Analysis of Protein Tyrosine Kinase Inhibitors in Recombinant Yeast Lacking the *ERG6* Gene

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Studies of small-molecule–protein interactions in yeast can be hindered by the limited permeability of yeast to small molecules. This diminished permeability is thought to be related to the unique sterol composition of fungal membranes, which are enriched in the steroid ergosterol. We report the construction of the novel Saccharomyces cerevisiae *yeast strain DCY250, which is compatible with yeast two-hybrid-based systems and bears a targeted disruption of the* ERG6 *gene to ablate ergosterol biosynthesis and enhance permeability to small molecules. The small-molecule inhibitors of protein tyrosine kinases (PTKs) PP1, PP2, herbimycin A, and staurosporine were investigated with yeast tribrid systems that detect the activity of the PTKs v-Abl and v-Src. These tribrid systems function by expression of the PTK, a B42 activation domain fused to the phosphotyrosine-binding Grb2 SH2 domain, a DNA-bound LexA-GFP-(AAYANAA)$_4$ universal PTK substrate, and a* lacZ *reporter gene. Yeast genetic systems that lack functional ERG6 were found to be as much as 20-fold more sensitive to small-molecule inhibitors of PTKs than systems with ERG6, and these deficient systems may provide a useful platform for the discovery and analysis of small-molecule–protein interactions.*

KEYWORDS:

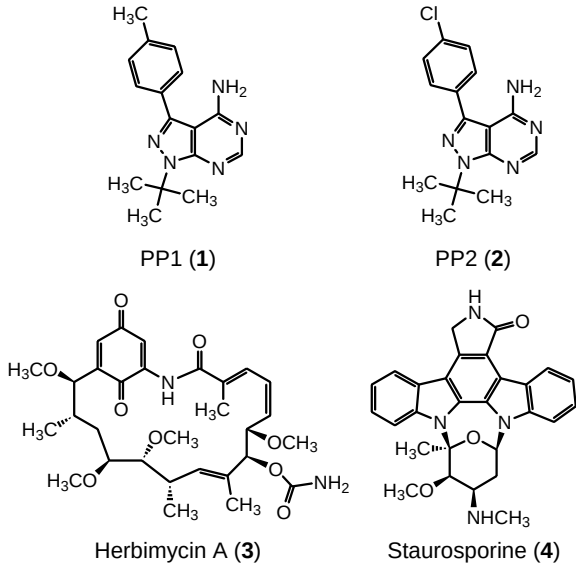
antitumor agents · high-throughput screening · molecular recognition · signal transduction · yeast

Introduction

Protein tyrosine kinase (PTK) enzymes activate complex intracellular signal transduction pathways by catalyzing the transfer of the gamma phosphoryl group of adenosine triphosphate (ATP) to tyrosine residues of target proteins. The resulting phosphotyrosine residues typically serve as docking sites for cognate phosphotyrosine-binding proteins that participate in the transmission of signals to the cell nucleus. These signals control diverse cellular functions, which include cellular proliferation, differentiation, and apoptosis.[1, 2] Up-regulation of specific signaling pathways by members of the Abl and Src families of PTKs control the proliferation of certain cancers by activating Ras oncoproteins.[1, 3, 4] Aberrant activation of Ras is implicated in a large number of human malignancies[5] and PTKs therefore represent major therapeutic drug targets.[6]

Given the importance of PTKs in human disease, novel classes of potent and selective PTK inhibitors are a widely sought after class of drug candidates (reviewed in refs. [6–8]). The majority of PTK inhibitors described to date are mimics of ATP, target the enzyme active site, and block phosphoryl transfer to target proteins. As shown in Scheme 1, this class of inhibitors includes the structurally divergent small molecules PP1 (**1**), PP2 (**2**), herbimycin A (**3**), and staurosporine (**4**).[9–12] Although numerous inhibitors of PTK activity are known,[6–8] the common mechanism of action of ATP mimicry has limited the identification of the highly specific compounds necessary for effective chemotherapy.[12] Hence, novel approaches to evaluate diverse PTKs in a high-throughput screening format are sought to discover alternative types of small-molecule inhibitors.

Inhibitors of PTKs are typically investigated through evaluation of cell extracts by ELISA techniques or through analysis of compounds against purified kinases in vitro.[6] However, these methods can often be expensive and time-consuming, and often require radioactive probes. As a potentially inexpensive, rapid, and nonradioactive alternative, we are investigating the utility of



Scheme 1. *Structures of protein tyrosine kinase inhibitors that engage the ATP binding site.*

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yeast two-hybrid-based assays for detection and analysis of PTK inhibitors. Towards this end, we recently reported the construction of a yeast tribrid system that employs a potentially universal PTK substrate that is efficiently phosphorylated by the v-Abl and v-Src enzymes (Figure 1).[13] Here, we report modifications of this yeast genetic system to enable analysis of inhibitors of this important class of enzymes.

Yeast genetic systems have emerged as powerful tools for the study of small-molecule – protein interactions(reviewed in refs. [14 – 17]). Recent reports have described the use of yeast one- and two-hybrid systems to investigate ligand – receptor interactions,[18–27] and to discover inhibitors of protein – protein interactions.[28–30] Other reports utilize yeast three-hybrid systems that employ dimeric small-molecule ligands that serve to dimerize protein ligand-binding domains and reconstitute functional transcriptional activators that activate

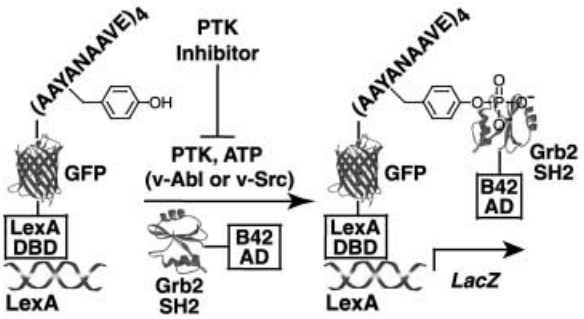
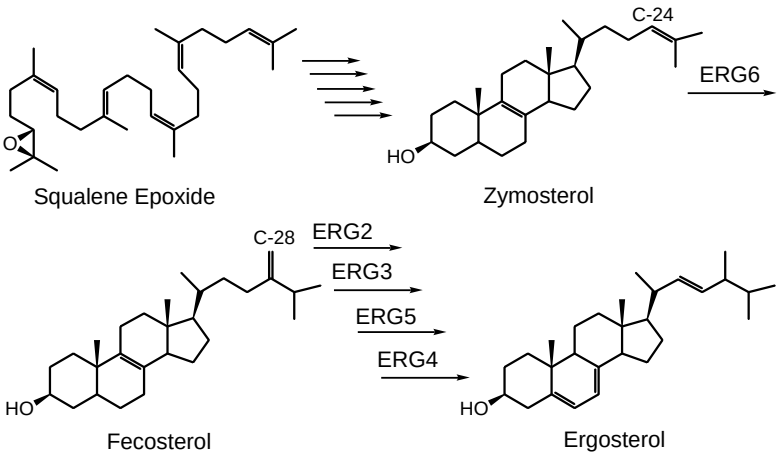


Figure 1. *Schematic depiction of a yeast tribrid system incorporating a universal PTK substrate. This assay was designed to detect cell-permeable PTK inhibitors by reduction of* lacZ *reporter gene expression.*

reporter gene expression.[31–36] Yeast have also been recently reported as sensors for the detection of small molecules that stabilize receptor folding.[37] However, a major limitation associated with yeast-based analysis of small molecules involves the limited permeability of yeast compared with mammalian cells.[15] This difference in permeability has been largely attributed to differences in cellular membrane composition. Mammalian cell membranes are highly enriched in the steroid cholesterol, whereas fungal membranes substitute the structurally related steroid ergosterol (Scheme 2), which primarily differs from cholesterol by the presence of a methyl substituent on C-24. Previous studies have elucidated the components of the ergosterol biosynthetic pathway in *Saccharomyces cerevisiae*.[38–41] As shown in Scheme 2, one particularly interesting gene in this pathway is *ERG6*, which encodes the C-24 sterol methyltransferase responsible for installation of the C-28 methyl group late in the sterol biosynthetic pathway. The *ERG6* gene product catalyzes the conversion of zymosterol to fecosterol, and deletion of *ERG6* has been shown to ablate ergosterol production without dramatically affecting cellular viability.[42] Moreover, yeast that lack functional *ERG6* (*erg6Δ* strains) have been reported to be more permeable to small molecules,[25, 43–50]



Scheme 2. *Key elements of the yeast ergosterol biosynthetic pathway.*

which has recently been attributed to increased rates of passive diffusion across cell membranes.[50] Here, we describe analysis of PTK inhibitors in a modified yeast tribrid system that bears a targeted deletion of the *ERG6* gene carried out to enhance permeability to small molecules. Deletion of this gene was found to enable detection of specific PTK inhibitors with more than 20-fold greater sensitivity than the parent yeast strain, FY250. This approach may be useful for high-throughput identification and analysis of pharmaceutically relevant PTK inhibitors.

Results and Discussion

Targeted deletion of *ERG6* in *S. cerevisiae* strain FY250

The *ERG6* gene of yeast is critical for the biosynthesis of ergosterol.[42] Targeted disruption of this gene to yield *erg6Δ* yeast strains can enhance permeability to a variety of small molecules.[25, 43–50] Yet, to our knowledge, only one report has described inactivating mutations in this gene in conjunction with yeast two-hybrid assays used to detect pharmacologically relevant small molecules.[25] We hypothesized that an *erg6Δ* yeast strain harboring the tribrid (modified two-hybrid) PTK assay shown in Figure 1 would enable improved detection of functionally diverse small-molecule inhibitors of the v-Abl and v-Src PTKs.

As shown in Figure 2, the *ERG6* gene was disrupted in yeast strain FY250 to yield the *erg6Δ* yeast strains DCY001 and DCY250. Gene deletion involved homologous recombination of a *TRP1* cassette flanked with *loxP* recombination sites into the *ERG6* locus. This insertion resulted in deletion of more than 75% of the *ERG6* gene to afford the strain DCY001. *TRP1* was chosen for selection of this initial *erg6Δ* strain because FY250 yeast are auxotrophic for tryptophan, and *trp1Δ*, *erg6Δ* strains fail to grow under normal (30 °C) aerobic culture conditions due to decreases in tryptophan uptake.[42] However, this growth impairment is known to be obviated by aerobic culture at 37 °C.[42] Recovery of the tryptophan selection marker by Cre – *loxP* site-specific recombination[51] to afford strain DCY250 was found to confer this temperature-sensitive phenotype when the yeast were grown in 5 – 10 mL cultures under aerobic conditions. Fortu-

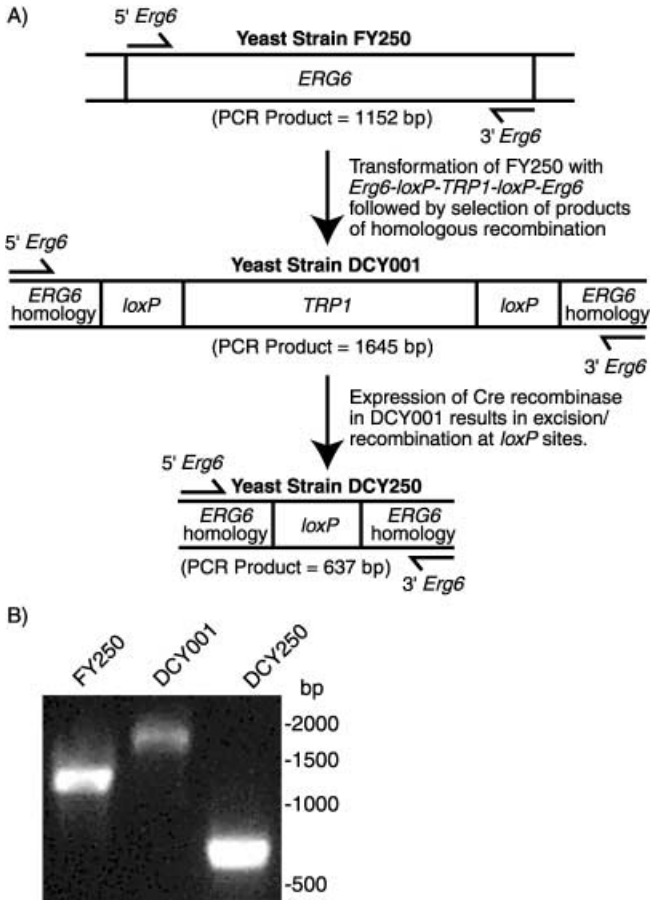


Figure 2. *Disruption of the* ERG6 *gene in yeast strain FY250. A) Schematic depiction of insertion of* TRP1 *at the* ERG6 *locus to afford strain DCY001 followed by Cre-mediated excision of* TRP1 *at* loxP *sites to yield strain DCY250. B) Analytical PCR analysis of* ERG6 *genes from genomic DNA of FY250, DCY001, and DCY250 yeast. (bp = base pairs).*

itously however, this temperature-sensitivity was not observed with cells grown on 96-well microtiter plates under aerobic conditions at 30 °C, and under these conditions growth was indistinguishable from that of the parent strain FY250. The ability of DCY250 yeast to grow on microtiter plates at 30 °C enabled direct comparisons of the activities of small molecules in FY250 and DCY250 systems. These genetic modifications were validated by analytical PCR methods (Figure 2).

To compare the permeability of DCY250 and FY250 to small molecules, their sensitivity to various antimicrobial agents, such as the protein synthesis inhibitors cycloheximide and G-418, and their resistance to the antibiotic nystatin were examined. Cycloheximide and nystatin are known to exhibit differential effects on *erg6Δ* yeast strains compared with *ERG6* competent strains.[42] As shown in Table 1, DCY250 was found to be significantly more sensitive than FY250 to the growth inhibitory effects of cycloheximide and G-418. In addition, DCY250 was substantially more resistant to nystatin, which exerts antifungal effects by binding to ergosterol in yeast membranes. These results confirmed that disruption of *ERG6* in FY250 yeast to yield DCY250 provided a yeast strain with enhanced permeability to small molecules.

Table 1. *Comparison of the growth inhibition effects of antimicrobial agents on yeast strains DCY250 and FY250.*[a]

	DCY250[b]	FY250[b]	Fold Δ[c]
Cycloheximide	0.0037	0.045	12.2
G-418	1.3	22.1	17.0
Nystatin	56.9	2.6	− 21.9

[a] Data shown represent the mean of duplicate experiments. Typical standard errors of the mean (SEM) were $< 3\%$. [b] Values represent concentrations ($\mu g\,mL^{-1}$) that inhibit growth by 50% (IC_{50}), calculated from dose–response curves. [c] Fold Δ represents growth inhibitory effects of compounds with respect to DCY250.

Modification of the yeast tribrid system

We previously reported a yeast tribrid system that detects intracellular tyrosine phosphorylation by the v-Abl and v-Src PTKs by using a universal substrate.[13] This system was modified to facilitate the detection of small-molecule inhibitors by reducing the expression level of the two enzymes. Toward this end, the high-copy (20–50 copies per cell) episomal 2μ-origin plasmids previously employed for kinase expression were substituted with low-copy (1–2 copies per cell) centromeric plasmids (CEN4/ARS1) bearing the galactose-inducible *GAL1* promoter and *LEU2* selection marker. These low-copy kinase expression vectors were transformed into the yeast strains DCY250 and FY250 with the previously described plasmids, which express the B42-Grb2 SH2 protein, LexA-GFP-$(AAYANAA)_4$ protein, and a *lacZ* reporter gene.[13] Expression of PTKs from either episomal or centromeric plasmids in tribrid assays did not result in significant differences in overall *lacZ* reporter gene expression (data not shown).

Analysis of PTK inhibitors with yeast tribrid systems

The small molecules PP1 (**1**), PP2 (**2**), herbimycin A (**3**), and staurosporine (**4**) were chosen as the known inhibitors of v-Abl and v-Src enzymes to be investigated. The former two inhibitors (**1**, **2**) are synthetic compounds whereas the latter two (**3**, **4**) are natural products. Literature IC_{50} values[52–55] for inhibition of these two enzymes are shown in Table 2. These compounds were chosen to encompass diverse functionality, such as the presence of a protonated amine at physiological pH values (staurosporine) and different molecular weights ranging from 281 daltons (PP1) to 574 daltons (herbimycin A), factors deemed likely to influence compound permeability to yeast cells.

The yeast tribrid system employed here for analysis of PTK inhibitors is illustrated schematically in Figure 1. In this system, reporter-gene expression is controlled by PTK-mediated phos-

Table 2. *IC_{50} values obtained from literature sources.*[52–55]

Inhibitor	IC_{50} (v-Abl)	IC_{50} (v-Src)
PP1 (**1**)	5 μM	0.3 μM
PP2 (**2**)	N/A	N/A
Herbimycin A (**3**)	5 μM	12 μM
Staurosporine (**4**)	80 nM	6 nM

phorylation of a LexA – GFP tetrameric universal substrate bound to DNA via the LexA DNA-binding protein. Conversion of tyrosine into phosphotyrosine in the substrate produces a high-affinity ligand of the B42-Grb2 SH2 protein, which results in recruitment of the B42 activation domain to LexA DNA binding sites of the reporter gene and activation of reporter gene expression. This gene expression should be reduced in a dose-dependent fashion by addition of a cell-permeable PTK inhibitor.

Four separate yeast tribrid assays (Figure 1) expressing the v-Abl or v-Src PTKs in FY250 or DCY250 (*erg6Δ*) yeast were investigated with the PTK inhibitors shown in Scheme 1. These inhibitors were also analyzed with control yeast strains that constitutively activate reporter gene expression by expression of a LexA – B42 fusion protein. These control experiments enabled nonspecific effects on reporter gene expression to be distinguished from specific effects on tyrosine kinase activity. As shown in Figure 3, dose-dependent effects of inhibitors on reporter gene expression were quantified, and IC_{50} values were determined (Table 3).

The results shown in Figure 3 and Table 3 demonstrate that deletion of the *ERG6* gene renders the DCY250 yeast strain substantially more sensitive to PTK inhibitors than strain FY250. Whereas only the low-molecular-weight PP1 (**1**) and PP2(**2**) compounds had significant effects against FY250, all of the inhibitors (**1** – **4**) conferred dose-dependent reductions in reporter gene expression in the *ERG6*-deficient DY250 strain. Importantly, all four inhibitors exhibited only minor effects on the control LexA – B42 assay at the highest concentrations examined (Table 3). Remarkably, although herbimycin A exhibits the lowest potency in vitro (Table 2) and is of the highest molecular weight, this compound proved to be the most potent compound against the DCY250 PTK assays. Herbimycin A was approximately 20 times more potent in the DCY250 strain than in FY250. This observation may relate to irreversible inhibition of PTKs by this compound.[11] In contrast, the other compounds (**1** – **3**) are reversible PTK inhibitors.

Table 3. *IC_{50} values measured in yeast systems.*[a]

Inhibitor	DCY250 Yeast v-Abl	DCY250 Yeast v-Src	DCY250 Yeast LexA – B42	FY250 Yeast v-Abl	FY250 Yeast v-Src	FY250 Yeast LexA – B42
PP1 (**1**)	12	7.6	> 100	20	> 100	> 100
PP2 (**2**)	4.2	4.5	> 100	25	> 100	> 100
Herbimycin A (**3**)	5.2	0.58	> 10	> 10	> 10	> 10
Staurosporine (**4**)	4.4	3.1	> 10	> 10	> 10	> 10

[a] Values represent concentrations (μM) required to inhibit reporter gene expression in yeast PTK tribrid assays by 50% (IC_{50}), calculated from the data shown in Figure 3.

The effects of compounds **1** – **4** on yeast growth were quantified in order to examine whether inhibition of cellular growth might contribute to effects on reporter gene expression. In these experiments (Table 4) cells were grown in the presence of test compounds for 16 hours and cell density was determined from absorbance values. Over this time interval, the growth of DCY250 was affected by all of the inhibitors, but most profoundly by staurosporine (**3**), which was highly toxic to both DCY250 and FY250. However, these effects on cellular growth are not likely to significantly influence the inhibitor data shown in Figure 3 and Table 3 because the inhibitors were analyzed after treatment of yeast for only 4 hours. This conclusion is supported by comparison with the corresponding control experiments with the LexA – B42 fusion protein.

To confirm that inhibitors **1** – **4** influence the activity of PTKs in whole yeast cells, the DCY250 strain that expresses v-Src was analyzed by immunoblotting with antiphosphotyrosine (Figure 4). These experiments re-

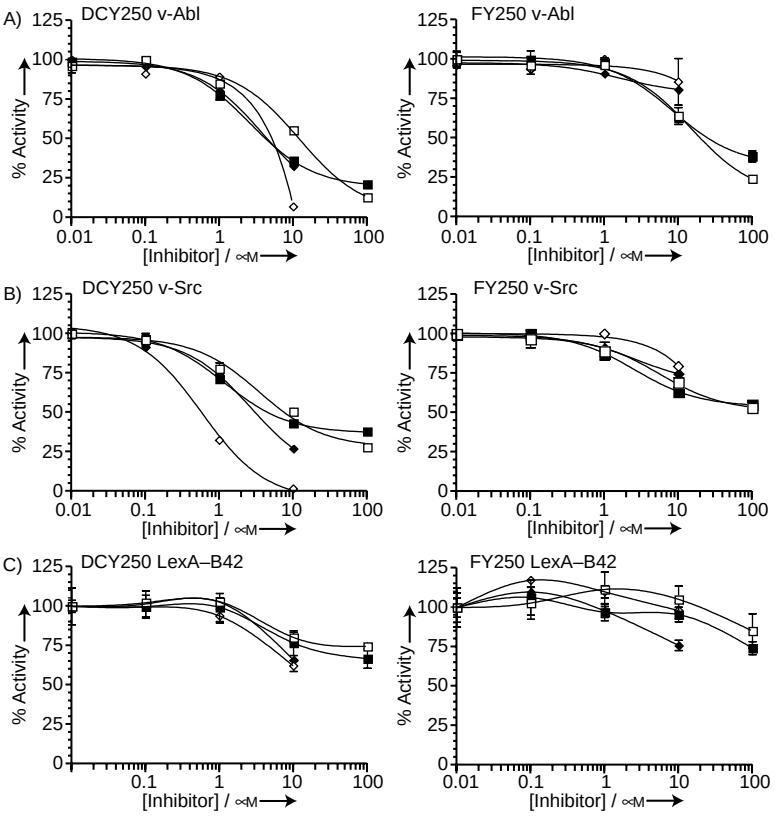


Figure 3. *Dose – response curves for yeast genetic systems treated with inhibitors □ PP1(**1**), ■ PP2(**2**), ◇ Herbimycin A(**3**), and ◆ Staurosporine (**4**). A) Expression of v-Abl in DCY250 (left) and FY250 (right) yeast tribrid systems. B) Expression of v-Src in yeast tribrid systems. C) Control yeast one-hybrid systems that constitutively activate reporter gene expression of a LexA – B42 fusion. Percentage activity represents normalized data with respect to the lowest concentration of inhibitor shown. Data presented are the average of at least duplicate experiments. Error bars represent SEM values. Typical errors were < 5%.*

Table 4. *Inhibition of yeast growth by PTK inhibitors.*[a]

	% Inhibition of growth DCY250	% Inhibition of growth FY250
PP1 (**1**, 100 μM)	78	0
PP2 (**2**, 100 μM)	43	0
Herbimycin A (**3**, 10 μM)	30	0
Staurosprorine (**4**, 10 μM)	100	95

[a] Data shown represent the mean of duplicate experiments with typical SEM values of < 1%.

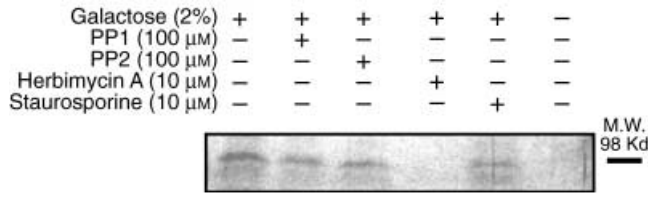


Figure 4. *Immunoblot analysis of the DCY250 v-Src tribrid assay by detection of phosphorylated proteins with antiphosphotyrosine. Each lane was loaded with equivalent amounts of cell extract after treatment with inhibitor for 4 hours.*

vealed that all of the inhibitors reduce v-Src activity and herbimycin A (10 μM) confers the greatest effect on phosphorylation. These results are consistent with the transcriptional tribrid dose-response data from Figure 3 and Table 3.

The results presented here demonstrate that yeast strains without the *ERG6* gene can be employed in two-hybrid-based assays to identify pharmacologically relevant small molecules that are normally impermeable to yeast. Given the importance of protein tyrosine kinases in human disease, the modified yeast tribrid assays reported here may be useful for the discovery of drug leads in high-throughput screening assays.

Experimental Section

General: Standard techniques for plasmid construction,[56] yeast transformation,[57] and amplification of yeast genomic DNA[58] were employed. The compounds PP1, PP2, and G-418 were obtained from Calbiochem. Herbimycin A, staurosporine, cycloheximide, nystatin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. 5-Fluoroorotic acid (5-FOA) was purchased from Toronto Research Inc. DNA oligonucleotides were from Integrated DNA Technologies. Restriction endonucleases and deoxynucleotide triphosphates were from New England Biolabs. GeneChoice *Taq* polymerase was purchased from PGC Scientific, and *Pfu* polymerase was purchased from Stratagene. DNA isolation and purification kits were from Qiagen. Microbiological media were from Difco and qBiogene. Prepoured solid yeast selection media was purchased from KD Medical. All new DNA constructs were confirmed by automated dideoxynucleotide sequencing at the Pennsylvania State University Nucleic Acid Facility. Data analysis was performed with the GraphPad Prism 3.0 program (GraphPad Software, Inc. San Diego, CA).

Bacterial and yeast strains: *Escherichia coli* DH5-α (Clontech) was employed for plasmid construction. *Saccharomyces cerevisiae* FY250 (*MAT*α, *ura3–52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63*) was a generous gift of Prof. M. Ptashne (Memorial Sloan-Kettering). Yeast strains DCY001 (*MAT*α, *ura3–52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63*, *erg6:loxP-TRP1-loxP*) and DCY250 (*MAT*α, *ura3–52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63*, *erg6:loxP*) were constructed as described within this study.

Plasmid construction: The yeast tribrid plasmids pJG4–5 Grb2 SH2, pAMLexA2 GFP-$(AAYANAA)_4$, pAMLexA2 GFP-$(AAFANAA)_4$, and the reporter vector pSH18–34 were described previously.[13] To reduce the expression levels of the v-Abl (amino acids 237–630) and v-Src (amino acids 137–526) PTKs, these genes were expressed from vector pYCDC. pYCDC is a *GAL1*-inducible, *LEU2*-selectable, centromeric plasmid constructed from pYCplac111[59] (a gift from R. D. Gietz) by digestion with *Eco*RI/*Hind*III and ligation to a *Mfe*I–*GAL1* promoter-HA tag-*Eco*RI/*Xho*I-*ADH1* terminator-*Hind*III (HA = hemagglutinin epitope tag) fragment derived from pRF4–6, a derivative of pJG4–5[60] that lacks the B42 activation domain and SV40 nuclear localization sequence[61]. The control plasmid for expression of the constitutively active LexA–B42 AD fusion protein was prepared by ligation of a *Mfe*I-SV40 NLS-B42 AD-HA tag-*Xho*I fragment derived from pJG4–5[60] into pAM423LexA.[13]

PCR from the template plasmid pDC2 ERG6-loxP2-TRP1-ERG6 generated the disruption cassette used to construct yeast strain DCY001. This plasmid included *loxP* sites amplified by PCR from the plasmid pU6H3HA[62] (a gift from A. De Antoni) and cloned into pDC2[13] to afford pDC2 loxP2. The forward *loxP* site included the hexameric 6 × His and trimeric HA tag sequences of pU6H3HA[62] flanked by in-frame 5′ *Kpn*I and 3′ *Mfe*I restriction sites. The reverse *loxP* site was amplified to include flanking in-frame 5′ *Xho*I and 3′ *Pst*I restriction sites. The *ERG6* gene sequences of the disruption cassette were generated by PCR amplification of yeast genomic DNA. Homology to N-terminal *ERG6* sequences included 270 bp upstream and 129 bp downstream of the *ERG6* start codon, flanked by in-frame 5′ *BamH*I and 3′ *Kpn*I restriction sites. Homology to C-terminal *ERG6* sequences included 129 bp upstream and 270 bp downstream of the *ERG6* stop codon, flanked by in-frame 5′ *Pst*I and 3′ *Hind*III restriction sites. These *ERG6* gene fragments were cloned into pDC2 loxP2 to create pDC2 ERG6-loxP2-ERG6. The *TRP1* selection marker was amplified from pJG4–5[60] flanked by in-frame 5′ *Eco*RI and 3′ *Xho*I restriction sites and cloned into *Eco*RI/*Xho*I-digested pDC2 ERG6 loxP2 to afford the final disruption template.

Targeted disruption of *ERG6*: Yeast strain FY250 was transformed with a DNA cassette amplified by PCR from plasmid pDC2-ERG6-loxP2-TRP1-ERG6 to include 149 bp of *ERG6* homology flanking each of the *loxP* sites. Homologous recombination yielded colonies that grew on solid synthetic-defined (SD) media (2% glucose) that lacked tryptophan (trp^-). Isolation and PCR analysis of individual yeast colonies provided the DCY001 strain.

DCY001 was transformed with the *Cre* expression plasmid pSH47[51] and transformants were selected on solid SD media (2% glucose) lacking uracil (ura^-) and tryptophan (trp^-). Selected transformants were inoculated into liquid yeast peptone media (2% galactose, YPGal) to induce Cre recombinase expression and incubated with shaking at 30 °C for 17 hours. Approximately 500 yeast cells were plated on solid SD media (2% glucose, ura^-) and incubated at 30 °C for 6 days. The most well-defined colonies were patched to solid SD media (2% glucose, trp^-, ura^-) to verify auxotrophic markers, and to solid SD media (2% glucose, ura^-) for further analysis, and were incubated at 37 °C for 1 day. Analytical PCR identified colonies with the appropriate genomic mutations. These colonies were patched to 5-FOA (1 mg mL^{-1}) plates and incubated at 37 °C for 2 days to remove the pSH47[51] plasmid. The resulting colonies were patched to solid yeast peptone media (2% dextrose, YPD) to obtain single colonies for final PCR analysis. A colony was selected based on PCR analysis and good growth characteristics and was patched to solid SD media (2% glucose, ura^-) and solid SD media (2% glucose, trp^-) and incubated at 37 °C to confirm auxotrophic markers. The strain derived from this colony was named DCY250. It should be noted that some of the media described above were supplemented with ergosterol (0.02 mg mL^{-1} final concentration added from a 1:1 ethanol/tergitol NP-40 200X stock solution) in an attempt to improve growth characteristics at 30 °C. However, aerobically cultured yeast cannot incorporate exogenously supplied ergosterol[38, 39] and ergosterol supplementation is not likely to have influenced yeast growth.

Yeast tribrid β-galactosidase reporter gene assay: Yeast tribrid assays for *S. cerevisiae* FY250 and DCY250 yeast were performed essentially as described previously.[13] Briefly, yeast harboring the appropriate plasmids were grown for 16 hours in SD liquid media

(2% raffinose, ura$^-$, trp$^-$, his$^-$, leu$^-$). Aliquots of saturated yeast cultures (80 μL) were added to a sterile 96-well microtiter plate followed by SD liquid media (100 μL, 4% galactose, ura$^-$, trp$^-$, his$^-$, leu$^-$) with a 10X stock solution of the PTK inhibitor (20 μL, 10% DMSO in H_2O). The plate was incubated with shaking at 30 °C for 4 hours and assayed for β-galactosidase reporter gene activity as previously described.[13] Values reported here represent the mean of at least duplicate experiments and were normalized to the activity calculated for wells containing the lowest concentration of the inhibitor shown. IC_{50} values were calculated by nonlinear regression with a one-site competition model (GraphPad Prism 3.0 software). Side-by-side comparisons with a nonphosphorylated phenylalanine analogue of the universal substrate (LexA-GFP-$(AAFANAA)_4$) typically provided less than 5% of the activity of the tyrosine-containing universal substrate. Error bars in the figures represent standard errors of the mean.

Immunoblotting: Yeast colonies picked for the DCY250 v-Src tribrid assays were grown 16 hours in SD media (2% raffinose, ura$^-$, trp$^-$, his$^-$, leu$^-$) and aliquotted (0.8 mL) onto a 24-well plate containing SD media (0.1 mL, 20% galactose, ura$^-$, trp$^-$, his$^-$, leu$^-$) and PTK inhibitors (0.1 mL, 10X stocks in 10% DMSO in H_2O) then incubated with shaking at 30 °C for 4 hours. Preparation of cell lysates and immunoblotting was performed as previously described,[13] but the immunoblots were developed with a CN/DAB substrate (Pierce).

Evaluation of growth inhibition: The growth inhibitory effects of compounds on FY250 or DCY250 yeast strains were determined by calculating the change in cell density after incubation with compounds for 16 hours. Briefly, aliquots of freshly inoculated YPD media (157.5 μL) and compounds (17.5 μL, 10X stocks in 10% DMSO in H_2O) were arrayed on 96-well plates and the initial cell density was determined by absorbance measurements at 590 nm. The plates were incubated with shaking at 30 °C for 16 hours and the final cell density was determined. Cell growth was calculated as the difference in cell densities. Values reported represent the mean of duplicate experiments and were normalized to cell growth in control wells (1% DMSO). IC_{50} values were calculated by nonlinear regression with a sigmoidal dose-response model (GraphPad Prism 3.0 software). Errors quoted correspond to standard errors of the mean.

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