Introduction

Protein kinases have a crucial role in most, if not all, signaling pathways and regulate diverse cellular functions, such as cell-cycle progression, apoptosis, metabolism, differentiation, cell morphology and migration, and secretion of cellular proteins [1]. Our present understanding of the majority of cellular signal transduction takes the form of wiring diagrams in which many of the component parts have been identified, and to some extent the relative position of the components in a given pathway, but beyond this static snapshot view, little is known about the details of their dynamic operation. A critical piece of this puzzle is an understanding of how external and internal inputs are sensed in a time-dependent manner to effect a given signaling output. Highly selective, cell-permeable and fast-acting inhibitors of individual kinases are sought-after as tools for studying the cellular function of kinases in real time. A combination of small molecule synthesis and protein mutagenesis, identified a highly potent inhibitor (1-Isopropyl-3-(phenylethynyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine) of a rationally engineered Hog1 serine/threonine kinase (Hog1T1085E). This inhibitor has been successfully used to study various aspects of Hog1 signaling, including a transient cell cycle arrest and gene expression changes mediated by Hog1 in response to stress. This study also underscores that the general applicability of this approach depends, in part, on the selectivity of the designed inhibitor with respect to activity versus the engineered and wild type kinases. To explore this specificity in detail, we used a validated chemogenetic assay to assess the effect of this inhibitor on all gene products in yeast in parallel. The results from this screen emphasize the need for caution and for case-by-case assessment when using the Analog-Sensitive Kinase Allele technology to assess the physiological roles of kinases.

Abstract

Highly selective, cell-permeable and fast-acting inhibitors of individual kinases are sought-after as tools for studying the cellular function of kinases in real time. A combination of small molecule synthesis and protein mutagenesis, identified a highly potent inhibitor (1-Isopropyl-3-(phenylethynyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine) of a rationally engineered Hog1 serine/threonine kinase (Hog1T1085E). This inhibitor has been successfully used to study various aspects of Hog1 signaling, including a transient cell cycle arrest and gene expression changes mediated by Hog1 in response to stress. This study also underscores that the general applicability of this approach depends, in part, on the selectivity of the designed inhibitor with respect to activity versus the engineered and wild type kinases. To explore this specificity in detail, we used a validated chemogenetic assay to assess the effect of this inhibitor on all gene products in yeast in parallel. The results from this screen emphasize the need for caution and for case-by-case assessment when using the Analog-Sensitive Kinase Allele technology to assess the physiological roles of kinases.

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adaptation which includes profound changes in gene expression. Specifically, Hog1 regulates gene expression by activation of specific transcription factors but also through chromatin binding. Hog1 recruits chromatin modifying/remodeling activities to stress-responsive genes altering their expression [8,10]. In addition, environmental stressors (e.g., changes in osmolarity) critically affect progression through the cell cycle [9,11,12].

To develop an analog-sensitive inhibitor of an engineered Hog1 kinase, we selected the pyrazolopyrimidines as they represent an excellent scaffold for targeting the protein kinase family due to their structural similarity to the adenine moiety of ATP. Furthermore, the scaffold has been shown to have activity against multiple kinase subfamilies. For example, different chemical substitutions around this scaffold result in increased selectivity in the inhibition of KDR [13], Src [14], and EGF [15] kinase families. Furthermore, this scaffold has previously been used to make orthogonal inhibitors [16]. We present here the design and synthesis of a novel orthogonal inhibitor based on the pyrazolopyrimidine that effectively inhibits a Hog1 as kinase, and is able to dissect the transient cell cycle arrest and regulation of gene expression mediated by Hog1 in response to stress.

Results and Discussion

Because of its central role in cellular homeostasis and the implication of human homologs in diverse disease states, we selected Hog1 as the target of our mutant kinase-inhibitor pair design. Sequence alignment analyses identified the conserved T100 as a gatekeeper residue in Hog1 [5,6]. Visual inspection of the binding pocket of an initial homology model of Hog1, using the structure of human p38 in the absence of a ligand (pdb code 1p38) for a template, indicated that a narrow path leads to a buried cavity within the ATP binding domain (Figure 1).

The cavity size and shape is comparable to that of a phenyl group, and mutation of T100 for a glycine would widen the pocket further (Figure 1). We therefore sought a compound that was based on the pyrazolopyrimidine structure, having a phenyl ring attached to it via a spacer of the appropriate length. Candidate compounds were manually docked into the binding site and the geometries were optimized in torsion space using an all-atom representation of both ligand and receptor, keeping the receptor fixed. 1-NM-PP1, a commercially available ATP competitive inhibitor was compatible with our model, but did not fit as well as other compounds into the ATP binding site of Hog1 as. The resulting model complex that best matched our specifications included a two-carbon, triple-bonded linker (compounds with the general structure 6, Figure 1). The triple bond would place the benzene ring in such orientation that it fills up the lipophilic pocket that becomes accessible upon mutation. At the same time, the heterocyclic moiety can make similar interactions with the hinge area as would ATP. In the wild-type kinase the non-mutated gatekeeper residue should block access to the lipophilic pocket (indicated in red).

Previous published synthetic approaches for making 1,3-disubstituted pyrazolopyrimidines involves at least five sequential reaction steps, but more importantly, the R1 substituent is introduced in the first step [17,18]. Therefore, the generation of analogues with varying C3 substituents is inefficient. We devised a convergent route for making 1,3-disubstituted pyrazolopyrimidines. This route involves the synthesis of a common intermediate, 4-amino-3-iodo-1H-pyrazolo[3,4-d]pyrimidine (3) that allows rapid derivatization of the heterocyclic core scaffold in two steps (Figure 2).

The common intermediate, 4-amino-pyrazolopyrimidine (2), was synthesized from (1) by a 4-step synthesis, on a multigram scale in 64% overall yield without the use of any chromatography. The corresponding 4-amino-3-iodopyrazolopyrimidine (3) was synthesized using N-iodosuccinimide (Figure 2) [19].

Starting from compound 3, a two-step derivatization process was developed. Initially, we found that alkylation of the N1 with 2-

![Figure 1. Inhibitor docked to Hog1](https://example.com/figure1.png)

Compound 6a docked to the homology models of wild-type (red mesh) and T100G mutant (ball-and-stick and yellow surface) Hog1, shown as cross sections from two angles, rotated 90 degrees around the horizontal axis. Compound 6a was designed to occupy the region available only for the T100G mutant (red mesh near phenyl ring) for specificity.

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![Figure 2. Synthesis of 1,3-disubstituted pyrazolopyrimidines.](https://example.com/figure2.png)

Figure 2. Synthesis of 1,3-disubstituted pyrazolopyrimidines.
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propanol using Mitsunobu conditions preceded only in moderate yield (50%). Treatment of 3 with 1.1 equiv of 2-chloropropan and 1.2 equiv of NaH in DMF for 3 h at 100 °C gave a mixture of the N1 and N4 alkylated products. However, when the base was exchanged with K₂CO₃ and the reaction was carried out with microwave assisted heating at 200 °C for 5 min, high regioselectivity was achieved and 4a was obtained in nearly quantitative yield. Using the same reaction conditions in combination with commercial available 2-(2-Chloroethoxy)tetrahydro-2H-pyran or 4-Chloromethyl-2,2-dimethyl-1,3-dioxolane compounds 4b and 4c were obtained in 70% and 79% yield respectively.

Compound 4 was then reacted with 4-halogenphenyl boronic acid (1.3 eq) Pd[PPPh₃]₂Cl₂, (3 mol %), 2M aq. K₂CO₃ (10 eq) in THF at reflux for 16 h to generate compounds with the general structure 5 [17] or with 1-ethenyl-4-halobenzene, Pd[PPPh₃]₂Cl₂, (3 mol %), Et₃N (2 eq) and CuI (6 mol %) in THF at reflux for 16 h to generate compounds with the general structure 6 [20]. In all cases the reactions proceeded with high turnover of the starting material and the target compounds were obtained in high yields (75–90%). Removal of the acid labile protection groups in 6d and 6e were carried out with 2N HCl in THF, resulting in compound 7a in 92% and 90% isolated yield respectively.

Using standard methods, we cloned, expressed and purified the glutathione-S-transferase (GST) fusion proteins of Hog1wt and Pbs2wt as well as the mutated kinases (Hog1as and Pbs2as). The analogue sensitive mutant allele of each kinase (as mutant) was created by the replacement of a conserved bulky residue with a glycine (T100G) in Hog1 or an alanine (M435A) in the active site of Pbs2.

The analogue sensitive mutant allele of each kinase (as mutant) was created by the replacement of a conserved bulky residue with a glycine (T100G) in Hog1 or an alanine (M435A) in the active site of Pbs2. Based on the analyses of their in vitro kinase activity, both Hog1as and Pbs2as were more active when the phosphodonor is the analogue Phenyl-Ethyl-ATP (PE-ATP) versus ATP, consistent with previous reports [5]. It is worth noting that, Hog1 expressed as a chaperone or that the conformation or posttranslational modification of the Hog1 protein in E. coli is different from that in yeast.

We tested the 1,3-disubstituted pyrazolopyrimidines (6a–6c, 5a, 5b), as well as SB203580 (8), a known inhibitor of p38α, p38β and AKT/PKB. p38 is the mammalian homologue of Hog1 and 1-NM-PP1 (9, Figure 3) [16,21,22,23], while Hog1 produced from yeast was sensitive to this inhibitor (data not shown). This observation suggests the E. coli protein is inactive/or partially active and that full function of Hog1 depends on association with another yeast protein, such as a chaperone or that the conformation or posttranslational modification of the Hog1 protein in E. coli is different from that produced in yeast.

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**Figure 3. In vitro inhibition of Hog1 and Pbs2.** In vitro inhibition of Hog1as (B) and Pbs2as (D) mutant variants by inhibitors, and effect in the wild type partners (A and C). The inhibitors used (final concentration 5 μM) were 6a–6c, 5a, 5b, as well as SB203580 (8) (a known inhibitor of p38γ, p38β and AKT/PKB). p38 is the mammalian homologue of Hog1 and 1-NM-PP1 (9) (a known inhibitor of the as kinases. Recombinant, tagged proteins were purified either from S. cerevisiae (Hog1) or E.coli (Pbs2) and were assayed for the phosphorylation of Sic1 (substrate of Hog1) or Hog1 (as substrate of Pbs2). Phosphorylated proteins were resolved by SDS-PAGE and their phospho-state detected by autoradiography. IC₅₀ values for in vitro inhibition of Hog1as and Pbs2as mutant variants by 6a and 9 (F). Recombinant tagged proteins were purified either from S. cerevisiae (Hog1) or E.coli (Pbs2) and were assayed for phosphorylation of Sic1 (substrate of Hog1) or Hog1 (as substrate of Pbs2). The results are the means ± S.D. of at least three independent experiments.

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using the $\text{STL1::LacZ}$ reporter (Figure 4). Cells were incubated with the different inhibitors for 8 hours at a fixed concentration of 5 mM, and $\beta$-galactosidase activity was assayed before ($-$NaCl) or after (+NaCl) osmotic stress (0.4 M NaCl for 30 min). $\beta$-galactosidase activity is presented in nanomols per minute per milligram. Data represent the mean of at least three independent experiments.

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To further characterize 6a we performed a time course at a concentration of 2 mM (endpoint analysis), a concentration that does not manifest a fitness defect in a $\text{HOG1}$ strain, but is sufficient to almost totally inhibit Hog1 (Figure 5 and data not shown). At 5 minutes of incubation with 6a the $\beta$-galactosidase activity was inhibit by 86%, with an IC$_{50}$ of 238 nM (Figure 5A and 5C). This rapid inhibition of gene expression by 6a suggests that this compound could be a key, fast-acting tool to study some of the aspects of the mRNA biogenesis regulated specifically by the Hog1 MAPK as by signaling kinases in general.

To complement the end-point analyses of the inhibitors effect on transcription we also carried out a time course experiment for both $\text{hog1as}$ and $\text{pbs2as}$ (Figure 6). The addition of 6a five minutes before stress completely blocked the expression of $\text{STL1}$, which in the absence of the inhibitor, increased up to 30 minutes in response to osmotic stress. Similar results were obtained by inhibition of Hog1as and Pbs2as.

To analyze whether the solubility of the molecule could affect the inhibition by 6a, we tested two derivatives with one or two hydroxyl functions (7a and 7b). We reasoned that increased solubility could result in better uptake and therefore more potent compounds. On the other hand, too polar compounds could be less bioactive if they are unable to cross the lipophilic cell membrane. However, we did not observe either a faster uptake or a faster inhibition with these two new derivates (Figure 7). Thus, although $\text{in vitro}$ these compounds were able to inhibit Hog1as as efficiently as 6a (Figure 7A), they did not improve the inhibition of the MAPK $\text{in vivo}$ (Figure 7B).

The Stress-Activated Protein Kinase (SAPK) Hog1 elicits a program for cell adaptation that includes the control of gene expression and the modulation of cell-cycle progression. As recent studies have shown that monitoring SAPKs activity $\text{in vivo}$ by reversible inhibition, we wanted to know if 6a, is a suitable tool to study the transient cell cycle arrest mediated by Hog1 activation in response to stress.
Both high osmolarity and inactivation of Sln1 activity will result in activation of Hog1. It is known that cells manifest a transient cell cycle arrest in response to Sln1 inactivation, a phenotype that can be followed by flow cytometry [12,24]. A temperature sensitive allele of SLN1, (sln1ts4) arrests at G1 phase following synchronization at G1/S with mating pheromone and release into the restrictive temperature (Figure 8, lane A). This arrest can be circumvented by mutations on the HOG1 gene (in hog1 cells) or if cells are pre-incubated with 6a for as little as 10 min.

Our results demonstrate that 6a is a powerful tool to study transient cell cycle arrest or gene expression mediated by Hog1 in response to stress. In addition, 6a was recently used to demonstrate that dynamic signaling in the Hog1 pathway relies on high basal signal transduction [7]. However, the general applicability of this approach depends, in part, on the selectivity with 6a inhibit the mutant protein kinases compared with the other wild-type protein kinases that are expressed endogenously in the same cells [25]. We therefore examined the specificity of 6a by chemical genetic profiling of the yeast deletion mutant collection and scored for mutants with reduced growth in the presence of 500 µM 6a and without osmotic stress (Figure 9). It should be noted that the concentration of 6a used in this experiment was 100 times higher than what was required to get efficient inhibition of Hog1as (5 μM) in osmotic stressed cells and only off-target effects as well as secondary effects of these was expected to be identified. This analysis revealed that 60 strains that showed a significant depletion from the pool of 1200 essential heterozygotes and 4800 non-essential homozygous diploids (i.e. with a log2 ratio greater than 1) when cultured competitively in the presence of 500 µM 6a.

Notably, of the 60 gene deletion strains (see table S1, Supporting Information), 50 could be classified into five functional groups; kinases (6), other enzymes 14), cytoskeleton (17), transcription regulation (10), and cell wall (3), clearly demonstrating that several off-target effects takes place at this concentration of 6a. A similar experiment has been reported using compound 9 (500 nM) targeting cdc28-as, showing excellent selectivity for the targeted kinase [26]. However, when the inhibitor concentration was increased to 5 µM several strains came up as sensitive, with several of these having a catalytic/nucleoside triphosphate binding role. Our results are consistent with these data. Together, these results...
on wet packed silica (0.040–0.063 mm) using flash chromatography. Microwave reactions were performed in a Biotage Initiator reactor with fixed hold time. Amino-3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine was prepared following a literature procedure [19].

**1H-pyrazolo[3,4-d]pyrimidin-4-amine (2).** A solution of malononitrile (22.6 g, 0.344 mol), triethyl orthoformate (83 mL, 0.499 mol), and acetic anhydride (77 mL, 0.791 mol) was heated to 100 °C for 5 h. After cooling to rt the solution was concentrated on the rotary evaporator. The solution was left to crystallize at rt overnight and the yellow solid was recrystallized from EtOH to give 36.0 g (98.5%) of 1,1-dicyano-2-ethoxylethene as yellow crystals. This material (36.0 g, 0.339 mol) was added carefully and in small portions to cold (0 °C) hydrazine hydrate (99.5%, 26 g, 0.807 mol). The solution was heated to reflux for 1 h and then left to cool at rt whereupon the contents of the flask solidified. Water (25 mL) was added to the solid material and the mixture was left in the refrigerator overnight. The mixture was filtered and the solid was washed with 10 mL of cold water and suction dried for about 5 min. The product was dried in a vacuum desiccator over calcium chloride and was obtained in 75.5% (27.7 g). This material (27.6 g, 0.255 mol) was added to formamidine (42 mL). The solution was vigorously boiled for 30 minutes (216 °C oil bath). The creamy suspension was left to cool at rt, diluted with water (70 mL), and filtered. The light tan colored solid was washed with water, suction dried, and dried in a vacuum desiccator over calcium chloride to give the target compound in 86% yield (29.6 g) that gives an overall yield of 64% (calculated over three steps). NMR was in agreement with published data [27].

**General procedure A; alkylation of N-1.** Compound 3 (1 eq) and K$_2$CO$_3$ (2 eq) were suspended in 12 ml of dry DMF in a 20 ml microwave vessel. To this mixture, R$_1$-Cl (1.1 eq) was added and the sealed tube was heated to 200 °C for 5 min. (20 s of presstirring and fixed hold time: on). After cooling of the reaction mixture to room temperature, additional R$_1$-Cl (0.5 eq) was added and the microwave vessel was heated again to 200 °C for 5 min. After cooling to ambient temperature, the reaction mixture was diluted with DMF and filtered. The solvent was removed in vacuo at 80 °C and the residue was co-distilled with toluene three times. The crude product was purified by flash column chromatography on silica gel.

**3-Iodo-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4a).** Compound 3 (2.00 g, 7.66 mmol) was converted to the target compound using general procedure A. The crude product was purified by flash column chromatography on silica gel (MeOH:CHCl$_3$ = 1:20) to give 97% (2.25 g) of 4a as fine yellow needles. $^1$H NMR (400 MHz, CDCl$_3$) δ 1.55 ppm (d, 6H), 5.09 ppm (d, 6H), 5.09 ppm (m, 1H), 6.17 ppm (bs, 2H), 8.32 ppm (s, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 22.27 ppm, 49.89, 85.62, 104.25, 153.17, 155.38, 157.63. Anal. Calcd for C$_{12}$H$_{16}$IN$_5$O$_2$: C, 37.03; H, 4.14; N, 47.26, 61.71, 64.85, 86.59, 98.12, 103.77, 155.79, 157.89. Found: C, 37.04; H, 4.15; N, 18.04.

**3-Iodo-1-((2,2-Dimethyl-1,3-dioxolan-4-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4c).** Compound 3 (2.00 g, 7.66 mmol) was converted to the target compound using general procedure A. The crude product was purified by flash column chromatography on silica gel (MeOH:CHCl$_3$ = 1:20) to give 70% (2.09 g) of 4c as a yellow powder. $^1$H NMR (400 MHz, CDCl$_3$) δ 1.27–1.64 ppm (m, 6H), 3.10–3.39 ppm (m, 1H), 3.48–3.58 ppm (m, 1H), 3.77–3.86 ppm (m, 1H), 3.99–4.09 ppm (m, 1H), 4.42–4.59 ppm (m, 3H), 8.22 ppm (s, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 18.93 ppm, 24.29, 29.41, 30.21, 47.26, 61.71, 64.85, 86.59, 98.12, 103.77, 154.14, 155.79, 157.89. Anal. Calcd for C$_{32}$H$_{36}$IN$_5$O$_2$: C, 37.03; H, 4.14; N, 17.99. Found: C, 37.04; H, 4.15; N, 18.04.

Figure 8. 6a is a suitable tool to study the transient cell cycle arrest mediated by Hog1. The sln1-ts4 hog1as or hog1as sln1-ts4 strains were synchronized with z-factor for 1 h, incubated with 5 μM of 6a for 10 minutes (B), shifted to 37 °C for 10 minutes and then released into YPD medium at 37 °C plus the inhibitor (time 0). Total DNA content was assessed by flow cytometry and presented as cell counts (y-axis) versus C and 2C DNA content (x-axis).

**Materials and Methods**

**Synthesis**

$^1$H and $^{13}$C NMR spectra were obtained from a JEOL JNM-EX 400 spectrometer. Column chromatography was performed...
An Inhibitor for Analog-Sensitive (as) Kinases
Figure 9. Chemical genetic profiling validation. (A) Visualization of genes sensitive to 500 μM 6a. The fold change (log2(6a-treated/untreated)) in microarray signal intensity is plotted on the y-axis for ~6000 genes (arranged alphabetically on the x-axis). (B) A network showing Kegg Pathways (Blue nodes) and related GO Terms (Red nodes) that are significantly enriched (FDR q-value < 0.15) in the genes that are sensitive to the compound in the chemogenomic profile. Nodes are connected based on mutual overlap. Node size is proportional to the total number of genes in each set and edge thickness represents the number of overlapping genes between sets.

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7.66 mmol) was converted to the target compound using general procedure A. The crude product was purified by flash column chromatography on silica gel (MeOH:CHCl₃ = 1:20) to give 73% (2.09 g) of 4c as fine yellow needles. ¹H NMR (400 MHz, CDCl₃) δ 1.33 ppm (d, 6H), 1.55 ppm (d, 6H), 1.59 ppm (d, 6H), 1.69 ppm (bs, 2H), 1.78–7.24 (m, 2H), 1.76–7.71 (m, 2H), 3.41–3.52 (m, 1H), 3.91–3.99 (m, 1H), 4.56–4.65 (m, 2H), 6.24 (bs, 2H), 8.32 (s, 1H), 8.43 (s, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 22.19 ppm, 24.16, 98.71, 129.70, 130.03, 135.28, 142.78, 173.77, 153.62, 158.10. Anal. Calcd for C₁₄H₁₄ClN₅ (287.75): C, 58.44; H, 4.90; N, 24.34. Found: C, 58.46; H, 4.91; N, 24.36.

3-(4-Chlorophenyl)-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4d). Compound 4a (2.00 g, 6.60 mmol) was converted to the target compound using general procedure C. The crude product was purified by flash column chromatography on silica gel (MeOH:CHCl₃ = 1:30) to give 87% yield (179 mg) of 6c as a white powder. ¹H NMR (400 MHz, CDCl₃) δ 1.46 ppm (d, 6H), 5.04 (m, 1H), 7.54 (d, 2H), 7.78 (d, 2H), 8.25 (s, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 21.71 ppm, 48.76, 82.22, 91.69, 120.54, 124.37, 127.87, 136.61, 134.11, 152.29, 152.21, 157.37. Anal. Calcd for C₁₉H₁₉N₅O₂ (295.30): C, 61.64; H, 4.53; N, 22.46. Found: C, 62.46; H, 4.53; N, 22.42.

3-[4-Chlorophenyl]-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (6d). Compound 4b (0.20 g, 0.66 mmol) was converted to the target compound using general procedure C. The crude product was purified by flash column chromatography on silica gel (MeOH:CHCl₃ = 1:30) to give 81% yield (194 mg) of 6d as a slightly yellow powder. ¹H NMR (400 MHz, CDCl₃) δ 1.38–1.75 ppm (d, 6H), 3.41–3.52 (m, 1H), 3.62–3.70 (m, 1H), 3.91–3.99 (m, 1H), 4.14–4.22 (m, 1H), 4.56–4.71 (m, 3H), 5.93 (bs, 2H), 7.38–7.47 (m, 3H), 7.58–7.63 (m, 2H), 8.40 (s, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 19.10 ppm, 25.48, 30.42, 47.47, 61.94, 65.00, 80.93, 94.26, 98.40, 101.85, 121.67, 126.74, 128.79, 129.64, 131.94, 134.04, 158.60, 158.10. Anal. Calcd for C₂₀H₂₁N₅O₂ (363.17): C, 66.12; H, 5.83; N, 19.30. Found: C, 66.12; H, 5.83; N, 19.30.

1-((2,2-dimethyl-1,3-dioxolan-4-yl)methyl)-3-(phenylethynyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (6e). Compound 4c (0.20 g, 0.66 mmol) was converted to the target compound using general procedure C. The crude product was purified by flash column chromatography on silica gel (MeOH:CHCl₃ = 1:30) to give 81% yield (194 mg) of 6e as a white powder. ¹H NMR (400 MHz, CDCl₃) δ 1.38–1.75 ppm (d, 6H), 3.41–3.52 (m, 1H), 3.62–3.70 (m, 1H), 3.91–3.99 (m, 1H), 4.14–4.22 (m, 1H), 4.56–4.71 (m, 3H), 5.93 (bs, 2H), 7.38–7.47 (m, 3H), 7.58–7.63 (m, 2H), 8.40 (s, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 19.10 ppm, 25.48, 30.42, 47.47, 61.94, 65.00, 80.93, 94.26, 98.40, 101.85, 121.67, 126.74, 128.79, 129.64, 131.94, 134.04, 158.60, 158.10. Anal. Calcd for C₂₀H₂₁N₅O₂ (363.17): C, 66.12; H, 5.83; N, 19.30.
60°C for 2 h. The solvent was evaporated and the residue co-evaporated with toluene three times. The product was purified by crystallization from MeOH/Et2O.

2-(4-amino-3-(phenylethynyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)propane-1,2-diol (7b). Compound 6d (0.15 g, 0.013 mmol) was converted to the target compound using general procedure C and was obtained in 90% yield (0.13 mg) of 7b as a white powder. 1H NMR (400 MHz, DMSO) δ 3.37–3.50 ppm (m, 2H), 3.96–4.05 (m, 1H), 4.32–4.43 (m, 2H), 7.45–7.54 (m, 2H), 7.74–7.80 (m, 3H), 8.39 (s, 1H). 13C NMR (100 MHz, DMSO) δ 51.17 ppm, 63.61, 69.95, 79.57, 94.67, 99.82, 120.94, 127.55, 128.67, 129.82, 132.09, 149.35, 151.66, 152.99. Anal. Calcd for C16H16ClN5O2 (345.10): C, 55.58; H, 3.54; N, 12.65, 12.96, 13.27, 13.58, 13.89, 14.20, 14.51. 1H NMR (100 MHz, DMSO) δ 3.84 ppm (t, 2H), 4.22 (t, 2H), 7.45–7.54 (m, 3H), 7.73–7.79 (m, 2H), 8.49 (s, 1H). 13C NMR (100 MHz, DMSO) δ 51.17 ppm, 63.61, 69.95, 79.57, 94.67, 99.82, 120.94, 127.37, 128.68, 129.79, 132.06, 132.16, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51. Anal. Calcd for C15H14ClN5O (315.09): C, 57.05; H, 9.99; 120.98, 127.37, 128.68, 129.79, 132.06, 135.51.

Cytometry analyses. For flow cytometry analyses, cells were fixed in ethanol, treated with RNase A, stained with propidium iodide and analysed in a FACScan flow cytometer (Becton Dickinson) in the FL3 channel. A total of 10000 cells were analysed for each time point.

Chemical Genetic Profiling. Yeast profiling was performed exactly as described by Ericson et al [30]. Gene set enrichment was carried out on the non-essential yeast genes in the chemogenomic profile using GSEA [31] and the results were visualized as a network using the enrichment map plugin [32] for cytoscape [33].

Modeling MolIDE/ICM modeling. The initial model of Hog1 was created using the standard procedure built into the program ICM, based on the structure of human p38 (pdb code 1p38) [34]. The model was refined by regularization, which imposes ideal geometry onto the model, followed by geometry optimization by Monte Carlo simulated annealing in torsion space. (ICM manual, Molsoft, CA) Ligands were manually docked into the site and geometry optimized by minimization and Monte Carlo conformation sampling. In the early phase of optimization, ligand atoms were tethered to their original positions using harmonic potentials that were gradually decreased in strength.

Eight additional wild-type and mutant Hog1 models were created based on four structure templates (pdb codes 1cm8, 1m7q, 1p38 and 3erk) to provide a better understanding of the orthogonal ligands' affinities. Template selection was carried out using the MolIDE program [35], which is an interface to programs that performs psi-blast searches against the nr database and stores the generated alignment profiles, searches the pdb sequences using the profiles, constructs a backbone model for aligned residues, adds and geometry optimizes side-chains and builds loops. Pairwise sequence alignments were manually edited before model building.

Kinase models built by MolIDE were regularized by ICM before docking and quality assessment. The local quality of protein models was checked using ICM's calcEnergyStrain, which identifies bad regions by reporting the relative energy of each residue.

Docking of a small library of the designed and known kinase inhibitors was performed using the standard protocol in ICM (data not shown), and the representative mutant and wild-type Hog1 models were manually chosen based on the accuracy of ligand docking poses.

Supporting Information Table S1 Gene deletion strains significantly sensitive to 500 uM 6a. (PDF)

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Author Contributions
Conceived and designed the experiments: MK MM AV LB M. Gebbia IMW GG CN FP M. Gottli. Performed the experiments: MK MM AV LB M. Gebbia IMW. Analyzed the data: MK MM AV LB M. Gebbia IMW GG CN FP M. Gottli. Contributed reagents/materials/analysis tools: LB GG CN FP M. Gottli. Wrote the paper: GG CN FP M. Gottli.

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