is added in the presence of larger amounts of magnesium. Eluate from this stage contains a mixture of $[\gamma$ - $^{32}\text{P}]N^6$ (benzyl)ATP and N^6 (benzyl)ADP, and can be used directly in kinase reactions. This protocol can also be used to generate γ - ^{35}S -labeled nucleoside triphosphates.

**BASIC
PROTOCOL 3**

Materials

- 1:1 slurry of cobalt affinity resin (IDA-Co²⁺-Sepharose) in HBS/0.02% sodium azide (see Support Protocol 1)
- HEPES buffered saline (HBS): 150 mM NaCl/100 mM HEPES, pH 7.4
- Purified NDPK-6×His (see Support Protocol 2)
- Phosphate-buffered saline (PBS): 150 M NaCl/100 mM sodium phosphate pH 7.4 (see APPENDIX 2 for sodium phosphate buffer)
- [γ -³²P]ATP (~3000 Ci/mmol or ~7000 Ci/mmol)
- 1 mM N⁶(benzyl)ADP (Shah et al., 1997; Shah and Shokat, 2002)
- PBS (see above) containing 5 mM MgCl₂
- 2-mm glass beads (VWR)
- 1-ml disposable pipet tip
- Stand and clamp to accommodate 1-ml disposable pipet tip

NOTE: All steps are performed at room temperature.

Prepare cobalt affinity column

1. Place a 2-mm glass bead inside a 1-ml disposable pipet tip and flick the tip to make sure the bead is firmly lodged near the end. Use scissors to remove the end of the tip where it extends beyond the glass bead. Mount this miniature column in a clamp on a stand.
2. Add 200 μ l of 1:1 slurry of cobalt affinity resin in HBS/0.02% sodium azide (prepared as in Support Protocol 1) to the column prepared in step 1.
3. Add 1 ml HBS and allow the buffer to drain until level with the top of the beads.

If desired, a 1-ml pipet can be used to drive fluid flow through the beads. If this is done, be careful to avoid introducing a negative relative pressure in the column when removing the pipet, as this can disturb the beads.

Add NDPK-6×His and radiolabeled ATP to column

4. Add ~165 μ g purified NDPK-6×His.

If the NDPK is in a buffer other than the recommended HKG buffer (see Support Protocol 2), it must be freed of any chelators or reducing agents, including EGTA, before addition to the column.

5. When this has flowed into the column, follow with 1 ml PBS. If desired, retain the eluate to quantify protein retention.

PBS is used in place of HBS in all steps subsequent to addition of NDPK. IDA-Co²⁺ appears to have a weak affinity for the various nucleoside phosphate species; PBS competitively blocks these interactions. A slight lavender color may be noticed on the initial addition of PBS. This is insoluble cobaltous phosphate. It has not been found to interfere with any subsequent steps, nor has the use of PBS caused any significant loss of enzyme from the column.

NDPK-6×His has an A₂₈₀ extinction coefficient of 1.31 for a 1 mg/ml solution, as calculated using the Gill equation.

6. Dilute 833 pmol [γ -³²P]ATP (2.5 mCi at 3000 Ci/mmol, 5.8 mCi at 7000 Ci/mmol) with PBS to contain no greater than 1 mM reducing agent.

Commercial [γ -³²P]ATP may contain an appreciable quantity of DTT or other reducing agent. This should be diluted so that enzyme is not lost from the column due to reduction of cobalt.

The reactions can be scaled for use of smaller quantities of label.

7. Add the radioisotope to the column and allow it to flow through, driving the flow if necessary.

A moderate darkening of the column due to a low level of metal reduction may be observed, and a small amount of reduced metal may be present in the eluate. This should not normally be cause for concern. Small samples of the load and eluate may be taken to quantify label retention on the column by liquid scintillation counting.

8. Wash the column at least twice, each time with 1 ml PBS.

The eluate should be sampled to quantify label release in these steps, to ensure that residual [γ - ^{32}P]ATP has been cleared from the column.

Add N^6 (benzyl)ADP to column and elute N^6 (benzyl)ATP

9. Add 8 μl of 1 mM N^6 (benzyl)ADP (8 nmol) to 32 μl PBS containing 5 mM MgCl_2 .

This ADP analog and others can be synthesized using published procedures (Shah et al., 1997; Shah and Shokat, 2002).

10. Carefully add this to the column, and then gently force it into the beads until the liquid is again level with the top of the beads.

11. Add 250 μl PBS containing 5 mM MgCl_2 and gently force it through the beads until level, collecting the eluate.

A small sample of the eluate should be taken for liquid scintillation counting to determine yield.

SUPPORT PROTOCOL 1

PREPARATION OF COBALT AFFINITY RESIN (IDA- Co^{2+} -SEPHAROSE)

Materials

Iminodiacetic acid (IDA)–Sepharose slurry (Sigma)
200 mM cobalt chloride
HEPES-buffered saline (HBS): 150 mM NaCl/100 mM HEPES, pH 7.4
HBS (see above) containing 0.02% sodium azide
15-ml conical tubes

1. Place ~3 ml of IDA-Sepharose bead slurry in a 15-ml conical tube.
2. Wash several times with water, each time by filling the 15-ml conical tube and then decanting.
3. Wash once with 200 mM cobalt chloride.
The binding is essentially instantaneous.
4. Wash four times with HBS.
5. Store beads at 4°C in a bead volume equivalent of HBS containing 0.02% sodium azide.

SUPPORT PROTOCOL 2

EXPRESSION AND PURIFICATION OF NDPK

A 6 \times His–tagged NDPK from *S. cerevisiae* is expressed in BL21(DE3) *E. coli* and purified by metal-affinity chromatography.

Materials

pJDB1 expression plasmid: available from the Shokat Laboratory
(justinb@itsa.ucsf.edu) or Weiss Laboratory (elweiss@northwestern.edu)
E. coli strain BL21(DE3) (Novagen)
IPTG
Cobalt affinity resin (IDA- Co^{2+} -Sepharose; see Support Protocol 1)
HIK200 buffer (see recipe), 4°C

HEK10 buffer (see recipe), 4°C
HKG buffer (see recipe), 4°C
Liquid nitrogen
30°C shaking incubator
5-ml chromatography column
MWCO 15,000 dialysis membrane

Additional reagents and equipment for transformation of *E. coli* (UNIT 1.8), growing bacterial cultures (UNIT 1.2), preparation of bacterial lysates (UNIT 1.7), dialysis (APPENDIX 3C), and determination of protein concentration (UNIT 10.1A)

Grow transformed bacteria

1. Transform (UNIT 1.8) the pJDB1 expression plasmid into *E. coli* strain BL21(DE3).

This plasmid is a derivative of pET19b containing the S. cerevisiae gene YNK1 with a C-terminal 6×-His tag. This plasmid confers resistance to kanamycin. Cells carrying this plasmid should be cultured in medium containing 30 μg/ml kanamycin for the transformation procedure (see UNIT 1.2).

2. Grow transformed strain to mid-log phase ($OD_{600} = 0.5$ to 1.0) at 37°C (UNIT 1.2).
3. Add IPTG to the culture for a final concentration of 0.4 mM.
4. Incubate with shaking at 37°C for 3 hr.

Prepare bacterial lysate

The following steps are performed at 4°C, using chilled buffers.

5. Isolate *E. coli* cells by centrifugation (UNIT 1.7).
6. Produce clarified lysate by method of choice (UNIT 1.7), ensuring that the salt concentration is not less than 150 mM in the final extract.
7. Prepare a 5-ml column of IDA-Co²⁺-Sephrose.

A 5-ml column is appropriate for a 1-liter-scale expression.

Purify lysate by cobalt affinity chromatography and dialysis

8. Apply the lysate (from step 7) to the column.
9. Wash with 4 column volumes HIK200 buffer.
10. Elute with 2.5 column volumes HEK10 buffer.
11. Dialyze (APPENDIX 3C) against three changes of HKG buffer, each time for 2 hr, using a MWCO 15,000 dialysis membrane.
12. Determine protein concentration by measuring A_{280} (UNIT 10.1A), divide into aliquots, and freeze using liquid nitrogen. Store at -80°C.

NDPK-6×His has an A_{280} extinction coefficient of 1.31 for a 1 mg/ml solution, as calculated using the Gill equation (also see UNIT 10.1A).

The purified protein has a tendency to precipitate in buffers containing <250 mM salt. Highly concentrated solutions may exhibit some precipitation at even higher salt concentrations. If precipitation is observed during dialysis, the precipitated protein may be resolubilized by slow addition of 2.5 M KCl. If necessary; a final KCl concentration as high as 800 mM is acceptable.

Storage buffer should be free of DTT or other reducing agents, as these will complicate subsequent use of the enzyme.

IN VIVO INHIBITION OF ANALOG-SENSITIVE KINASES IN YEAST

If the activity of a protein kinase of interest is required for growth under some or all conditions, halo assays can be used to evaluate inhibition of *-as* alleles of this kinase. These simple experiments involve placement of a small disc of filter paper saturated with a compound of interest onto a solid-medium plate that has been uniformly spread with yeast cells. If the compound inhibits growth of the cells, a clear zone or “halo” will be evident around the filter disc. The size of this halo is a function of the sensitivity of the cells to the compound. These assays are a convenient way to evaluate sensitivity to a number of different compounds. Alternatively, a traditional liquid culture and inhibitor assay may be performed. Both methods are included here.

Materials

Inhibitors: INM-PP1 (see Basic Protocol 1) and INA-PP1 (see Basic Protocol 2)

DMSO

Yeast cells of interest containing wild-type (control) and analog-sensitive kinase alleles

Appropriate liquid and solid yeast media (*UNIT 13.1*)

5-mm sterile filter discs

Additional reagents and equipment for growing yeast cells (*UNIT 13.2*)

For the halo assay

- 1a. Make 10 mM, 1 mM, and 0.5 mM working solutions of inhibitors (1NM-PP1 and 1NA-PP1) in DMSO.
- 2a. Grow yeast cells of interest (containing *-as* analog-sensitive kinase gene; also include wild-type control) to log phase in liquid medium (*UNIT 13.2*).
- 3a. Dilute cultures to $OD_{600} = 0.2$. Place 2 to 3 ml of a diluted culture onto a solid medium plate (*UNITS 13.1 & 13.2*) such that the entire surface of the plate is covered with excess liquid medium. Allow cells to settle for 2 to 5 min.
- 4a. Tilt the plate so excess liquid pools along one edge. Remove most of this liquid with a pipet. Rock the plate a few times to ensure that remaining liquid is evenly distributed. Allow the plate to dry.
- 5a. Place 5-mm sterile filter discs onto the plate.
Four or five evenly spaced discs can generally be placed on a single plate.
- 6a. Gently pipet 10 μ l of inhibitor solution onto the middle of a filter disc. Include a DMSO-only control. Allow cells to grow and measure diameter of growth inhibition zones.

For the liquid culture assay

- 1b. Make a stock solution of the inhibitor of interest (1NM-PP1 or 1NA-PP1) dissolved in DMSO, containing between 10 mM and 25 mM inhibitor.

The solution can be stored at room temperature in a dark box.

- 2b. Grow yeast cells of interest (containing *-as* analog-sensitive kinase gene; also include wild-type control) to mid-log phase in liquid medium (*UNIT 13.2*).

Remember to grow enough cells to perform inhibitor treatment and a DMSO-only control. If planning to use conditioned medium, grow enough excess culture to provide medium for subsequent incubations with and without inhibitor.

Fresh and conditioned media generally differ in pH and nutrient concentration. The use of conditioned medium avoids effects caused by adaptation to new medium.

- 3b. Add inhibitor stock solution to the liquid medium. When assaying a range of inhibitor concentrations, ensure that the final concentration of DMSO is the same in all incubations. If fresh nonconditioned medium is used, add the inhibitor stock to fresh medium while actively mixing, either by swirling in a flask or stirring with a magnetic bar. Add the same amount of DMSO to a second portion of fresh medium for a no-inhibitor control. Harvest the cells from the culture grown in step 2 by filtration or centrifugation, and resuspend them in the inhibitor-containing and control medium, respectively. If conditioned medium is used, remove cells from an appropriate volume of culture by filtration or centrifugation. Collect the cleared conditioned medium (filtrate or supernatant), add inhibitor to one portion and DMSO for control to a second portion, then resuspend the cells back into the inhibitor- (or DMSO)-containing conditioned medium.

Direct addition of concentrated inhibitor to growing cultures is not recommended.

- 4b. Incubate and perform phenotypic analysis as desired.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

HEK10 buffer

20 mM HEPES, pH 7.4
10 mM EDTA
250 mM KCl
Store indefinitely at room temperature

HIK200 buffer

20 mM HEPES, pH 7.4
200 mM imidazole
50 mM KCl
Store indefinitely at 4°C

HKG buffer

20 mM HEPES, pH 7.4
250 mM KCl
20% (v/v) glycerol
Store indefinitely at room temperature

COMMENTARY

Background Information

Understanding the function of any protein kinase requires identification of its direct phosphorylation targets and evaluation of effects caused by its inactivation. This kind of characterization is among the most challenging in contemporary molecular biology. The study of protein kinase function has been greatly advanced through the use of cell-permeable compounds that inhibit the enzymatic activity of these proteins. Protein kinase inhibitors with relatively high specificity have been identified in screens of chemical compound libraries (Gray et al., 1998). Such compounds have already proven useful in treat-

ment of cancers caused by deranged protein kinase signaling (Druker et al., 2001). However, kinase inhibitors generally block the activity of a group of closely related enzymes. This lack of perfect specificity can prove problematic if closely related kinases control different processes in the cell type being studied. Acquisition of specific kinase inhibitors through exhaustive screening of compound libraries is furthermore a laborious process, and is beyond the capabilities of most molecular biology laboratories.

In the absence of perfectly specific protein kinase inhibitors, it is necessary to use alternative means to inactivate a kinase of interest.

Despite their power, each of these approaches has drawbacks. Depletion techniques (such as RNAi) do not address the function of the kinase's enzymatic activity. Furthermore, the kinetics of depletion can be very slow, which may allow homeostatic mechanisms to compensate for loss of enzyme function through activation of alternate pathways. Endogenous kinase genes can be replaced with catalytically inactive alleles in organisms that are easy to manipulate genetically. This is not straightforward with essential kinases, and is also subject to concerns about phenotypes that arise through adaptation. It is possible to generate conditional alleles that are rapidly inactivated under restrictive conditions (most often elevated temperature). These alleles are usually generated through random mutagenesis of the kinase of interest, and the biochemical reason for their effect on activity is generally obscure. More importantly, the phenotype of any conditional allele must be weighed against the effects of the restrictive conditions on wild-type cells. Temperature shifts can cause major perturbation of cell physiology, significantly complicating interpretation of conditional mutant phenotypes.

Given limitations in the above approaches, chemical and genetic approaches have been combined to allow highly specific inhibition of many protein kinases. The ATP-binding site of many protein kinases can be mutated to create kinase variants that are inhibited by cell-permeable compounds that do not appreciably affect the activity of wild-type protein kinases (Bishop et al., 2000; Weiss et al., 2000; Carroll et al., 2001; Sekiya-Kawasaki et al., 2003; Wang et al., 2003; reviewed in Bishop et al., 2001). Allele-specific inhibition of these kinases *in vivo* can be used to determine an individual protein kinase's physiological function without the confounding effects of temperature shift and adaptation. These "analog-sensitive" or "*-as*" mutant enzymes can often use derivatized forms of ATP that are poorly used by wild-type kinases (Shah et al., 1997; Bishop et al., 1998; Polson et al., 2001; Shah and Shokat, 2002). Specific use of ATP analogs by *-as* kinases *in vitro* can be exploited to directly identify kinase phosphorylation targets in complex protein mixtures (Shah and Shokat, 2002; Ubersax et al., 2003). Substrate specificity of *-as* kinase alleles does not appear to differ from corresponding wild-type alleles (Witucki et al., 2002). This is consistent with the physical separation between the mutated region of the

ATP-binding pocket and the substrate-recognition domain.

Design of analog-sensitive protein kinase alleles

The primary sequences and three-dimensional structures of protein kinase ATP-binding sites are conserved (Hanks and Hunter, 1995), and it is therefore straightforward to design candidate analog-sensitive kinase alleles. Analog-sensitive protein kinase alleles are constructed by substitution of glycine or alanine for an amino acid in kinase subdomain V, which is typically bulky and hydrophobic. This "gatekeeper" residue forms part of a hydrophobic pocket that interacts with the adenosine moiety of ATP; many competitive inhibitors of protein kinases bind in this site. Replacing the large side chain at this position with a smaller one alters the shape of the pocket, allowing molecules that do not interact with wild-type kinases to bind. Glycine substitutions at this position are referred to as "*-as1*" alleles and alanine substitutions as "*-as2*" alleles. The rationale behind this approach is supported by analysis of crystal structures of Hck-PP1 complex and the c-Src(*-as1*)-*N*⁶(benzyl)ATP (Schindler et al., 1999; Witucki et al., 2002). The nature of these alleles is illustrated in Figure 18.11.4, which schematizes some of the kinase-inhibitor contacts that are important for association of PP1 with the ATP binding site of Hck, as well as the engineered extra space present in Hck1-*as1* that allows binding of 1NA-PP1.

Figure 18.11.5 shows an alignment of amino acids in subdomains V and VII that are relevant to the construction of analog-sensitive kinases. The gatekeeper amino acid, which is mutated to alanine or glycine to create the *-as1* and *-as2* alleles, is shaded in gray. The amino acid that must be changed to construct *-as* kinase alleles can be most easily identified using a freely available online resource at <http://kinase.ucsf.edu/ksd/>. This Web site includes thorough instructions for use of the database. It is advisable to use the GenBank GI number of the protein kinase of interest to search for the appropriate alignment using the "Search by: family name" option. Alignments are generated as Microsoft Excel format files, for download to a computer, or in HTML format for viewing using a Web browser. In these alignments, ATP-contacting amino acids are highlighted green, and the gatekeeper residue is highlighted red.

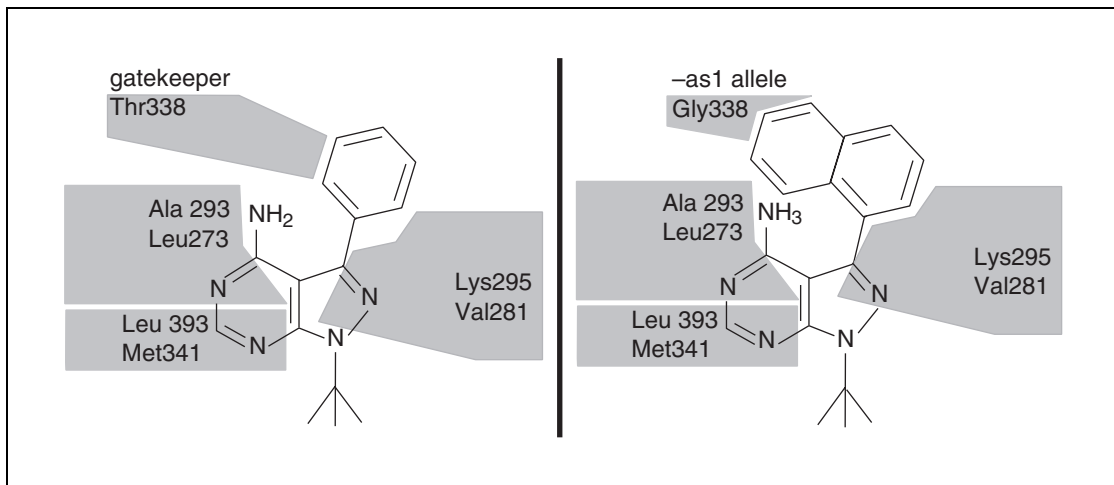


Figure 18.11.4 Inhibitor binding. Important contacts between PP1 and the ATP binding site of the Src-family protein kinase Hck (left) and the space created in the *-as1* allele that allows 1NA-PP1 binding (right). The right panel is inferred from the structure of c-Src(*-as1*).

	Subdomain V Gatekeeper	Subdomain VII -as3 position
v-Src	[332] EPIYIVTLEYMSKGSLLDFLKGEMG---//---LVCKVADFGLARLIEDNEY	
Cdk2	[074] NKLYLVFEFLHQ-DLKKFMDASAL---//---GAIKLADFGLARAFGVVPR	
Cdc28	[082] HKLYLVFEFLDL-DLKRYMEGIPK---//---GNLKLGFDFGLARAFGVPLR	
Fus3	[093] NEVYIIQELMQT-DLHRVISTQML---//---CDLKVCDFGLARIIDESAA	
Cla4	[634] DDLWVVMFEMEGSLTDI IENSPT---//---ARVKITDFGF ^{DF} CARLTDKRS	
JNK	[102] QDVYLVMEELMDA-NLCQVIHMELD---//---CTLKILDFGLARTACTNFM	
PKA α	[115] SNLYMVM EYVAGGEMF ^{DF} SHLRRIGR---//---GYIQVTDGFGFAKRVKGRTW	

Figure 18.11.5 Subdomain V and VII alignments. Analog-sensitive alleles of these protein kinases, whose wild-type sequences are shown, have been engineered. The gatekeeper residue in each kinase and the site of the second mutation (immediately amino terminal to the DFG motif) in Cla4 and JNK are shaded in gray.

Troubleshooting

Specific activity of *-as* kinases

The ideal analog-sensitive kinase allele is functionally silent (i.e., still recognizes ATP, is not a hypermorph or hypomorph, is localized to the same cellular compartments as the wild-type kinase, etc.) and yet has features not found in any wild-type protein kinases: it preferentially uses unnatural ATP analogs, and is selectively inhibited by low concentrations of compounds that do not affect wild-type kinases. In practice, *-as* kinase alleles may require relatively high concentrations of inhibitor to block activity, and they may exhibit substantially reduced catalytic activity. It is advisable to construct both *-as1* and *-as2* alleles: reduction of enzyme activity is generally more severe in *-as1* alleles than in *-as2* alleles, while *-as2* alleles are sometimes less

sensitive to inhibitor than *-as1* alleles. Sensitivity to inhibitor can be enhanced in some cases by an additional mutation outside the adenosine-binding pocket: kinases mutable to optimal analog sensitivity generally have the sequence (L/I)ADFG in subdomain VII. In Figure 18.11.4, this residue is shown shaded in gray. If in the sequence XDFG the amino acid at position X is anything other than glycine or alanine, introduction of alanine at this position may greatly enhance sensitivity to inhibitor. This amino acid was identified as an important determinant of PP1 binding to Hck (Schindler et al., 1999). Changing the sequence TDFG in the yeast PAK Cla4p ADFG greatly enhances sensitivity of the *-as2* allele (Weiss et al., 2000); such double-mutant kinases are called *-as3* alleles. The *prk1-as3* allele exhibits similarly enhanced sensitivity to inhibitor (Sekiya-Kawasaki et al., 2003).

While *-as* alleles of some protein kinases exhibit minor reduction in enzymatic activity, *-as1* and *-as2* alleles of some kinases have greatly reduced catalytic activity. The structural features that determine tolerance for the *-as* alleles are presently poorly understood. However, alignment of the ATP-binding sites of *-as* kinases that work well with those that do not may suggest compensatory mutations that ameliorate loss of activity in *-as1* and *-as2* alleles. MEKK1-*as1* (I1288G), for example, exhibits low kinase activity using both ATP and *N*⁶-phenethyl-ATP as substrates. MEKK1 contains cysteine at position 1238 on the β 2 strand, which should be in direct contact with the oxygen atom in the ribose ring of ATP; the vast majority of kinases contain Val at corresponding positions. A Cys1238 to Val mutation introduced into MEKK1-*as1* restored the kinase activity of MEKK1-*as1* (using ATP) to wild-type level, and this double-mutant enzyme was also able to use *N*⁶-(phenethyl)-ATP more efficiently than MEKK1-*as1* (Dennis Templeton, pers. comm.). If one constructs a *-as* allele that is severely compromised, it is advisable to change highly divergent residues in the ATP binding site to the consensus amino acid.

Heterologous expression systems for protein kinases (e.g., bacterial or insect cell expression) can produce active wild-type forms of mammalian kinases. However, the authors have found that these heterologous expression systems often do not provide active forms of *-as1* or *-as2* alleles of protein kinases. The reasons for this are particular to each protein kinase, but some common problems have been encountered. Many protein kinases require phosphorylation of an activation site (often in the activation segment). These residues may or may not be phosphorylated in a *trans*-auto-phosphorylation-dependent manner, or may be phosphorylated by an "upstream" kinase. In heterologous expression systems, such upstream kinases are absent and the function of activation-site phosphorylation must be carried out by the kinase itself in a nonoptimal reaction. Since the *-as* alleles of a given kinase may already be slightly less active with ATP as a phosphodonor than wild-type enzyme, this non-natural phosphorylation reaction may proceed extremely inefficiently, resulting in production of an inactive *-as* kinase allele. This can be corrected by incubation of the *-as* kinase with 1 mM ATP for 1 to 3 hr prior to assay, to greatly favor any phosphorylation activity to "autoactivate" the kinase.

Naturally, if the normal upstream kinase is known, it is preferable to activate the *-as* kinase by direct incubation with this enzyme. Interestingly, a number of *-as* kinases that cannot be activated by these means and never shown sufficient activity via heterologous expression systems are fully able to complement the function when expressed in the homologous cell where it normally carries out its function. Therefore, heterologous expression results with *-as* alleles should be interpreted with caution.

Phosphorylation target identification by allele-specific ATP analog use

While ATP analogs like *N*⁶-(benzyl)ATP are poorly used by most wild-type kinases, they are apparently accepted moderately well by some (Kraybill et al., 2002). This can result in background labeling that is independent of *-as* kinase addition. It is therefore important to characterize the level of background labeling that occurs when γ -³²P labeled ATP analogs are added to the protein mixture of interest; in some cases it will be necessary to reduce the level of background. The nature and magnitude of endogenous kinase activity in cell extracts and fractions varies significantly depending on the extract source, preparation, and fractionation strategy. Therefore, optimal strategies for background reduction are likely to be case-specific. However, the inclusion of significant concentrations of unlabeled ATP is often helpful. Since *-as* kinases can have higher *K_m*'s for ATP than wild-type kinases, ATP will often more effectively compete for ATP analog binding to wild-type kinases than to the *-as* kinase of interest. Consequently, background labeling will be reduced to a greater extent than *-as* kinase-dependent signal.

ATP is rapidly depleted in many extracts. In most cases, it is preferable to maintain a constant ATP concentration through the use of an ATP regenerating system. Addition of ATP, a molar excess of phosphocreatine, and creatine kinase will serve this purpose: the excess phosphocreatine acts as a substrate for creatine kinase in the conversion of ADP to ATP. If an ATP regenerating system is to be used in *-as* kinase substrate labeling experiments, it is advisable to first determine the ATP concentration that gives the greatest inhibition of background labeling without significantly compromising *-as* kinase-dependent signal. To this end, one should set up a series of extract labeling reactions in which

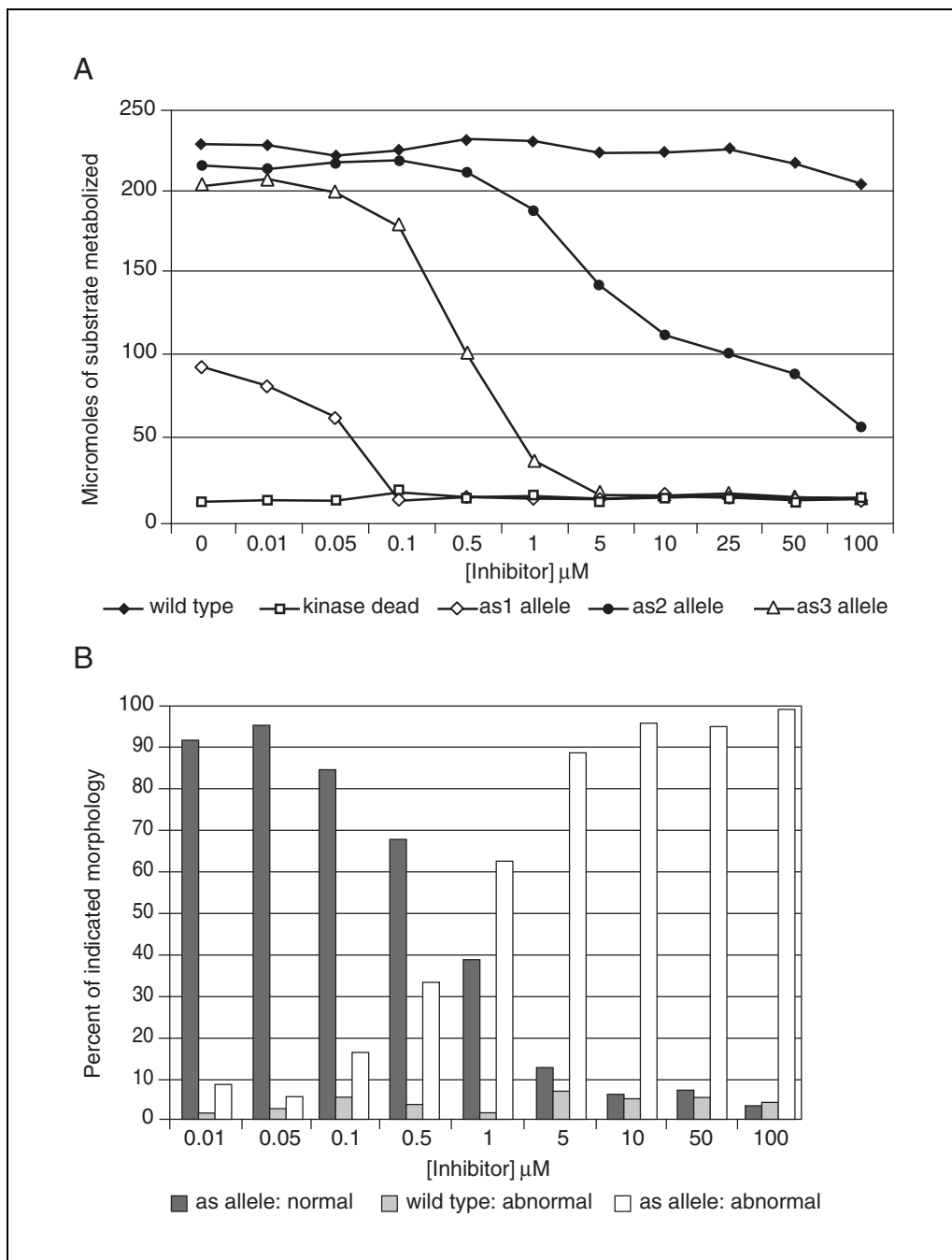


Figure 18.11.6 Hypothetical data from $-as$ kinase inhibition experiments. **(A)** Inhibition curves for a kinase-dependent metabolic process in which the amount of metabolite consumed in a fixed time can be measured. **(B)** Simple morphometric analysis of the inhibition of a kinase required for normal cell morphogenesis. Percent of normal wild-type cells is omitted for clarity.

the ATP concentration is varied from 100 μM to 2 mM.

It may not be possible to detect specifically labeled proteins in complex mixtures directly if the $-as$ kinase of interest has low specific activity, the extract exhibits high background labeling, or the phosphorylation targets of that $-as$ kinase are very rare. If a protein or complex of interest can be affinity purified, how-

ever, specific labeling by $-as$ kinases may be easier to demonstrate. In this approach, labeling reactions are performed using $[\gamma\text{-}^{32}\text{P}]\text{N}^6(\text{benzyl})\text{ATP}$ (or other radiolabeled analog) in a complex protein mixture with and without $-as$ kinase. The protein or complex of interest is then isolated from the two reactions and incorporation of label compared (Ubersax et al., 2003).

Anticipated Results

Analog-sensitive kinase alleles should ultimately be useful for *in vivo* analysis of kinase function in a wide range of model systems. There is, therefore, no general method for their use, but rather a set of principles for good experimental design. Ideally, the endogenous kinase of interest should be replaced by the *-as* allele. Once this is accomplished, two major questions must be answered in any *-as* kinase experiment: (1) how well does the mutant kinase supply function in the absence of inhibitor; and (2) how completely is the *-as* kinase activity of interest blocked by addition of allele-specific inhibitor? These questions can be answered through quantitative evaluation of phenotypic responses to a wide range of inhibitor concentrations.

Figure 18.11.6 shows hypothetical examples of dose-response experiments that evaluate *-as* kinase function and inhibition. Ideally, the *in vivo* function of the *-as* kinase in question should be significantly inhibited at PP1 derivative concentrations $<10 \mu\text{M}$. When a kinase is required for a process or enzyme activity with a measurable steady-state rate, the progress of this process or activity over a fixed time should be measured in the presence of increasing inhibitor concentrations (Fig. 18.11.6A). If the kinase is required for cell morphogenesis, organization of intracellular structures, or cell-cycle progress, the percentage of cells with abnormal (or cell-cycle-arrested) morphology should be scored (Fig. 18.11.6B). Naturally, inhibitor treatment of wild-type cells should be used as a control. When possible, the effects of *-as* kinase inhibition should also be measured relative to the phenotype of a catalytically inactive kinase allele. With extended PP1 analog treatment, the phenotype of a fully inhibited *-as* allele should be identical to a catalytically inactive kinase allele.

Figure 18.11.6A shows hypothetical inhibition curves for wild-type, catalytically inactive, *-as1*, *-as2*, and *-as3* alleles of a kinase required for a cell's consumption of a measurable compound. The measurements involved in such assays should be performed on cells that have been pretreated with inhibitor, allowing time for the treatment to have maximal effect. While the *-as1* allele in this experiment is sensitive to low concentrations of inhibitor, it is also severely compromised relative to wild-type and is therefore of limited utility. The *-as2* allele is less compromised, but is not sufficiently sensitive to inhibitor.

The *-as3* allele is sufficiently sensitive while retaining adequate *in vivo* activity in the absence of inhibitor. The phenotype of this allele "saturates" at $\sim 5 \mu\text{M}$ inhibitor. It may be advisable to use slightly higher concentrations in future assays to ensure inactivation. Figure 18.11.6B shows morphological phenotypes over a range of inhibitor concentrations. A number of researchers have published examples of dose-response analysis with *-as* kinases (Bishop et al., 2000; Weiss et al., 2000; Weiss et al., 2002; Sekiya-Kawasaki et al., 2003).

Thus far, most *in vivo* inhibition studies using *-as* kinases have been performed in the budding yeast *Saccharomyces cerevisiae*. The considerable pace of yeast experiments is in part due to the ease of gene replacement in this organism; *-as* kinase inhibition is also clearly useful in metazoans (Wang et al., 2003). The authors have included guidelines for *-as* kinase inhibition in yeast. The specific assay conditions will, of course, depend on the nature of the process of interest.

Literature Cited

- Bishop, A.C., Shah, K., Liu, Y., Witucki, L., Kung, C., and Shokat, K.M. 1998. Design of allele-specific inhibitors to probe protein kinase signaling. *Curr. Biol.* 8:257-266.
- Bishop, A., Kung, C., Shah, K., Witucki, L., Shokat, K.M., and Liu, Y. 1999. Generation of monospecific nanomolar tyrosine kinase inhibitors via a chemical genetic approach. *J. Am. Chem. Soc.* 121:627-631.
- Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., Wood, J.L., Morgan, D.O., and Shokat, K.M. 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* 407:395-401.
- Bishop, A.C., Buzko, O., and Shokat, K.M. 2001. Magic bullets for protein kinases. *Trends Cell Biol.* 11:167-72.
- Carroll, A.S., Bishop, A.C., DeRisi, J.L., Shokat, K.M., and O'Shea, E.K. 2001. Chemical inhibition of the Pho85 cyclin-dependent kinase reveals a role in the environmental stress response. *Proc. Nat. Acad. Sci. U.S.A.* 98:12578-12583.
- Druker, B.J., Sawyers, C.L., Capdeville, R., Ford, J.M., Baccarani, M., and Goldman, J.M. 2001. Chronic myelogenous leukemia. *Hematology (Am. Soc. Hematol. Educ. Program)* pp. 87-112.
- Gray, N.S., Wodicka, L., Thunnissen, A.M., Norman, T.C., Kwon, S., Espinoza, F.H., Morgan, D.O., Barnes, G., LeClerc, S., Meijer, L., Kim, S.H., Lockhart, D.J., and Schultz, P.G. 1998. Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. *Science* 281:533-538.

- Hanks, S.K. and Hunter, T. 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J.* 9:576-596.
- Kraybill, B.C., Elkin, L.L., Blethrow, J.D., Morgan, D.O., and Shokat, K.M. 2002. Inhibitor scaffolds as new allele specific kinase substrates. *J. Am. Chem. Soc.* 124:12118-12128.
- Mourad, N. and Parks, R.E. Jr. 1966. Erythrocytic nucleoside diphosphokinase. II. Isolation and kinetics. *J. Biol. Chem.* 241:271-278.
- Polson, A.G., Huang, L., Lukac, D.M., Blethrow, J.D., Morgan, D.O., Burlingame, A.L., and Ganem, D. 2001. Kaposi's sarcoma-associated herpesvirus K-bZIP protein is phosphorylated by cyclin-dependent kinases. *J. Virol.* 75:3175-3184.
- Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. 1999. Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. *Mol. Cell* 3:639-648.
- Sekiya-Kawasaki, M., Groen, A.C., Cope, M.J., Kaksonen, M., Watson, H.A., Zhang, C., Shokat, K.M., Wendland, B., McDonald, K.L., McCaffery, J.M., and Drubin, D.G. 2003. Dynamic phosphoregulation of the cortical actin cytoskeleton and endocytic machinery revealed by real-time chemical genetic analysis. *J. Cell Biol.* 162:765-772.
- Shah, K. and Shokat, K.M. 2002. A chemical genetic screen for direct v-Src substrates reveals ordered assembly of a retrograde signaling pathway. *Chem. Biol.* 9:35-47.
- Shah, K., Liu, Y., Deirmengian, C., and Shokat, K.M. 1997. Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proc. Natl. Acad. Sci. U.S.A.* 94:3565-3570.
- Ubersax, J.A., Woodbury, E.L., Quang P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. 2003. Targets of the cyclin-dependent kinase Cdk1. *Nature* 425:859-864.
- Wang, H., Shimizu, E., Tang, Y.P., Cho, M., Kyin, M., Zuo, W., Robinson, D.A., Alaimo, P.J., Zhang, C., Morimoto, H., Zhuo, M., Feng, R., Shokat, K.M., and Tsien, J.Z. 2003. Inducible protein knockout reveals temporal requirement of CaMKII reactivation for memory consolidation in the brain. *Proc. Natl. Acad. Sci. U.S.A.* 100:4287-4292.
- Weaver, R.H. 1962. Nucleoside diphosphokinases. *Enzymes* 6:151-160.
- Weiss, E.L., Bishop, A.C., Shokat, K.M., and Drubin, D.G. 2000. Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. *Nat. Cell Biol.* 2:677-685.
- Weiss, E.L., Kurischko, C., Zhang, C., Shokat, K., Drubin, D.G., and Luca, F.C. 2002. The *Saccharomyces cerevisiae* Mob2p-Cbk1p kinase complex promotes polarized growth and acts with the mitotic exit network to facilitate daughter cell-specific localization of Ace2p transcription factor. *J. Cell Biol.* 158:885-900.
- Witucki, L.A., Huang, X., Shah, K., Liu, Y., Kyin, S., Eck, M.J., and Shokat, K.M. 2002. Mutant tyrosine kinases with unnatural nucleotide specificity retain the structure and phospho-acceptor specificity of the wild-type enzyme. *Chem. Biol.* 9:25-33.

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