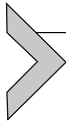

CHAPTER EIGHT

The Logic and Design of Analog-Sensitive Kinases and Their Small Molecule Inhibitors

Michael S. Lopez, Joseph I. Kliegman, Kevan M. Shokat
Howard Hughes Medical Institute and Department of Cellular & Molecular Pharmacology, University of California, San Francisco, California, USA

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Abstract

Analog-sensitive AS Kinase technology allows for rapid, reversible, and highly specific inhibition of individual engineered kinases in cells and in mouse models of human diseases. The technique consists of two parts: a kinase containing a space-creating mutation in the ATP-binding pocket and a bulky ATP-competitive small molecule inhibitor that

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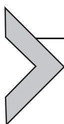
complements the shape of the mutant ATP pocket. This strategy enables dissection of phospho-signaling pathways, elucidation of the physiological function of individual kinases, and characterization of the pharmacology of clinical-kinase inhibitors. Here, we present an overview of AS technology and describe a stepwise approach for generating AS Kinase mutants and identifying appropriate small molecule inhibitors. We also describe commonly encountered technical obstacles and provide strategies to overcome them.



1. INTRODUCTION

1.1. Overview of analog-sensitive-kinase technology

Analog-sensitive (AS) Kinase technology is a chemical-genetic technique that enables systematic generation of highly specific inhibitors for individual kinases (Shokat et al., 2000). This approach combines the advantages of a small molecule inhibitor (temporal resolution, reversibility, etc.) with the specificity of a genetic perturbation. When a residue at a structurally conserved position in the kinase active site, termed the gatekeeper (Liu et al., 1999), is mutated from the natural amino acid (methionine, leucine, phenylalanine, threonine, etc.) to a residue bearing a smaller side chain (glycine or alanine), a novel pocket not found in wild-type (WT) kinases is created within the ATP-binding site. The engineered kinase is termed AS because it can be potently and specifically inhibited with ATP analogs containing a bulky substituent that complements the enlarged ATP-binding pocket (Bishop et al., 1999). AS Kinases are powerful tools for deciphering phospho-signaling networks, elucidating the cellular function of individual kinases, and characterizing the pharmacology of clinical therapeutics.

Since nearly all kinases contain a bulky gatekeeper residue, extending AS Kinase technology to the entire kinome is possible in principle. Toward this end, we developed a systematic approach for engineering functional AS Kinases and identifying suitable inhibitors from a small panel of rationally designed small molecules (Fig. 8.1A). This strategy has enabled the successful application of the AS technique to more than 80 kinases reported to date. Here, we describe the stepwise protocol for the identification of the gatekeeper residue in diverse kinases, the proper choice for gatekeeper substitution, and several strategies to rescue the activity of compromised AS Kinases (Fig. 8.1A–D). We also explain the logic of AS Kinase inhibitor design, describe the strengths and limitations of common AS Kinase inhibitors, and detail protocols for their synthesis and characterization. Throughout the text, we also review key applications of this method in order to highlight experiments in which this technique may prove most useful.

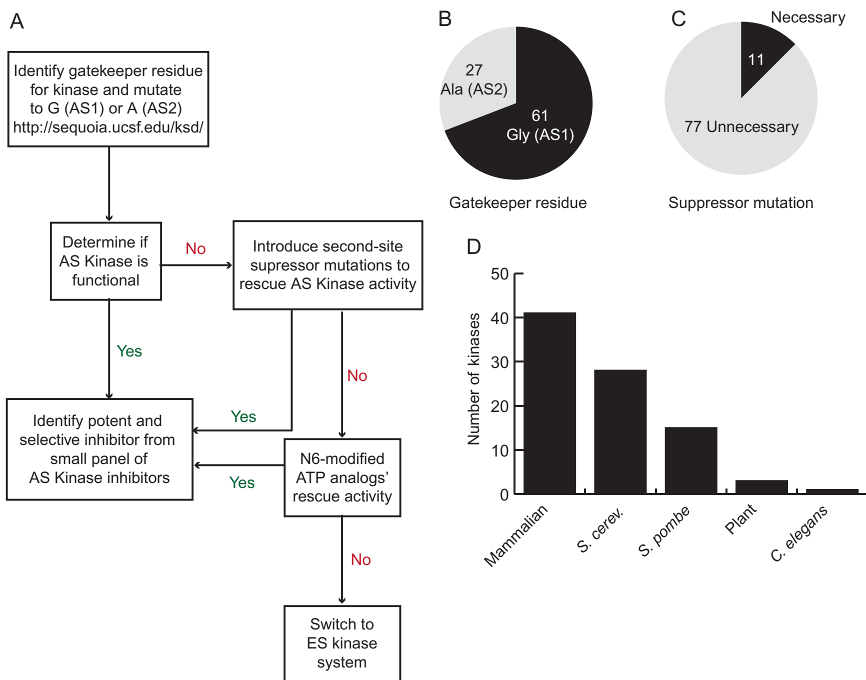


Figure 8.1 Overview of AS Kinase technology. (A) Flowchart outlines systematic approach for identifying functional AS Kinases. (B) Distribution of Ala and Gly gatekeeper residues used in reported AS Kinases. (C) Fraction of reported AS Kinases requiring second-site suppressor mutations. (D) Distribution of reported AS Kinases according to species.

1.2. The gatekeeper governs access to the ATP-binding pocket of protein kinases

The amino acid position analogous to c-Src T338 is called the gatekeeper because the size of this residue's side chain governs the volume and shape of the ATP-binding site and thereby determines the size of ATP-competitive ligands that can be accommodated. Kinases with small gatekeeper residues allow binding of molecules with large bulky groups such as the C3-tolyl ring of PP1 that cannot bind to kinases with larger gatekeepers such as methionine or phenylalanine. This phenomenon was first observed with PP1 (Fig. 8.2A), a potent ATP-competitive inhibitor of Src-family kinases (Hanke et al., 1996). Liu, Shah, Yang, Witucki, and Shokat (1998) used bioinformatics and site-directed mutagenesis to demonstrate that PP1 is capable of binding any kinase with a small amino acid residue (Thr, Val, Ala, Gly) at the position analogous to c-Src T338 (Liu et al.,

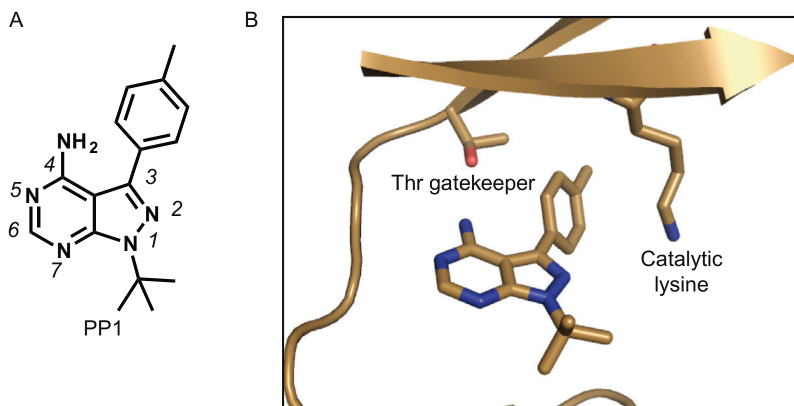
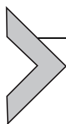


Figure 8.2 Structural basis of PP1 activity. (A) Chemical structure of PP1. (B) X-ray cocrystal structure of PP1 bound to Hck kinase.

1998). Importantly, Liu and Shokat recognized the potential for any kinase to be made sensitive to inhibitors such as PP1 by exchanging the native gatekeeper with a smaller amino acid by site-directed mutagenesis. They also noted that kinases sensitive to PP1 become resistant upon mutation of the gatekeeper residue to isoleucine, thereby predicting resistance mutations at the gatekeeper position that would later emerge in the clinic (Branford, Rudzki, Walsh, & Grigg, 2002).

Soon after the discovery of the gatekeeper and the characterization of PP1 selectivity by Liu and Shokat, the structure of PP1 bound to the Src-family kinase Hck showed the pyrazolopyrimidine core of PP1 mimics the adenine ring of ATP in its binding to the nucleobase pocket while the *p*-tolyl group at the C3 position projects into a deep hydrophobic cleft situated between the gatekeeper T338 and the catalytic lysine residue K295 (Fig. 8.2B; Schindler et al., 1999). Based on these structural features, it was predicted that PP1 analogs with an enlarged C3 substituent would suffer a steric clash with the WT gatekeeper residue while mutation of the gatekeeper to glycine or alanine, which are exceedingly rare at this position, would create space to accommodate the enlarged C3 substituent. Thus, the same phenomenon that results in the selectivity of PP1 for a subset of WT kinases can be used to design inhibitors with selectivity for kinases engineered to contain glycine or alanine gatekeepers. It has been demonstrated that potent and specific inhibitors can be readily identified for various AS Kinases by screening a small panel of PP derivatives with enlarged C3 substituents (Shokat et al., 2000).



2. CONSTRUCTING AS KINASES

The challenge of constructing a novel AS Kinase varies; however, the technique has become a robust and rapid method for kinase characterization and the steps to engineering an AS Kinase is now routine. The following steps outline a straightforward strategy to identify the gatekeeper residue by primary or structure-based sequence alignment, mutate the gatekeeper, and find a selective inhibitor analog from a small panel of molecules developed in the Shokat Lab. In cases where the AS Kinase mutant suffers compromised activity, there are standard second-site suppressor mutations that may restore kinase activity to the AS Kinase. Additionally, bulky ATP analogs are more efficiently used by AS Kinases and can be employed to restore the activity of hypomorphic AS Kinase mutants in cells. Finally, a complementary technique that relies on covalent targeting of a cysteine gatekeeper kinase (termed an electrophile-sensitive (ES) kinase) offers the unique experimental advantages of irreversible inhibition.

2.1. Identifying the gatekeeper residue

As illustrated in Fig. 8.1A, there is a systematic approach for engineering functional AS Kinases. The first step is to correctly identify the gatekeeper residue of the kinase of interest (KOI). This can be done by submitting the amino acid sequence of the KOI-kinase domain to the Kinase Database at http://sequoia.ucsf.edu/ksd/. Alternatively, the amino acid sequence of the kinase domain can be aligned with c-Src and other protein kinases to identify the position equivalent to c-Src T338 (Fig. 8.3). Typically, the gatekeeper residue is preceded by two hydrophobic amino acids (I336 and V337 in c-Src) and followed by an acidic residue (E339 in c-Src) and another hydrophobic amino acid (Y340 in c-Src). While atypical kinases may require additional structural information to accurately identify the gatekeeper position, this simple sequence alignment is usually sufficient for canonical tyrosine and serine/threonine protein kinases.

To identify the gatekeeper by structural alignment, you must first download appropriate protein-modeling software such as MODELLER (http://salilab.org/modeller/). Then, identify the structure that will be used as a template to model your KOI. This may be done with an integrated feature of MODELLER that searches the PDB (rcsb.org) for solved structures with sequence homology to your KOI. If this feature is used, the quality of the homology can be evaluated with an integrated script that outputs statistics

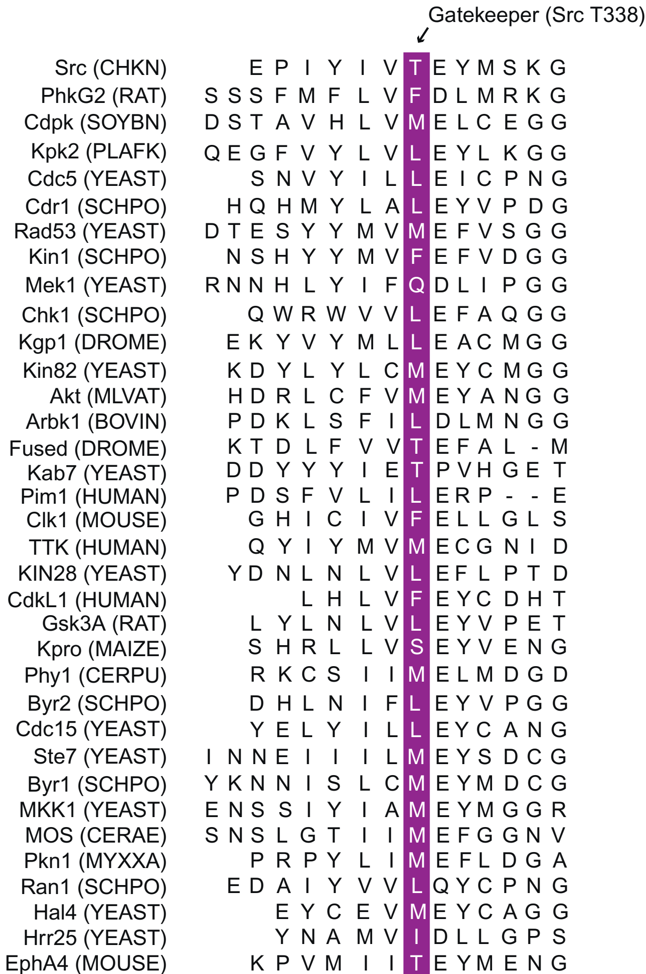


Figure 8.3 Representative alignment of the hinge region of kinase domains from diverse organisms and kinase families.

that inform the choice of template. Alternatively, the phylogenetic tree of kinases may be used to determine the most related kinase with a published structure. Once the appropriate template is selected, model building is a simple two-step process of (1) aligning your KOI to the published structure and (2) building the model. The commands for accomplishing these steps are clearly described (http://salilab.org/modeller/tutorial/basic.html). Thus, structural alignment using MODELLER allows for the identification of gatekeeper residues in atypical kinases with low sequence conservation.

2.2. Second-site suppressor mutations

A challenge that is sometimes encountered in the construction of AS Kinases is the loss of activity that results from mutation of the gatekeeper to glycine or alanine. The gatekeeper residue of protein kinases contributes to the hydrophobic spine of the kinase domain and plays an important role in promoting enzymatic activity (Azam, Seeliger, Gray, Kuriyan, & Daley, 2008). Thus, mutation of this position to a smaller amino acid may disrupt this structural feature and lead to a reduction in the catalytic efficiency of the AS Kinase. For example, mutation of the budding yeast kinase Cdc28 from the native phenylalanine gatekeeper to glycine results in a 10-fold reduction in ATP affinity and a sixfold reduction in k_{cat} (Shokat et al., 2000). Fortunately, this reduced activity is often acceptable and does not result in biological defects. The activity of the kinase may be easily evaluated by testing the ability of the AS allele to complement loss of the WT gene. In cases where compromised kinase activity is prohibitive, our lab has developed a series of steps to remedy this limitation.

The glycine gatekeeper mutation (termed AS1) creates the maximal expansion of the ATP-binding pocket and typically maximizes the difference in sensitivity between AS and WT alleles to AS Kinase inhibitors. However, if the AS1 kinase suffers a prohibitive reduction in activity, the alanine gatekeeper (termed AS2) can be employed to increase enzyme activity while retaining sufficient sensitivity to AS Kinase inhibitors (Fig. 8.1B). In cases where the KOI is intolerant of both glycine and alanine gatekeepers second-site suppressor mutations may be introduced to restore AS Kinase activity (Fig. 8.1C). Mutation of residues in the antiparallel β-sheet of the N-terminal lobe of the kinase domain to branched aliphatic residues such as valine or isoleucine can restore activity in the context of the AS gatekeeper (Zhang et al., 2005). For example, Cdc5 and MEKK1 contain cysteine residues in place of the typical valine at the position analogous to Src V284. Mutation of this position to valine restores the activity of both Cdc5-AS2 and MEKK1-AS1 to near-WT levels.

In certain cases, the activity of an impaired AS Kinase can be rescued by rational substitution of residues that are not conserved in closely related but well-behaved AS Kinases. For example, Niswender et al. observed that the homologous kinases PKAα and PKAβ have differential tolerance for the gatekeeper mutation (Niswender et al., 2002). PKAα-AS2 (M120A) retained greater than 75% activity relative to PKAα-WT, while PKAβ-AS1 (M120G) and PKAβ-AS2 (M120A) retained only 10% and 40% of

PKAβ-WT activity, respectively. The authors observed that most amino acid differences between the two kinases were relatively conservative substitutions with the exception of position 46, an isoleucine residue in PKAα and a lysine in PKAβ. Thus, combining the gatekeeper mutation M120A with the K46I substitution yields an AS PKAβ mutant that retains greater than 75% activity. As the number of AS Kinases described in the literature continues to grow, they will increasingly be used as a roadmap for indentifying mutations that rescue the activity of kinases intolerant of alanine and glycine gatekeepers.

2.3. Unnatural ATP analogs rescue enzyme activity

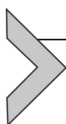
An effect of the smaller gatekeeper residue is a reduction in affinity of the kinase for its substrate ATP. Thus, introduction of an ATP surrogate with enhanced affinity for the expanded ATP-binding pocket rescues the activity of impaired AS Kinases. AS Kinases have enhanced catalytic activity when ATP analogs bearing hydrophobic N6 substituents complementing the gatekeeper pocket are used in the place of ATP (Shah & Shokat, 2002). Recently, Merrick et al. (2011) used this strategy to rescue the hypomorphic Cdk2-AS mutant in human epithelial cells. In this case, the gatekeeper mutation resulted in a structural defect that disrupted the proper binding of Cdk2 to one of its activating subunits cyclin A. The addition of 3MB-PP1 corrected the structural defect as evidenced by proper binding of cyclin A to Cdk2, but inhibited kinase activity as one would expect. In contrast, the addition of N6-benzylaminopurine (6-BAP) rescued cyclin A binding but also retained kinase activity as evidenced by unperturbed cell proliferation. Importantly, the cellular activity of 6-BAP suggests that this nucleobase is converted into the active nucleotide in cells. This hypothesis is supported by work showing conversion of a related N6-modified nucleobase kinetin into kinetin tri-phosphate in HeLa cells (Hertz et al., 2013).

2.4. Cysteine gatekeeper alternative

In extreme cases where glycine and alanine residues are not tolerated at the gatekeeper position, our lab has developed an approach that utilizes a cysteine gatekeeper (Garske, Peters, Cortesi, Perez, & Shokat, 2011). This engineered kinase is termed an ES kinase and relies on inhibitors that target the cysteine gatekeeper through covalent chemistry. The cysteine gatekeeper has a larger, nonpolar side chain that maintains the integrity of the hydrophobic spine and thereby retains greater enzymatic activity. For

example, mutation of the threonine gatekeeper of c-Src to glycine reduces the catalytic efficiency from 4.99 to 0.59 min/μ*M*, while mutation to cysteine retains robust activity of 8.34 min/μ*M* (Garske et al., 2011). The expansion of the ATP-binding pocket of ES kinases is minimal; however, the unique reactivity of the cysteine thiol side chain allows selective covalent targeting of the gatekeeper with inhibitors bearing appropriate electrophiles. Like glycine and alanine, cysteine gatekeepers are exceedingly rare in the human kinome thereby reducing the chance of off-target activity of ES-kinase inhibitors.

The irreversible nature of covalent inhibitors also provides unique experimental advantages. For example, an ES kinase might be titrated with inhibitor to identify the precise activity threshold required for a specific function. Blair et al. (2007) used a similar approach to determine the quantitative relationship between EGFR stimulation by its ligand EGF and its downstream-signaling outputs (Blair et al., 2007). Another exciting advantage of this technique is the potential to target two different engineered kinases with orthogonal inhibitors. Taken together, glycine, alanine, and cysteine gatekeeper residues can be used in combination with appropriate second-site suppressor mutations to engineer nearly any kinase into a functional enzyme that is sensitive to specific small molecule inhibition.



3. AS KINASE INHIBITORS

Once a functional AS Kinase is generated, the next step is to identify a suitable inhibitor from a small panel of molecules specifically designed to have broad generality toward diverse AS Kinases and minimal activity toward WT kinases or other cellular proteins. There are two major classes of AS Kinase inhibitors: PPs are derived from the semipromiscuous kinase inhibitor PP1 and staralogs are derivatives of the pan-kinase inhibitor staurosporine. The only (ES-kinase inhibitors reported to date are based on the PP and quinazoline scaffolds) (Garske et al., 2011). A new class of inhibitors based on BEZ235 that target AS mutants of PI3-like kinases in yeast have recently been described (Kliegman et al., 2013). These molecules function based on the same logic as staralogs and PP analogs with a larger hydrophobic moiety creating a steric clash with WT PI3-like kinases, but binding to the engineered kinase with an expanded affinity pocket due to a size reduction of the gatekeeper residue. Here, we describe the limitations and advantages of each class of inhibitors and provide protocols for their synthesis and use.

3.1. Pyrazolo[3,4-*d*]pyrimidine inhibitors

Initially, 1NA-PP1 and 1NM-PP1 (Fig. 8.4A) were identified as the most efficacious AS Kinase inhibitors and are the most commonly used in peer-reviewed publications. These molecules also have the advantage that they are now commercially available from several companies. However, one limitation of 1NA-PP1 is that it inadvertently inhibits a small set of WT human kinases (Zhang et al., 2013). 1NM-PP1 is more selective but lacks potency against some AS Kinase. To develop molecules that overcome these limitations we recently reported the development of 3-substituted benzyl PPs (Fig. 8.4A) with improved selectivity and potency for AS Kinase. These new molecules have found widespread utility and allowed the application of the AS technique to 10 kinases that were previously recalcitrant to inhibition with 1NA-PP1 and 1NM-PP1 (Zhang et al., 2013).

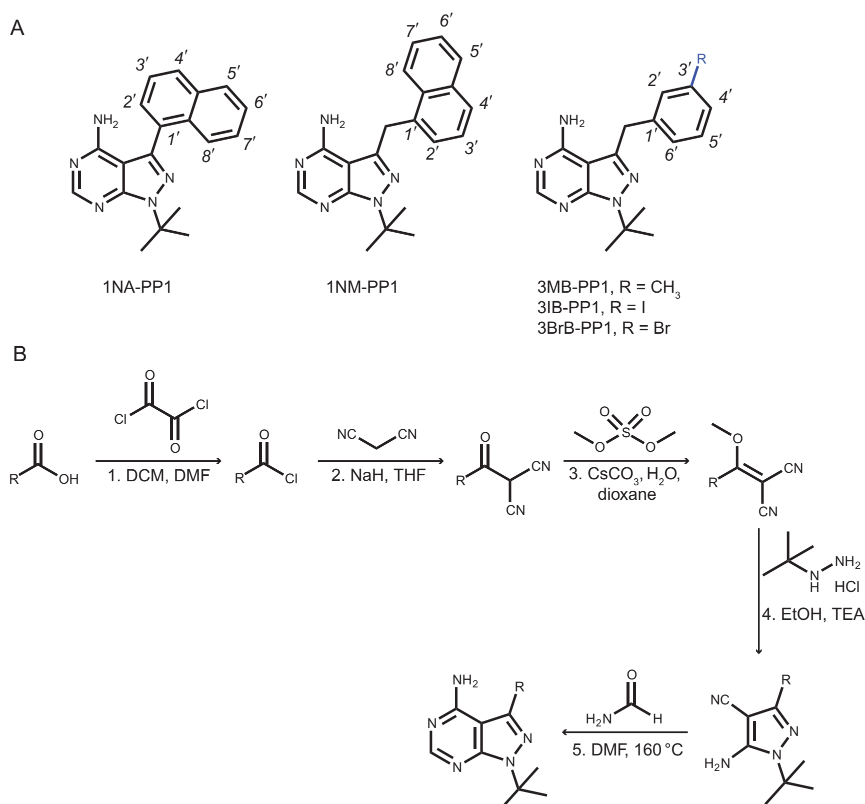


Figure 8.4 PP inhibitors. (A) Chemical structures of commonly used PP inhibitors. (B) Synthetic scheme for the synthesis of PP analogs.

3.2. Synthesis of PP inhibitors

Pyrazolo[3,4-*d*]pyrimidine (PP) inhibitor synthesis protocol (Fig. 8.4B):

(1) Dissolve 1 equiv. of the appropriate carboxylic acid in dry CH_2Cl_2 (0.2% DMF) in a clean dry, round-bottom flask (rbf) under argon (Ar). Slowly add 3 equiv. oxalyl chloride (dissolved 1:4 in CH_2Cl_2) over 15 min via an addition funnel, and allow the reaction to stir for 1 h. Remove the solvent and volatiles *in vacuo* and carry the crude concentrate to the next step.

(2) Dissolve 1 equiv. of malononitrile in THF, cool to 0 °C, and add 2 equiv. of sodium hydride. Next, redissolve the crude product 1 in a minimal volume of THF and add to the stirring reaction. Allow the reaction to warm to RT and proceed for ~30 min. Concentrate the reaction and carry the crude product on to the next step.

(3) Dissolve crude intermediate 2 in 1:1 dioxane:H_2O then add 8 equiv. $CsCO_3$ and 7 equiv. dimethylsulfate. Heat the reaction to 70 °C for 4 h, cool to RT, and allow it to proceed overnight. Dilute the reaction into brine and extract with several portions of EtOAc. Combine the organics, dry with $MgSO_4$, filter, and concentrate. Triturate the yellow concentrate with diethyl ether to yield a solid.

(4) Combine 1 equiv. of intermediate 3, 1 equiv. *t*-butyl hydrazine HCl, and 2 equiv. TEA in EtOH and allow the reaction to proceed for 1 h at RT. Concentrate and purify by SiO_2 chromatography ($CH_2Cl_2 \rightarrow$ 9:1 CH_2Cl_2:MeOH).

(5) Dissolve intermediate 4 in formamide in a clean, dry rbf equipped with water-cooled condenser and purge with Ar gas. The solid may not dissolve completely until heated. Heat the reaction at 165 °C overnight. Allow the reaction to cool to RT then transfer it dropwise to ice-cold water whereupon the product should precipitate. Purify by C18 HPLC (H_2O + 0.1% formic acid $\rightarrow$ 1:4 H_2O:MeCN + 0.1% formic acid).

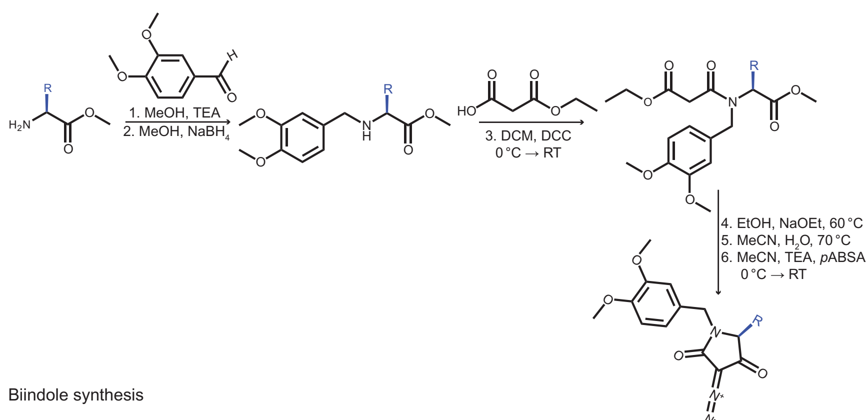
3.3. Staralog inhibitors

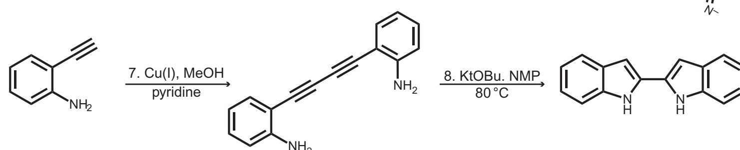
Staralog inhibitors are C7-substituted indolocarbazole kinase inhibitors (Lopez et al., 2013). The primary advantage of these molecules is their high level of selectivity. For example, 1 μ*M* Star 12 retains potency against most AS Kinases but does not inhibit greater than 60% activity of a single WT kinase *in vitro* (308 tested). A similar analog, Star 17, was found to be the ideal inhibitor for targeting EphA4-AS1 in cells; this is because EphA4 is inherently sensitive to PP-based inhibitors thereby preventing complete

inhibition of EphA4-AS1 without also inhibiting WT activity. In contrast, 10 μ*M* Star 12 is able to completely inhibit EphA4-AS1 without affecting the activity of EphA4-WT in 293T cells.

3.4. Synthesis of staralog inhibitors

Staralog inhibitor synthesis protocol (Fig. 8.5):





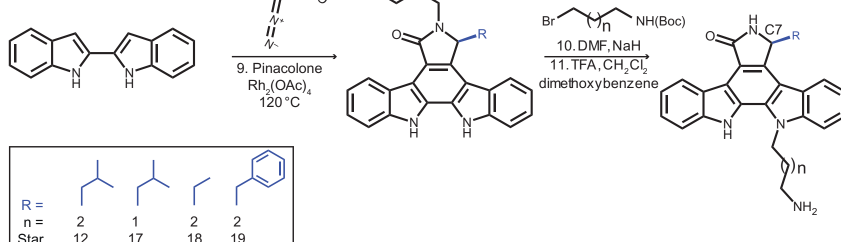


Figure 8.5 Synthetic scheme for the synthesis of staralogs.

(1) Place appropriate amino acid methyl ester, dimethoxybenzaldehyde, and dry methanol into a clean, dry rbf-containing Teflon stir. Add dry triethylamine (TEA) and stir at room temperature for 4–12 h.

(2) Add $NaBH_4$ in small portions over a period over 1 h. This reaction is exothermic and tends to boil over if $NaBH_4$ is added too quickly. After $NaBH_4$ addition is complete, stir for 1 h at RT, concentrate, redissolve concentrate in EtOAc, and wash with several portions of aqueous $NaCO_3$ in a separation funnel. Dry the organics with $MgSO_4$, filter, and concentrate. Redissolve concentrate in EtOAc, and purify by SiO_2 chromatography (hexanes → 1:1 hexanes:EtOAc).

(3) In a clean, dry rbf, dissolve ethyl hydrogen malonate and purified product from step 2 in CH_2Cl_2 and cool to 0 °C with stirring. In a separate vial, dissolve DCC in a small volume of CH_2Cl_2 at RT. Add the DCC/CH_2Cl_2 solution to the stirring, cooled reaction dropwise. The DCU byproduct should precipitate with 30 s. Allow the reaction to warm to room temperature and stir for 1 h. Remove DCU by filtration through a plug of celite, concentrate, redissolve in a minimal volume of CH_2Cl_2, and filter once more. Remove solvent *in vacuo* to yield a yellow oil substance. Redissolve oil in EtOAc and purify by SiO_2 chromatography (hexanes → 1:1 hexanes/EtOAc).

(4) Wash ~1.2 equiv. of sodium metal (in mineral oil) with hexanes and place in a preweighed glass vial. Quickly evaporate the residual hexanes with a stream of Ar gas, close vial screwcap, and record mass of sodium. Prepare a fresh solution of sodium ethoxide by dissolving 1.2 equiv. of sodium metal in dry ethanol. This reaction is exothermic and evolves hydrogen gas. In a separate vial, dissolve purified product 3 in a small volume of dry ethanol and add dropwise to the stirring solution of sodium ethoxide. Fit the reaction vessel with a water-cooled condenser and stir in 60 °C oil bath for 10 min, allow reaction to cool to RT, and remove ethanol *in vacuo* to yield a white solid. Suspend the white solid in 1 *M* HCl and extract with three portions of CH_2Cl_2. Combine the organic portions and remove solvent *in vacuo* to yield an orange colored oil. Carry the crude reaction on to the next step.

(5) Dissolve the crude product 4 in 95:5 MeCN:H_2O in a rbf fitted with a water-cooled reflux condenser and heat at 70 °C for 1 h. Allow the reaction to cool to RT then remove solvent *in vacuo*. Carry the crude concentrate on to the next step.

(6) Dissolve the crude product 5 and 1.1 equiv. of *p*ABSA in MeCN and cool to 0 °C. Add 1.1 equiv. of TEA and continue to stir reaction until ice melts and the reaction warms to RT (2–3 h). The solution should change color from pale yellow to deep red-orange upon the addition of TEA. Remove the solvent *in vacuo*, redissolve in 0.1 *M* NaOH, and extract with several portions of EtOAc. Combine the organic portions and concentrate to yield a dark red solid. Redissolve solid in EtOAc and purify by SiO_2 chromatography (hexanes → 3:7 hexanes/EtOAc) to yield an orange, gummy solid.

(7) Dissolve 2-alkynylaniline in 1:1 pyridine/MeOH and add 1 equiv. of $Cu(OAc)_2$. Stir the reaction overnight, remove MeOH *in vacuo*, and partition between H_2O and EtOAc. Remove the aqueous layer and wash with three more portions of 0.1 *M* HCl, followed by three portions of 1 *M* copper sulfate (to remove pyridine) and another three portions of H_2O. Concentrate the organics and carry the crude product on to the next step.

(8) Dissolve the crude product 7 in NMP, add KOtBu, and stir the reaction at 80 °C overnight. Dilute the reaction into EtOAc and wash with 0.1 *M* NaCl, followed by several portions of H_2O. Remove the organic solvent *in vacuo*, purify by recrystallization from EtOAc.

(9) Combine purified products 6 and 8 in a clean, dry-sealed reaction vessel and dissolve in freshly distilled pinacolone. Thoroughly degas this solution by purging under Ar or N_2. Add 0.1 equiv. of $Rh_2(OAc)_4$ and degas once more, then seal reaction vessel and move to 120 °C oil bath for 2–3 h. Allow reaction to cool to RT then remove pinacolone *in vacuo*. Redissolve concentrate in EtOAc and wash with 0.1 *M* NaCl and several portions of H_2O. Retain the organic portion, dry with $MgSO_4$, filter, and concentrate. Redissolve the concentrate in EtOAc and purify by SiO_2 chromatography (0–100% EtOAc in hexanes).

(10) Dissolve purified product 9 in dry DMF and cool to 0 °C. Add 1.2 equiv. of NaH and allow reaction to proceed for ~10 min at 0 °C. Add appropriate alkyl halide and allow the ice to melt and the reaction to warm to RT. If reaction progress stalls after several hours, dilute reaction into water, extract with EtOAc, dry, concentrate, and resubject. Once reaction is complete, dilute in H_2O, extract with several portions of EtOAc, dry with $MgSO_4$, filter, and concentrate. Carry crude concentrate on to the next step.

(11) Dissolve crude product 10 in a solution of 95:5 TFA:H_2O and 100 equiv. of dimethoxybenzene precooled to 0 °C. Allow the reaction to warm to RT then stir for an additional 1 h. Remove the solvent *in vacuo* and purify by C18 High-Performance Liquid Chromatography with a gradient of 20% MeCN → 100% MeCN in H_2O + 0.1% formic acid.

3.5. ES-kinase inhibitors

While AS Kinase inhibitors such as 1NA-PP1 project a bulky nonpolar group that complements the shape of the glycine or alanine gatekeeper pocket, ES-kinase inhibitors project groups that complement the chemical reactivity of the cysteine gatekeeper. To date, the only ES-kinase inhibitors reported are based on the PP and quinazoline scaffolds (Garske et al., 2011). It is important to remember that ES-kinase inhibitors are sensitive to thiol nucleophiles such as β-mercaptoethanol and dithiothreitol and should not be stored with or unnecessarily exposed to high concentrations of these reagents (Fig. 8.6).

3.6. Synthesis of ES-kinase PP inhibitors

The ES-kinase inhibitor 3VS-PP1 is closely related to other PP inhibitors in structure and synthetic design. A procedure similar to that described in Section 3.3 can be used to prepare the 3-nitrophenyl PP intermediate. The following protocol outlines the conversion of this intermediate to the final compound 3VS-PP1.

3VS-PP1 inhibitor synthesis protocol:

(1) Dissolve 1 equiv. of 3-nitrophenyl PP and 30 equiv. of zinc dust (<10 μm) in a clean, dry rbf and purge with Ar gas. Add 20 equiv of glacial acetic acid and stir reaction overnight. Filter the reaction through a plug of celite, dilute into sat. $NaHCO_3$, and extract with several portions of EtOAc. Combine the organics, dry with $MgSO_4$, filter,

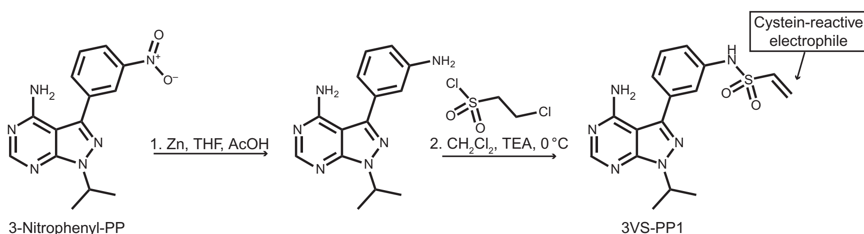


Figure 8.6 Synthetic scheme for the synthesis of the ES-kinase inhibitor 3VS-PP1.

concentrate, and purify by SiO_2 chromatography (100% $CH_2Cl_2 \rightarrow$ 9:1 CH_2Cl_2:MeOH).

(2) Dissolve 1.1 equiv. of intermediate 1 and 1.2 equiv. of TEA in dry CH_2Cl_2 and cool to 0 °C. Add 1 equiv. 2-chloro-1-ethane sulfonylchloride and allow the reaction to proceed for 1 h at RT. Quench reaction by dilution into sat. $NaHCO_3$ and extract with three portions of CH_2Cl_2. Combine the organic portions, dry with $MgSO_4$, filter, and concentrate. Purify by C18 HPLC (H_2O + 0.1% formic acid $\rightarrow$ 1:1 H_2O:MeCN + 0.1% formic acid).

3.7. AS Kinase inhibitors for PI3-like kinases

For PI3-like kinases, structural consensus is emerging that a functionalized quinoline scaffold can act as an effective hinge-binding element in addition to projecting into the pocket toward the gatekeeper residue. Kliegman et al. found that in at least two cases in *Saccharomyces cerevisiae* (TOR2-as2 and MEC1-as2), commercially available BEZ235 was selective for the AS Kinase over the WT enzyme in cells (Kliegman et al., 2013). QL-IX-55 is similar in structure to BEZ235 but has a smaller amino pyridine substituent in place of the quinoline group. This difference in substituent size explains why BEZ235 but not QL-IX-55 forms a steric clash with the yeast TOR2-WT gatekeeper and is selective for the AS kinase. In contrast, QL-IX-55 is a potent inhibitor of TOR1-WT and TOR2-WT (Hsieh et al., 2012) (Figure 8.7). This BEZ-based scaffold represents a new structural class of inhibitors of engineered PI3-like kinases that should be explored further.

In contrast to yeast, PI3 kinase-like kinases in mammals are intolerant of the typical gatekeeper mutation, rendering mTOR catalytically inactive. However, the nontraditional gatekeeper mutant (Y2225A) retains activity and is potently inhibited by typical PP1-based inhibitors. While it was formerly understood that PI3-like kinases were functionally inaccessible using the AS Kinase strategy (Alaimo & Shokat, 2001), recent work has shown that expanding the selection of analogs as well as creative alternatives to the canonical gatekeeper residue can be employed to make these proteins amenable to the technique (Kliegman et al., 2013).

3.8. Protocol for measuring inhibitor potency

In order to identify the most efficacious inhibitor for a particular AS Kinase, a simple assay should be devised to measure the potency and selectivity of a panel of inhibitors. If the purified WT and AS Kinases are available, it may be ideal to carry out this experiment *in vitro* using a peptide or protein substrate and ^{32}P-ATP. The half-maximal inhibitory concentration

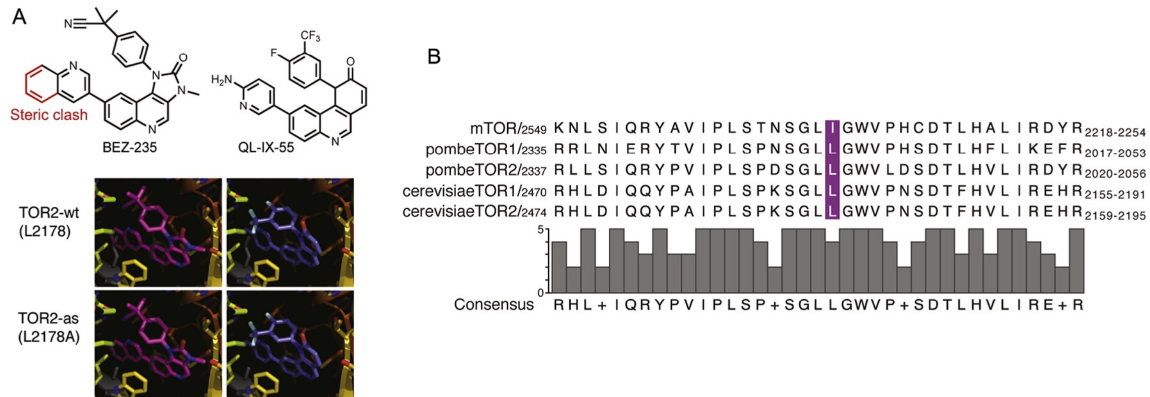


Figure 8.7 (A) Homology model of mTOR based on the structure of PI3Kγ shown with the gatekeeper residue in gray. The known *S. cerevisiae* TOR1/TOR2 inhibitor QL-IX-55 (purple) and BEZ235 (magenta) were oriented based on a typical H-bonding interaction with the backbone carbonyl of valine in the active site at VanDer Waals distances away from other residues that form the ATP-binding pocket. The isoleucine gatekeeper clash with BEZ235 is exacerbated by mutation to leucine and alleviated by mutation to alanine. The smaller QL-IX-55 does not sense this residue. (B) Sequence alignment shows that the gatekeeper residue (in purple) is isoleucine in mTOR and leucine in all other cases. The active site is highly conserved.

(IC_{50}) of each molecule can be determined by measuring the inhibitor dose-dependent activity of the kinase. Typically, appropriate AS Kinase inhibitors have *in vitro* IC_{50} values below 100 n*M* for the AS mutant and above 1 μ*M* for the WT kinase. The advantage of this approach is that precise IC_{50} values can be measured for several inhibitors in one experiment. For example, one 96-well plate can accommodate eight concentrations of four inhibitors in triplicate. The major disadvantage is that many kinases are difficult to express and purify.

If the purified proteins are unavailable, an alternative way to measure kinase inhibition is by overexpressing the WT and AS Kinases in cells and analyzing for substrate protein phosphorylation by western blot or measuring an appropriate phenotypic readout. For example, many kinases can be transiently transfected into 293T cells and probed for substrate or autophosphorylation using commercially available antibodies. One disadvantage of this strategy is that it may difficult to scale this experiment to allow measuring dose–response curves for multiple inhibitors.

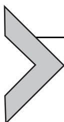
Protocol for IC_{50} determination with Src-AS1 *in vitro*:

1. Prepare the following three solutions:

   2.5 × Reaction mix (5 n*M* Src kinase, 125 m*M* Tris (pH 8.0), 25 m*M* $MgCl_2$, 0.25 mg/mL BSA, 250 μ*M* substrate peptide (IYGEFKKK)),
   2.5 × ATP mix (250 μ*M* ATP, 0.2 μCi ^{32}P ATP), and
   5 × Inhibitor dilution in 10% DMSO.
2. In a 96-well plate, combine 10 μL of reaction mix with 5 μL drug dilution for each reaction, centrifuge to ensure removal of air bubbles, and incubate at room temperature for 15 min.
3. Combine 10 μL of ATP mix with the drug dilution/reaction mix to initiate the kinase reaction.
4. Quench 3 μL of the reaction by spotting onto P31 ion exchange paper and quickly evaporating under heat lamp. Wash the paper 3 × with 1% phosphoric acid and dry under the heat lamp. Expose dried P31 paper to a phosphorimaging screen and analyze using a Typhoon FLA 9500 (GE Healthcare Life Sciences).
   - It is preferable to quench samples at several time points (15, 30, and 45 min).
5. Quantify the signal of each reaction spot using imaging software such as ImageQuant (GE Healthcare Life Sciences), plot values as a function of log(drug concentration), and fit to a sigmoid to determine the IC_{50} values.

Protocol for testing inhibitors against EphA4-AS1 in 293T cells:

1. Plate 0.3×10^6 293T cells in 2 mL DMEM (10% FBS) into each well of a 6-well tissue culture plate and grow cells 12–18 h at 37 °C in 5% CO_2.
2. Transfect 0.1 μg of pCS2+ plasmid-bearing full-length EphA4-WT or EphA4-AS1 using lipofectamine®, LTX, and PLUS reagent (Life Technologies) and return cells to 37 °C 5% CO_2 incubator for 12–18 h.
3. Remove medium and replace with 1.5 mL of fresh DMEM (10% FBS) containing inhibitor and a final concentration of 1% DMSO. Incubate for 1–2 h at 37 °C 5% CO_2.
   - Typical concentration ranges (10 μ*M* to 39 n*M*, fourfold dilutions).
4. Remove drug medium and wash cells with cold PBS.
5. Add 150 μL cold lysis buffer, incubate on ice for 10 min, and pipette up and down several times to ensure complete lysis.
   - Lysis buffer: 50 m*M* Tris (pH 7.4), 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* Na_3VO_4, 10 m*M* sodium-β-glycerophosphate, 1% triton, 50 m*M* NaF, 5 m*M* sodium pyrophosphate, 0.27 *M* sucrose, 50 m*M* benzamidine, one complete mini protease inhibitor tablet (Roche), 1 m*M* PMSF, one PhosSTOP (Roche) phosphatase inhibitor tablet, 0.1 mg/mL RNAse A, and 0.1 mg/mL DNAse I.
6. Centrifuge lysates to precipitate insoluble debris then transfer the soluble lysate to fresh tubes.
7. Normalize sample protein concentration, separate lysate proteins by SDS–PAGE, and transfer to a nitrocellulose membrane.
8. Probe cells for phopsho-Eph and total EphA4 using appropriate primary antibodies and IRDye® 800 secondary antibodies (LiCOR). Image and quantify results using a LI-COR Odyssey Quantitative Imaging System and plot the ratio of pEph/EphA4 as a function of drug concentration. EC_{50} values can be determined by plotting the pEph/EphA4 ratio as a function of the log(drug concentration) and fitting the data to a sigmoid function using graphing software.



4. AS KINASES IN CELLS

AS technology is most commonly used to study kinase-signaling pathways in single-cell eukaryotes, such as yeast, or in cell lines derived from multicellular organisms such as mice and humans. This allows ease of genetic manipulations and avoids the complication of pharmacokinetics (PK) and pharmacodynamics (PD) that are encountered when using small molecules

in intact multicellular organisms. The advantages of the approach are most apparent *in vivo* since the technique permits rapid and tunable interrogation of catalytic function that is not possible using genetic manipulations alone. Many processes in biology (signaling events in particular) occur on time-scales that genetic perturbations (which are subject to adaptation at the transcriptional and translational timescales) are unable to capture. Chemical perturbation offers precise experimental control over cellular events such as cell division that occur within minutes.

4.1. AS Kinases in yeast

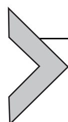
The genetic tractability of yeast and their ability to exist in a stable haploid state has lead to widespread use of AS technology in both *S. cerevisiae* and *Schizosaccharomyces pombe*. For example, there are 113 conventional protein-kinase genes in budding yeast (Hunter & Plowman, 1997) and the AS technique has been applied to 26 individual kinases thereby covering 23% of the yeast kinome. More recently, Gregan and coworkers developed functional AS alleles and identified suitable inhibitors for 13 of the 17 essential kinases in *S. pombe* (Cipak et al., 2011). Some of the key discoveries made with various AS Kinases in yeast include the large-scale identification of Cdc28 substrates (Holt et al., 2009) and the discovery of allosteric activation of the RNAse activity of Ire1 upon ligand binding to the ATP-binding site within its kinase domain (Papa, Zhang, Shokat, & Walter, 2003).

Recently, Kliegman and coworkers combined AS technology with high-throughput yeast genetics in order to measure dose-dependent genetic interactions between the catalytic function of a kinase and each deleted gene. This technique offers a new systematic approach that addresses the functional connectivity of individual enzymes without the destruction of protein complexes that is typically queried by traditional gene deletion studies. The ease of using the AS approach in yeast chemical-genetic applications offers a systematic way to use this technology to elucidate eukaryotic kinase-signaling pathways and generate testable hypotheses for kinase–substrate relationships.

4.2. AS Kinases in mammalian cells

Over 40 mouse or human AS Kinases have been reported in peer-reviewed publications making mammalian cells the most commonly studied with AS technology. In many cases, a disease-causing kinase allele may be dominant, allowing simple overexpression of the AS mutant in an appropriate cell line

for investigation. In other cases, the WT-kinase gene must be replaced with the AS allele. Although genetic manipulation of mammalian cells is often more difficult than in yeast, they offer the opportunity to address many questions most relevant to human disease. For example, Wong et al. used an AS mutant of the disease-causing fusion protein Bcr-Abl to demonstrate that inhibition of this kinase alone was not sufficient to eliminate all myeloproliferative disorder cell populations (Wong, 2004). This was an important discovery as it revealed the off-target effects of the clinical therapeutic imatinib are, in fact, required for effective treatment of heterogeneous chronic myeloid leukemia cell populations. AS Kinases have also been used to shed light on fundamental processes of mammalian biology and development. An AS mutant of Zap-70, for example, has been used to identify its role in T-cell receptor and CD28 super agonist signaling and to determine which functions are dependent on kinase activity. Thus, the use of AS Kinases in mammalian tissue culture is a powerful method for investigating fundamental biological processes and the molecular basis of human disease.



5. AS KINASES IN LIVING MULTICELLULAR ORGANISMS

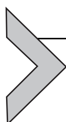
Many developmental processes can only be studied in the context of an intact living plant or animal. Furthermore, therapeutic drug targets can only truly be validated by inhibition *in vivo*. Thus, several AS Kinases have been introduced into mice and used to study the *in vivo* biology or pharmacology of kinase-signaling pathways. The *in vivo* use of AS Kinases presents the additional challenges of achieving optimal PK and PD properties for small molecule inhibitors. While the properties of current AS Kinase inhibitors have not been optimized for *in vivo* efficacy, they are nevertheless sufficient for use with several AS Kinases in mice. For example, Wright and coworkers generated mice that are homozygous for an AS mutant of Ret kinase in order to study the role of GDNF signaling in spermatogenesis (Savitt et al., 2012). Taconic is a company that generates mice bearing homozygous AS Kinase alleles, which they refer to as ASKA mice. Although the effectiveness of their ASKA mice is not subject to peer review, they report to have generated functional ASKA mice for the following kinases: Akt1, Gskα, Gsk3β, Ntrk1, Ntrk2, Src, Btk, Map3k5, EphB4, Mapk14, PdgfrB, Met, and Rps6kb1 (http://www.taconic.com). It is likely that such a large number of ASKA mice suggest a strong unmet need in drug target validation. In both drug development enterprises and academic investigations, it is clear

that many important questions can only be addressed *in vivo* and that AS Kinase technology may play an important role in furthering these studies.

The methylene position of 1NM-PP1 and the 3-methyl group of 3MB-PP1 are likely to be susceptible to inactivating oxidation by P450 enzymes *in vivo* (Baer, DeLisle, & Allen, 2009; Lourido et al., 2013). This may explain why the inhibitor most commonly used to target AS Kinases in mice is 1NA-PP1, which has neither liability. However, since 1NA-PP1 is not the most potent or selective inhibitor for all AS Kinases, future work to design staralogs and 3-substituted PPs with enhanced PK properties will be of great importance to expanding the use of AS Kinases in mice. An important technical observation is that 1NA-PP1 is much more soluble as the HCl salt. Thus, it may be helpful to convert commercially available 1NA-PP1 to the HCl form in order to achieve the concentration necessary for *in vivo* efficacy.

Protocol for preparation of 1NA-PP1 HCl:

Dissolve 1.16 g of 1NA-PP1 in 50 mL of CH_2Cl_2 and stir at RT until completely dissolved. Add 1.2 equiv. of HCl (2 *M* in diethyl ether) and concentrate *in vacuo*. Wash the resulting off-white solid with cold ether. The HCl form can be distinguished from 1NA-PP1 by ^{1}H NMR as well as the fact that it has increased solubility (1NA-PP1 HCl but not 1NA-PP1 is soluble at 20 mg/mL in DMSO).



6. SUMMARY

This report provides a practical overview for constructing AS Kinases and their inhibitors as well as a theoretical framework for designing experiments that leverage the power of the AS technique. We outline how to identify the gatekeeper position of a kinase and describe several strategies to rescue the activity of AS Kinases' intolerant of glycine or alanine gatekeeper residues. Next, we explain the logic of AS Kinase inhibitor design and describe the strengths, limitations, and synthetic protocols for several of the most commonly used inhibitors. We highlight examples of how AS Kinases have been used to address questions about basic biological processes in yeast and in mammalian cell lines. Finally, we examined the use of AS Kinases in mice and explained the scope and limitations of current inhibitors *in vivo*.

Recently, Kawashima, Takemoto, Nurse, and Kapoor (2013) described a complementary chemical-genetic approach for studying protein kinases. This technique involves conducting a chemical screen to identify inhibitors

of the KOI and then generating a resistant kinase mutant to function as a negative control for off-target inhibitor activity. This strategy has the advantage that the WT kinase is targeted by the inhibitor and thus is the focus of study, while the engineered resistant mutant is only used as a negative control. This eliminates the possibility that processes under study might be perturbed by the reduced activity that is sometimes encountered with AS Kinases. However, this approach is limited in that it remains difficult to identify specific inhibitors for many kinases. In contrast, rescuing the activity of hypomorphic AS Kinases is straightforward using the steps we detail herein should it be necessary. In summary, the AS technique is a facile and general approach for probing kinase-signaling pathways with small molecule inhibitors. The stepwise strategy that we describe in this report allows for the identification of appropriate mutations and inhibitors and enables the application of AS technology to kinases from diverse families and organisms in cells and *in vivo*.

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