Chemical genetic strategy for targeting protein kinases based on covalent complementarity

Adam L. Garske[a], Ulf Peters[a], Arianna T. Cortesi[a], Jenny L. Perez[a], and Kevan M. Shokat[a,b,1]

[a]Howard Hughes Medical Institute and Department of Cellular and Molecular Pharmacology, University of California, 600 16th Street, MC 2280, San Francisco, CA 94158; and [b]Department of Chemistry, University of California, Berkeley, CA 94720

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The conserved nature of the ATP-binding site of the >500 human kinases renders the development of specific inhibitors a challenging task. A widely used chemical genetic strategy to overcome the specificity challenge exploits a large-to-small mutation of the gatekeeper residue (a conserved hydrophobic amino acid) and the use of a bulky inhibitor to achieve specificity via shape complementarity. However, in a number of cases, introduction of a glycine or alanine gatekeeper results in diminished kinase activity and ATP affinity. A new chemical genetic approach based on covalent complementarity between an engineered gatekeeper cysteine and an electrophilic inhibitor was developed to address these challenges. This strategy was evaluated with Src, a proto-oncogenic tyrosine kinase known to lose some enzymatic activity using the shape complementarity chemical genetic strategy. We found that Src with a cysteine gatekeeper recapitulates wild type activity and can be irreversibly inhibited both in vitro and in cells. A cocrystal structure of T338C c-Src with a vinylsulfonamide-derivatized pyrazolopyrimidine inhibitor was solved to elucidate the inhibitor binding mode. A panel of electrophilic inhibitors was analyzed against 307 kinases and MOK (MAPK/MAK/MRK overlapping kinase), one of only two human kinases known to have an endogenous cysteine gatekeeper. This analysis revealed remarkably few off-targets, making these compounds the most selective chemical genetic inhibitors reported to date. Protein engineering studies demonstrated that it is possible to increase inhibitor potency through secondary-site mutations. These results suggest that chemical genetic strategies based on covalent complementarity should be widely applicable to the study of protein kinases.

chemical genetics | irreversible inhibitor | SgK494

Reversible phosphorylation plays a paramount role in cell signaling processes and is regulated by kinases and phosphatases. Kinases, which constitute a large family of enzymes (>500 in humans), catalyze the transfer of the γ-phosphate of ATP to protein substrates. Aberrant kinase activity is linked to cancer as well as metabolic, immunological, and nervous system disorders. As a result, kinases have emerged as an important class of drug targets (1). The tyrosine kinase inhibitor imatinib, which blocks BCR-ABL (breakpoint cluster region-Abelson) in chronic myelogenous leukemia and c-KIT (Steel growth factor receptor) in metastatic gastrointestinal stromal tumors is a particularly effective therapeutic (2). While significant progress has been made in the kinase field, much of the kinome remains understudied. A recent analysis suggests that ∼200 kinases are poorly characterized and >100 are completely uncharacterized (3).

How can the functions of each individual kinase be determined? Genetic approaches for perturbing kinase activity have the advantage of being exquisitely specific, but are slow to exert their effects and cannot induce variable levels of inactivation. In contrast, small molecules induce kinase inactivation rapidly and in a dose-dependent manner. Notably, several cases have been documented in which genetic and pharmacological approaches yield strikingly differing phenotypes when used to target the same kinase (4). These differences are not necessarily contradictory and may have both biological and therapeutic implications. Pharmacological approaches to studying protein kinases can be challenging to implement because it is difficult to generate selective ATP-competitive inhibitors. In several serendipitous cases, an originally unwanted "off target" of a kinase inhibitor in the clinic turned out to drive therapeutic efficacy while the original target was irrelevant (5, 6).

From the perspective of research tools to understand the role of individual kinases a systematic means to modulate the activity of a single kinase of interest is highly desirable (7, 8). We have developed a chemical genetic approach based on engineered shape complementarity between the kinase active site and a small molecule inhibitor, which allows systematic discovery of an inhibitor for every kinase. A conserved hydrophobic residue in the kinase active site known as the "gatekeeper" is mutated to a small residue such as glycine or alanine to generate a uniquely targetable mutant kinase termed an analog-sensitive (*AS*) allele (9, 10). The engineered kinase can be targeted with sterically bulky analogs of natural kinase inhibitors, which are capable of occupying the enlarged engineered kinase pocket (Fig. 1). Wild type kinases are resistant to inhibition by the bulky analog as the result of a steric clash with naturally occurring gatekeeper residues.

In yeast, the *AS* strategy has been useful in determining the function of many kinases including Cdc28 (10, 11), Ime2 (11), Cla4 (12), and Snf1 (13). Several discoveries using this approach have also been made in mammalian systems including a noncatalytic role for Zap70 in regulatory T-cells (14), and the dependence of transformed cells on aberrant oncogenic signaling by the EGFR kinase (15). In addition, the *AS* method has led to new insights regarding inhibitor-induced conformational changes of kinases, which have important clinical implications. Examples include elucidation of the mechanisms of inhibitor-induced Akt hyperphosphorylation (16) and transactivation of RAF (Rapidly Accelerated Fibrosarcoma) dimers (17).

While the *AS* strategy works well in many cases, mutation of the gatekeeper residue to a small amino acid often impairs the activity of the kinase (sometimes completely), likely by disruption of a "hydrophobic spine," which stabilizes the active kinase conformation (18, 19). This loss of activity is acceptable in some situations where signaling is still reasonably robust, but in others precludes use of the analog-sensitive approach. In some instances

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[1]To whom correspondence should be addressed. E-mail: shokat@cmp.ucsf.edu.

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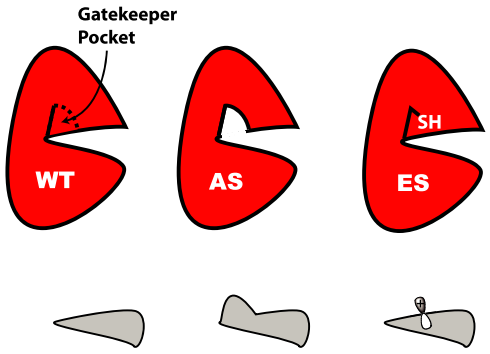


Fig. 1. Chemical genetic strategies for inhibiting protein kinases. Kinases are depicted in red and inhibitors types are represented by gray shapes. WT kinases generally harbor hydrophobic gatekeeper residues and are difficult to inhibit selectively. An *AS* protein kinase has an engineered glycine or alanine gatekeeper and may be selectively inhibited by a bulky inhibitor. An *ES* protein kinase contains an engineered cysteine gatekeeper and may be selectively inhibited by an electrophilic inhibitor.

a second-site suppressor can be identified in the N-terminal subdomain to mitigate activity loss (20) but a general solution has not been forthcoming. Besides reductions in activity (k_{cat}), mutation of the gatekeeper to a small amino acid can have significant effects on substrate binding (K_m). For example, the M486A gatekeeper mutation of PKCε causes a marked decrease in priming site phosphorylation relative to wild type PKCε, an observation that has been attributed to a reduced affinity for ATP (21).

Due to the attenuated catalytic activity in some kinases and potentially lower affinity for ATP, caused by mutation of the gatekeeper residue to a glycine or alanine residue, we sought to develop a new chemical genetic approach for targeting mutant kinases. Our goal was to identify a mutation that would enable specific and potent inhibition with a small molecule, without the need to enlarge the size of the ATP-binding pocket. We chose to explore introduction of a cysteine gatekeeper and use of electrophilic small molecule inhibitors that specifically target this mutation. We reasoned that introduction of a cysteine gatekeeper would better preserve the geometry of the ATP pocket than glycine or alanine, and would provide a reactive handle for targeting by an electrophilic inhibitor. This approach relies on covalent complementarity between the engineered kinase and the inhibitor analog and we refer to the mutant kinase as an electrophile-sensitive (*ES*) allele (Fig. 1). Previous studies have successfully exploited engineered cysteines for regulation of cyclophilin (22, 23) and kinases (24–26) with electrophilic small molecules.

Here, we report generation of a Src tyrosine kinase allele containing a cysteine gatekeeper residue (T338C c-Src) and compare its properties with a previously described glycine gatekeeper (T338G c-Src) allele. We show that T338C c-Src is an excellent mimic of the wild type kinase and can be specifically targeted with electrophilic inhibitors. X-ray crystallography and kinome-wide screening suggest that these are among the most selective chemical genetic kinase inhibitors developed to date. Additional studies revealed that inhibitor potency can be tuned with secondary mutations near the engineered cysteine residue in the ATP-binding site.

Results

Cysteine as a Gatekeeper. Recent work suggests that the hydrophobicity of the gatekeeper residue in protein kinases is correlated with stabilization of a hydrophobic spine, which is a signature of the active conformation (18, 19). The combination of hydrophobicity, unique reactivity, and the lack of gatekeeper cysteines in all but two of the human kinases (MAPK/MAK/MRK overlapping kinase and SgK494) made a cysteine gatekeeper an ideal candidate for chemical genetic studies. To evaluate the feasibility of employing a cysteine gatekeeper, we generated recombinant wild type (WT) and T338C c-Src and assayed for kinase activity (Table 1). For comparison, we also evaluated T338G c-Src (c-Src-*AS*1), the variant typically used for chemical genetic studies. The k_{cat} value for T338C c-Src (183 min^{-1}) closely approximated that of WT (159 min^{-1}) and was ~3.5-fold greater than that of c-Src-*AS*1 (51.9 min^{-1}). These effects translate to a 14-fold improvement in catalytic efficiency (k_{cat}/K_m) for T338C c-Src in relation to c-Src-*AS*1.

Table 1. Kinetic parameters for c-Src variants

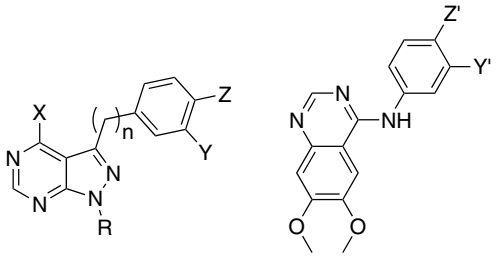
c-Src Variant	k_{cat} (min^{-1})	$K_{m,ATP}$ (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
WT	159 ± 4	31.9 ± 3.0	4.99 ± 0.40
T338C	183 ± 3	21.9 ± 1.7	8.34 ± 0.57
*AS*1	51.9 ± 1.9	87.5 ± 12.6	0.592 ± 0.072

Values were determined by fitting data to the Michaelis-Menten equation. Standard errors associated with the fits are reported.

Targeting a Cysteine Gatekeeper with Electrophilic Inhibitors. Encouraged by the robust activity exhibited by T338C c-Src, we sought to determine whether it was possible to target a cysteine gatekeeper with electrophilic inhibitors. Pyrazolopyrimidines have proven to be excellent scaffolds for designing ATP-competitive kinase inhibitors (9, 10). A panel of 3-phenyl-substituted pyrazolopyrimidines was synthesized with electrophilic groups at positions expected to be in close proximity to the gatekeeper residue (compounds **1**–**6**; Table 2). Structurally diverse electrophiles were installed at meta and para positions of the 3-phenyl ring and consisted of vinylsulfonamides, acrylamides, and chloroacetamides. A meta-substituted vinylsulfonamide, **3**, exhibited the most potent inhibition of T338C relative to WT c-Src (~10-fold increase), while a para-substituted version, **5**, elicited an ~8-fold improvement (Table 2). Acrylamides (**1**) and chloroacetamides (**6**) proved to be less potent inhibitors. Because the C4-exocyclic amine of pyrazolopyrimidine-based scaffolds may hydrogen bond with the side chain of threonine gatekeepers—an interaction that may occur with a cysteine gatekeeper and hamper nucleophilic reactivity—we also generated 4-*des*-amino versions of meta-substituted pyrazolopyrimidines (**2** and **4**). Under the assay conditions used, we did not detect IC_{50} values under 5 μM for either **2** or **4** for WT or T338C c-Src. The poor potency of the *des*-amino compounds is most likely due to loss of a hydrogen bonding interaction with the hinge region of the kinase.

Past studies reveal that while 3-phenyl-linked pyrazolopyrimidines such as PP1 inhibit kinases with relatively small gatekeeper residues such as threonine, bulkier substituents typically confer orthogonality to these kinases (9, 10). Because threonine and cysteine occupy similar volumes (93 $Å^3$ vs. 86 $Å^3$) (27), it was unclear whether it would be possible to achieve inhibition of T338C c-Src with a benzyl-derivatized pyrazolopyrimidine scaffold. An array of 3-benzyl-substituted pyrazolopyrimidines modified with electrophiles or isosteric and unreactive negative control groups at the meta and para positions was synthesized and screened against WT and T338C c-Src (compounds **7**–**17**, Table 2). As expected, the benzyl functionalized compounds were poor inhibitors of WT c-Src (IC_{50} values >5 μM). We were pleased to find that it was possible to achieve potent inhibition of T338C c-Src despite the similar size of cysteine and threonine. Interestingly, the best inhibitors of T338C c-Src possessed electrophiles at the meta position. One notable compound, **9**, which is functionalized with a vinylsulfonamide, exhibited an IC_{50} value of 114 nM. An unreactive control compound **11** resulted in a 31-fold drop in potency. A fluoromethylketone bearing compound, **13**, yielded an IC_{50} value of 317 nM, which was >14-fold more potent than the corresponding ketone, **14**. The fact that **13** was a potent

Table 2. IC_{50} values for electrophile-derivatized pyrazolopyrimidines and 4-anilinoquinazolines against WT c-Src and T338C c-Src



Compound	*n*	*R*	*X*	*Y*	*Z*	WT c-Src IC_{50} (nM)	T338C c-Src IC_{50} (nM)
1	0	*i*Pr	NH_2	$NHCOCHCH_2$	H	2,319	419
2	0	*i*Pr	H	$NHCOCHCH_2$	H	>5,000	>5,000
3	0	*i*Pr	NH_2	$NHSO_2CHCH_2$	H	1,004	111
4	0	*i*Pr	H	$NHSO_2CHCH_2$	H	>5,000	>5,000
5	0	*i*Pr	NH_2	H	$NHSO_2CHCH_2$	899	145
6	0	*i*Pr	NH_2	$NHCOCH_2Cl$	H	>5,000	817
7	1	*i*Pr	NH_2	$NHCOCHCH_2$	H	>5,000	2,762
8	1	*i*Pr	NH_2	H	$NHCOCHCH_2$	>5,000	>5,000
9	1	*i*Pr	NH_2	$NHSO_2CHCH_2$	H	>5,000	114 (306)
10	1	*i*Pr	NH_2	H	$NHSO_2CHCH_2$	3,083	1,759
11	1	*i*Pr	NH_2	$NHSO_2CH_2CH_3$	H	>5,000	3,497
12	1	*i*Pr	NH_2	$NHCOCH_2Cl$	H	>5,000	>5,000
13	1	*i*Pr	NH_2	$COCH_2F$	H	>5,000	317 (336)
14	1	*i*Pr	NH_2	$COCH_3$	H	>5,000	4,520
15	1	Me	NH_2	$NHSO_2CHCH_2$	H	>5,000	3,161
16	1	*t*Bu	NH_2	$NHSO_2CHCH_2$	H	>5,000	618
17	1	Cp	NH_2	$NHSO_2CHCH_2$	H	>5,000	196
Compound				*Y′*	*Z′*		
18				$NHCOCHCH_2$	H	>5,000	1,661
19				$NHSO_2CHCH_2$	H	>5,000	1,004
20				H	$NHSO_2CHCH_2$	2,170	560

In these assays, the inhibitors were preincubated with the Src variant for ten minutes prior to assay initialization by addition of ATP. Values in parentheses indicate IC_{50} values that were measured using reduced kinase concentration (0.5 nM as opposed to 5 nM) and a wider range of inhibitor concentrations (10,000-2.4 nM as opposed to 10,000-10 nM). These measurements were performed to better evaluate the potency of our most orthogonal inhibitors against T338C c-Src.

inhibitor is significant because fluoromethylketone electrophiles are known to have low reactivity with intracellular thiols (28). Meta-substituted acrylamides and chloroacetamides were relatively weak inhibitors.

Recent work suggests that the substitution pattern at the N1 position of pyrazolopyrimidines can modulate activity (29). In order to determine the effects of modifying the group at this position, a structure activity relationship analysis was performed on the pyrazolopyrimidine scaffold with a benzyl-linked meta-vinylsulfonamide (compounds **9**, **15**–**17**; Table 2). This analysis revealed that secondary alkyl groups such as isopropyl (**9**) and cyclopentyl (**17**) moieties elicited optimal activity against T338C c-Src. Relative to isopropyl substitution, *tert*-butyl (**16**) and methyl (**15**) derivatization resulted in 5- and 28-fold drops in potency, respectively. Collectively, these results indicate that substitution at N1 can be used to modulate potency against T338C c-Src.

Having demonstrated that electrophile-derivatized pyrazolopyrimidines can potently inhibit a kinase with a cysteine gatekeeper, we wanted to determine the feasibility of using other ATP-competitive scaffolds. To this end, Michael acceptor-derivatized 4-anilinoquinazolines were synthesized and evaluated as inhibitors (compounds **18**–**20**; Table 2). We found that meta- and para-substitution of the aniline moiety yielded inhibitors of poor potency and only 3- to 6-fold selectivity for T338C relative to WT c-Src (Table 2). However, there are several kinases (e.g., EGFR) for which quinazoline-type inhibitors exhibit remarkable potency (1). It is possible that the electrophilic quinazolines reported in this study would have increased potency against these kinases with engineered cysteine gatekeepers.

Evaluation of Reversibility. In order to determine whether the electrophilic inhibitors covalently interact with the cysteine gatekeeper, two compounds, **9** and **13**, were further evaluated. Both compounds inhibited T338C c-Src in a time-dependent manner (*SI Appendix: Table S1*). In addition, when T338C c-Src was treated with either inhibitor and purified by gel filtration, inhibition against the kinase was retained (*SI Appendix: Fig. S1*). In contrast, in the case of WT c-Src, inhibition was lost after gel filtration. Importantly, inhibition by PP1, a reversible Src inhibitor, was abrogated in the cases of both WT and T338C c-Src following gel filtration (*SI Appendix: Fig. S1*). Full protein mass spectrometry suggested specific labeling of T338C relative to WT c-Src for **9** (*SI Appendix: Fig. S2*). However, under similar conditions, we did not observe adduct formation with **13**, possibly due to a reversible covalent interaction. These types of interactions are well documented for several types of electrophilic protease inhibitors (30). The results suggest that covalent binding of the electrophilic inhibitors depend on the presence of a cysteine gatekeeper and we will hereafter refer to T338C c-Src as electrophile-sensitive c-Src1 (c-Src-*ES*1).

Crystal Structure of c-Src-*ES*1 with 9. In order to elucidate the binding mode for a kinase with a cysteine gatekeeper and an irreversible inhibitor, we solved an X-ray crystal structure of the catalytic domain of c-Src-*ES*1 (residues 251–533) bound to **9** (Fig. 2, *SI Appendix: Table S2*). Cocrystallization of c-Src-*ES*1 with **9** was performed using hanging-drop vapor diffusion. The complex was solved by molecular replacement and, contained two molecules in the crystallographic asymmetric unit of the *P*1 space group. The structure was refined to 2.2 Å and exhibited electron

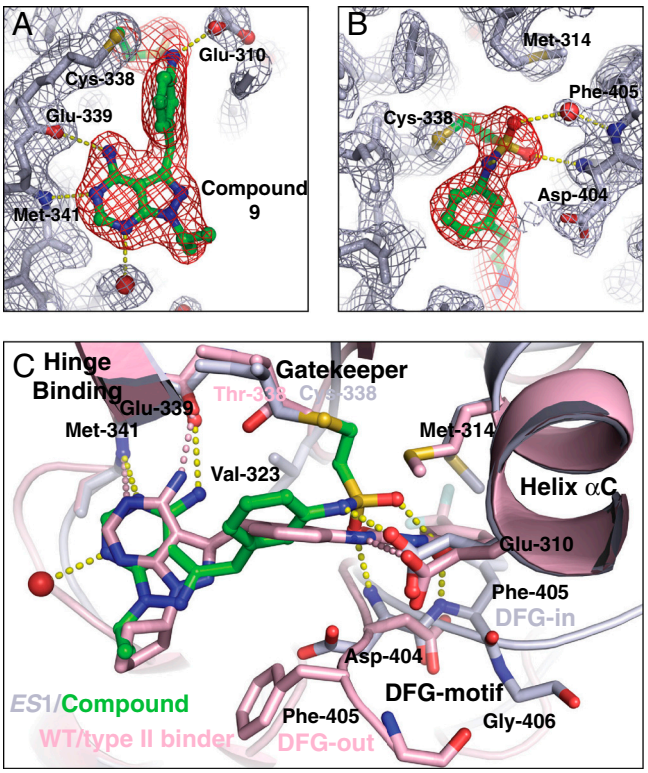


Fig. 2. Crystal structure of compound **9** bound covalently to c-Src-*ES*1. The experimental electron density of c-Src-*ES*1 at 2.20 Å resolution is shown ($2F_0$-F_c map at 1σ). (*A*) The pyrazolopyrimidine portion of compound **9** (green) interacts with the hinge region of c-Src (Met-341 and Glu-339), while the sulfonamide group makes a hydrogen bond with Glu-310 of the αC helix. (*B*) Electron density reveals a covalent linkage between Cys-338 and compound **9**. The oxygen atoms of the sulfonamide interact with the backbone of Asp-404 and via a water molecule with Phe-405, both of which are part of the DFG-motif of the kinase. (**C**) Comparison of structural features of compound **9** (green) bound to c-Src-*ES*1 (gray) and a published Type II pyrazolopyrimidine compound bound to WT c-Src (pink, pdb code: 3el7) (29). Both compounds engage the hinge region in a similar fashion and bind the αC helix in the "in" conformation. Furthermore, both compounds participate in hydrogen bonding interactions with Glu-310 and backbone amides of the DFG-motif. However while the Type II inhibitor binds in the "DFG-out" conformation, compound **9** engages the "DFG-in" orientation. In addition, while the positioning of the gatekeeper main chain is almost identical, the sulfhydryl of the Cys-338 points in the opposite direction relative to the hydroxyl group of Thr-338 in order to facilitate a covalent bond with compound **9**.

density for **9** covalently bound to Cys338. Poor electron density was observed near the N terminus (residues 251–256) and in flexible regions of the kinase such as the glycine-rich loop (residues 275–278) and the activation segment (residues 407–424). However, the DFG motif at the beginning of the activation segment (residues 404–406) was clearly resolved and was in the conformation associated with an active kinase (DFG-in).

In the **9**-c-Src-*ES*1 cocrystal structure, the pyrazolopyrimidine pharmacophore interacts with the backbone amides of Glu339 and Met341 of the hinge region (Fig. 2*A*). The oxygen atoms of the sulfonamide hydrogen bond directly to the backbone amide of Asp404 and to that of Phe405 via a water molecule (Fig. 2 *B* and *C*). Additionally, the nitrogen of the sulfonamide makes a direct hydrogen bond to the side chain Glu310 (Fig. 2 *A* and *C*). In crystal structures of WT Src, the hydroxyl of the gatekeeper threonine is often directed towards the C4-exocyclic amine of the adenine portion of ATP mimetics (29, 31) (Fig. 2*C*). However, in the **9** c-Src-*ES*1 cocrystal structure, the sulfhydryl of Cys338 adopts a distinct rotamer to accommodate the bulky C-3 benzyl group and facilitate a covalent bond (Fig. 2*C*). The flexible ethylsulfonamide moiety is situated to allow the covalent linkage with Cys338 (Fig. 2*B*). Interestingly, the side chain of Met314, a critical component of the hydrophobic spine (18, 19), is dramatically shifted relative to its position in other c-Src structures (Fig. 2 *B* and *C*). Movement of Met314 appears to prevent a steric clash with the ethylsulfonamide moiety of **9**.

Kinome-Wide Profiling of Inhibitors. In order to identify potential off-targets, we screened a panel of the electrophilic inhibitors that showed inhibition of c-Src-*ES*1 against 307 kinases (*SI Appendix: Table S3*). Compounds that were profiled include **3**, **4**, **9**, **13**, and **20**. Excluding **3**, all of the compounds had relatively few off-target effects. The fact that **4** had significantly fewer off-targets than **3** is not surprising as **4** lacks the exocyclic amino group that is present in **3**. The exocyclic amine mimics N6 of ATP and plays an important role in a hydrogen bonding interaction with the hinge region of kinases. It is interesting to note that several of the kinases for which >80% inhibition was achieved with vinylsulfonamide-based inhibitors was observed are those with exposed cysteines near the active site (e.g., EGFR, HER4, BMX, and TXK). It is likely that inhibition of these kinases is due to reaction with the cysteine residue near the active site, as they all share the same nonconserved cysteine. It is unclear whether these interactions represent true inhibition as the result of an alternative inhibitor binding mode or are due to nonspecific reactions at the kinase surface. Further analysis will be required to resolve this observation. The fluoromethylketone-type compound, **13**, had very few off-targets and is the most selective chemical genetic kinase inhibitor that we have reported to date.

Evaluation of Cysteine Gatekeeper Targeting Inhibitors for MOK Inhibition. To our knowledge, MOK (MAPK/MAK/MRK overlapping kinase) and SgK494 are the only human kinases with an endogenous cysteine gatekeeper (32). Unfortunately, neither of these kinases was represented in the kinome screen. We chose to further investigate MOK because it had been previously examined and an expression system was established (33, 34), while little is known about SgK494. MOK is a serine/threonine kinase of the MAPK superfamily that is expressed in several tissue types. Very little is known about the cellular role of MOK and no substrates have been identified to date. MOK was heterologously expressed in Cos7 cells, immunoprecipitated, and tested for inhibition by a small panel of inhibitors using myelin basic protein as a substrate (Fig. 3). The inhibitors evaluated included **9**, **11**, **13**, and **14**. While neither **9** nor **11** were particularly potent at a 1 μM concentration, both **13** and **14** brought MOK activity to near background levels. The unexpected potency achieved by the control compound **14**, may be due to the formation of a covalent reversible hemithioacetal species, because this compound contains a reactive ketone. These findings suggest that MOK may be an off-target of **13**, but is likely to be minimally impacted by **9**. However, this potential off-target should not be an impediment to use of **13** as it has significantly fewer off-targets than frequently used analog-sensitive inhibitors such as 1NA-PP1 and 1NM-PP1 (*SI Appendix: Table S4*).

Blockade of v-Src-*ES*1 Activity in Cells with Electrophilic Inhibitors. Having demonstrated in vitro activity and noted few off-targets, we asked whether the electrophilic inhibitors would function in cells. To this end, I338C (v-Src-*ES*1), I338T, I338G (v-Src-*AS*1), and WT v-Src-transformed NIH-3T3 cell lines were generated. Unlike c-Src, v-Src is constitutively active and harbors an isoleucine gatekeeper. The I338T v-Src variant was generated for consistency with the in vitro c-Src studies. For each cell line, global levels of phosphotyrosine were analyzed (*SI Appendix: Fig. S3*). Importantly, the v-Src-*ES*1 variant was an excellent mimic of WT v-Src, while the activity of v-Src-*AS*1 (the mutant used in previous chemical genetic studies) was markedly diminished as

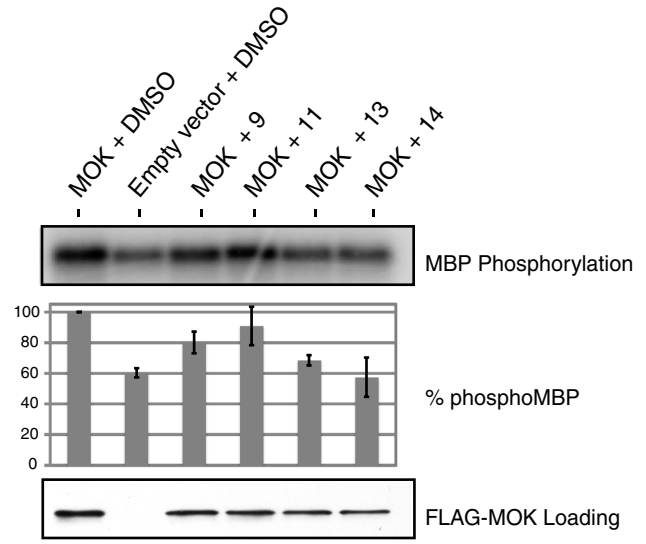


Fig. 3. Assay for MOK inhibition by cysteine gatekeeper-targeting compounds. (Top) FLAG-MOK expressed in COS7 cells was immunoprecipitated and assayed in vitro with a myelin basic protein (MBP) substrate and inhibitors at a concentration of 1 μM. Autoradiography is shown. (Center) Quantification of the percent MBP phosphorylated from three independent experiments with associated standard errors. All values are normalized relative to the MOK + DMSO lane. (Bottom) Western blot of loading controls for FLAG-MOK are shown.

judged by whole cell phosphotyrosine levels. To determine whether the electrophilic inhibitors function in cells, the v-Src-*ES*1 and I338T v-Src-transformed cell lines were treated with **9** and **13**. Both **9** and **13** inhibited v-Src-*ES*1 in a dose-dependent manner, while isosteric control compounds (**11** and **14**) showed no activity (Fig. 4). Furthermore, neither **9** nor **13** inhibited I338T v-Src even at levels as high as 10 μM. Collectively, these results suggest that a kinase with a cysteine gatekeeper can be selectively targeted in cells.

Second-Site Mutations to Modulate Inhibitor Potency. Naturally occurring cysteine residues in the active sites of enzymes often exhibit perturbed pK_a values as a result of specific acid/base containing amino acids which provide accentuated nucleophilic reactivity over surface cysteines or those in hydrophobic pockets. The gatekeeper residue in natural kinases is not positioned for nucleophilic chemistry and thus the engineered gatekeeper

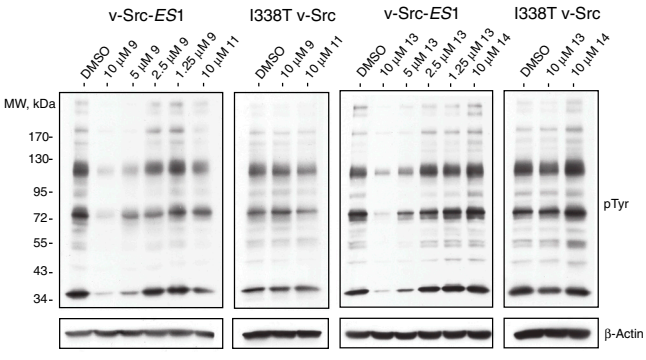


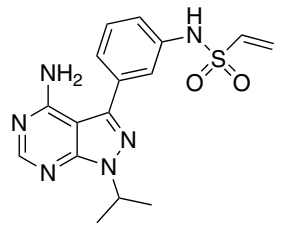
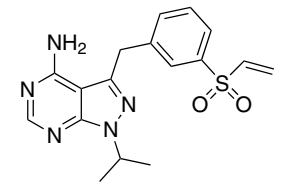
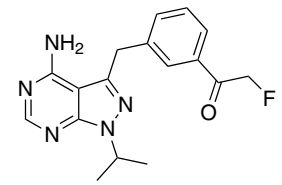
Fig. 4. Cellular dose response analysis for inhibition of v-Src-*ES*1 (I338C) with electrophilic inhibitors. NIH-3T3 cells stably transduced with either v-Src-*ES*1 or I338T v-Src were treated with electrophilic inhibitors or nonreactive analogs for one hour. Kinase activity was monitored by blotting for global phosphotyrosine levels. Actin blots were included to control for protein content.

cysteine is predicted to be in an unoptimized environment for heightened nucleophilic reactivity. Therefore, we sought to determine whether further kinase engineering could enhance potency. Our design strategy was to either enhance the reactivity of the cysteine by installing nearby hydrophilic/basic residues or to slightly enlarge the area around the cysteine to allow for additional rotational freedom to facilitate optimized thiol-electrophile attack geometry. Accordingly, mutations at Val323—a residue within 4 Å of the gatekeeper (Fig. 2*C*) in c-Src—were introduced in combination with T338C. Of the double mutants, V323A/T338C (c-Src-*ES*2) and V323S/T338C (c-Src-*ES*3) had substantial activity, while V323D/T338C (c-Src-*ES*4),V323E/T338C (c-Src-*ES*5), and V323H/T338C (c-Src-*ES*6) were inactive (*SI Appendix: Table S5*). Enhanced inhibitor potency was observed for both c-Src-*ES*2 and c-Src-*ES*3 when treated with **13** (Table 3). In the latter case, a 17-fold improvement was noted relative to c-Src-*ES*1. Interestingly, the potencies of **3** and **9** were not modulated appreciably upon introduction of the additional mutations. Taken together, these results indicate that the judicious placement of a secondary mutation can be an effective means for modulating inhibitor potency for an *ES* allele, but that this strategy needs to be evaluated on a case-by-case basis.

Discussion

Chemical genetics has proven valuable for determining the role of kinases in biology and disease. To date, >50 kinases have been evaluated using the widely employed version of this strategy, which takes advantage of shape complementarity. Mutation of the gatekeeper to a small amino acid such as glycine or alanine provides access to a nearby affinity pocket for bulky inhibitors, which are unable to inhibit the WT. While many kinases tolerate these mutations with little consequence, some kinases (~30%) suffer a significant or complete loss of activity. Furthermore, the noncovalent nature of the inhibitors limits their potency.

Table 3. IC_{50} values of a panel of electrophilic inhibitors against c-Src-*ES* variants with second-site mutations (*ES*1 =T338C; *ES*2 =T338C/V323A; *ES*3 =T338C/V323S)

Compound	c-Src variant IC_{50} (nM)		
	*ES*1	*ES*2	*ES*3
3 	138	69	163
9 	114	131	392
13 	317	57	19

In these assays, the inhibitors were preincubated with the Src variant for 10 min prior to assay initialization by addition of ATP.

To address these issues, we report a unique technique that makes use of a cysteine gatekeeper with the potential for covalent complementarity. A cysteine gatekeeper has the advantages that it is exceedingly rare in the kinome and is relatively hydrophobic, making it an excellent mimic of most WT gatekeeper residues.

A major advantage of the *ES* (i.e., Cys gatekeeper) approach to kinase chemical genetics over the commonly used *AS* (i.e., Gly or Ala gatekeeper) approach is that cysteine better maintains the geometry of the ATP-binding site and may thereby retain kinase stability and activity. Recent work suggests that more hydrophobic gatekeeper residues are linked to increased levels of catalytic activity for tyrosine kinases (19). This finding may explain why the drug resistant T315I and T790M mutations of BCR-ABL and EGFR (epidermal growth factor receptor), respectively, are particularly oncogenic and are present prior to drug therapy. The ability to recapitulate the activity conferred by hydrophobic gatekeepers is especially important because bulky residues such as methionine, leucine, phenylalanine, and threonine at this position account for ~90% of the kinome (35). Our kinetic measurements reveal that in the case of c-Src, the *ES*1 variant is an excellent mimic of WT activity. Furthermore, the *ES*1 variant of v-Src compares favorably to the WT, which contains a particularly hydrophobic (isoleucine) gatekeeper (*SI Appendix: Fig. S3*).

In addition to the turnover number (i.e., k_{cat}), the faithful mimicry of the WT affinity for ATP ($K_{m,ATP}$) is of physiological importance. Accumulating data indicate that the conformation of the ATP-binding pocket can have a significant impact on catalytic-independent functions of kinases. For example, recent studies with Akt (16) and PKCε (21) suggest that binding to an ATP-competitive inhibitor potentiates membrane recruitment and the induction/stabilization of priming phosphorylation events. In the case of PKCε, priming phosphorylation events for the M486A gatekeeper mutant are compromised upon PMA-induced translocation to the plasma membrane (21). This finding underscores the importance of the gatekeeper in modulating the conformation of the ATP pocket. Studies with RAF reveal that ATP-competitive inhibitors can also trigger membrane translocation and that this event is independent of kinase activity (17, 36). Together, these findings highlight the importance of the ATP-binding pocket and suggest that chemical genetic studies that preserve the native conformation will be useful in studying kinase function.

Targeting kinases with a cysteine gatekeeper is contingent on generating a high affinity ligand equipped with an electrophile within covalent bonding distance of the thiol. A major challenge inherent to this strategy is developing an inhibitor that minimizes reversible interactions with other kinase targets due to the similarity in size between cysteine and most endogenous gatekeepers. We were pleased to find that a benzyl-substituted pyrazolopyrimidine scaffold exhibited optimal activity, as it confers orthogonality to other kinases even those with relatively small gatekeeper residues such as threonine. High degrees of orthogonality are likely due to the fact that reversible binding to the benzyl portion causes a steric clash (even in the case of the cysteine gatekeeper; see Table 2 compounds **10** and **14**) but that transient binding followed by covalent bond formation are able to overcome this thermodynamic barrier. From our panel of compounds, selective inhibition was best accomplished with benzyl-substituted pyrazolopyrimidines bearing vinylsulfonamide (e.g., **9**, **17**) or fluoromethylketone (**13**) functionalities. It is interesting to note that other benzyl-substituted pyrazolopyrimidines bearing similar electrophiles such as an acrylamide (**7**) and an α-chloroacetamide (**12**) were poor inhibitors. In the former case, this fact may be because the double bond of the vinylsulfonamide (**9**) is expected to have greater rotational freedom than the planar acrylamide (**7**) as a result of the poor overlap between the sp^3/d orbitals of nitrogen and sulfur, respectively (37). In the instance of the α-chloroacetamide, it is possible that the electrophile/nucleophile pair is unable to assume an orientation compatible with S_N2 attack. This situation may not be the case for the fluoromethylketone (**13**), as the reaction may proceed by an initial attack of the cysteine at the carbonyl rather than via an S_N2 mechanism (38). As with the *AS* approach, the *ES* strategy may require screening of a small panel of compounds for a given kinase to determine an optimal inhibitor. This preliminary screen may be especially necessary for the *ES* approach because the inhibitors have multiple electrophiles and potentially different modifications at N1 of the pyrazolopyrimidine.

The binding mode of **9** (a vinylsulfonamide functionalized compound) with c-Src-*ES*1 is reminiscent of what has recently been dubbed Type I½ kinase inhibition (35). Like Type I inhibitors, Type I½ inhibitors bind the active conformation of the kinase (DFG-in) and engage in a series of hydrogen bonds in the hinge region. Type I½ are similar to Type II inhibitors in that they occupy the pocket situated behind the gatekeeper and hydrogen bond to the carboxylate of the conserved glutamate on the αC-helix and backbone amide of the DFG aspartate (Fig. 2*C*). The hydrogen bonds afforded by the tetrahedral arrangement of the sulfone may contribute to the increased potency of **9** relative to **7**, which contains an acrylamide. Another noteworthy feature of the cocrystal structure with **9** is that the pyrazolopyrimidine functionality is hydrogen bonded to the hinge region in a manner that would be expected for a reversible inhibitor. This observation suggests that the position of the electrophile is optimal relative to the introduced cysteine.

A particularly striking feature of the cocrystal structure with compound **9** is that in order to accommodate the inhibitor, the side chain of Met314 undergoes a substantial shift relative to its position in unliganded c-Src structures (39). This highly conserved residue is a critical component of the kinase hydrophobic spine (18, 19) and is almost always methionine or leucine (32). The residue may serve as a selectivity filter for especially bulky inhibitors such as **9** because the γ-branching of leucine may attenuate binding. Consistent with this hypothesis, **9** is a poor inhibitor of MOK, a kinase with a leucine at this position. This secondary selectivity filter is potentially advantageous as it reduces off-target activity against MOK. For kinases that harbor a leucine at this position, mutation to methionine may improve the potency of **9**. This mutation will be evaluated in future studies.

The ability to create reactive cysteine residues is a challenge in the protein design field. While most cysteine residues have pK_a values in the 8–9 range, it is likely that a gatekeeper cysteine has an elevated pK_a due to the surrounding hydrophobic microenvironment, rendering it especially unreactive. Nature exploits several strategies to depress the pK_a values of cysteine including the introduction of electrostatic interactions with nearby positively charged residues (40), hydrogen bonding with polar amino acid side chains/ backbone amides (41), and the use of helix-dipoles (42). Our results suggest that simple modification of the microenvironment around the cysteine gatekeeper by alteration of one nearby residue (i.e., Val323) is sufficient to have a significant impact on inhibitor potency. Liberating additional space with a V323A mutation resulted in a 6-fold increase in potency for **13**, while the V3232S mutation had a 17-fold effect. Interestingly, we did not note improvement in potency for **3** or **9** upon second-site mutation. This observation may be due to different arrangements of nucleophile and electrophile with respect to the second-site mutation.

Besides the uses reported in this study, *ES* kinase alleles should be useful for a host of other applications. For example, fluorescently labeled versions of the inhibitors could be used to quantitatively probe the occupancy of kinase active sites to determine the percent activity required for signaling events (25, 26, 43–45). This strategy may also be useful for studying the properties of pseudokinases, for which there is no good readout of active site

occupancy. The use of irreversible inhibitors for poorly studied members of large families allows rapid validation of target specificity when downstream assays are not known, as has been shown in the serine hydrolase family (46). In addition, the technology reported in this study may enable multiplex inhibitor studies, such as those requiring washing steps or the use of more than one chemical genetic inhibitor. We anticipate that further modifications to compounds like the fluoromethylketone (**13**) may allow highly specific inhibition of the two human kinases with endogenous cysteine gatekeepers (MOK and SgK494), the roles of which remain ambiguous. Furthermore, an essential kinase in *Toxoplasma gondii* was found to contain a natural glycine gatekeeper, allowing it to be selectively targeted with the *AS* kinase specific inhibitor, 3-MB-PP1 (47). As sequencing projects for microbes progress, we may discover potential kinase drug targets with cysteine as a gatekeeper. We are currently evaluating a cysteine gatekeeper-containing PAK kinase in *Plasmodium falciparum*, a microbe that causes malaria.

Methods

Methods for expression, purification, assay, mass spectrometry, and crystallization of c-Src are included in *SI Appendix*. Methods for synthesis of inhibitors, immunoprecipitation, and assay of MOK, as well as inhibition assays with v-Src transformed NIH-3T3 cells are also included in *SI Appendix*.

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