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A Three-Hybrid Approach to Scanning the Proteome for Targets of Small Molecule Kinase Inhibitors

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Summary

In this study, we explored the application of a yeast three-hybrid (Y3H)-based compound/protein display system to scanning the proteome for targets of kinase inhibitors. Various known cyclin-dependent kinase (CDK) inhibitors, including purine and indenopyrazole analogs, were displayed in the form of methotrexate-based hybrid ligands and deployed in cDNA library or yeast cell array-based screening formats. For all inhibitors, known cell cycle CDKs as well as novel candidate CDK-like and/or CDK-unrelated kinase targets could be identified, many of which were independently confirmed using secondary enzyme assays and affinity chromatography. The Y3H system described here may prove generally useful in the discovery of candidate drug targets.

Introduction

Organic small molecules have been widely used as cell permeable ligands to elucidate mechanisms of signal transduction and as therapeutic agents. The majority of small molecule drugs elicit their pharmacological action through binding to protein targets. The identification of such targets is of fundamental importance to our understanding of the molecular basis of drug action and of mechanisms underlying drug efficacy and side effects.

Traditionally, the identification of receptors for small molecules has relied on in vitro biochemical methods, such as photo-crosslinking, radiolabeled ligand binding, and affinity chromatography. Recent technological advances in protein separation and analytical methods have significantly improved affinity chromatography-based approaches for the detection of small molecule-protein interactions. However, these approaches remain laborious and time consuming. Furthermore, the cloning of cDNAs encoding candidate targets is uncoupled from the target identification process. Several functional cloning methods have been developed in recent years to circumvent these problems. These include yeast three-hybrid (Y3H) [1], drug-western [2], phage display cloning [3], and mRNA display cloning [4]. All of these methods link the identification of ligand binding proteins to the selection of cDNAs encoding these proteins. The Y3H system distinguishes itself from the other methods in that the interaction between a small molecule and a target protein occurs in intact cells rather than in vitro.

Y3H is based on the yeast two-hybrid system (Y2H), which has proven to be a powerful tool for detecting protein-protein interactions [5–7]. Adaptation of Y2H to Y3H required the use of small molecule hybrid ligands, sometimes also referred to as chemical inducers of dimerization (CIDs) or "dimerizers" [8]. CIDs have been widely used to crosslink ("dimerize") at will proteins in living cells that have been engineered to express hybrid proteins with appropriate ligand binding and signaling domains (three-hybrid systems). In this manner, numerous biological processes, such as activation of specific signaling pathways, protein subcellular localization, or gene expression, were placed under the control of chemical dimerizers [8–10]. In contrast, small molecule hybrid ligands can be used in Y3H to identify and characterize small molecule-protein interactions.

Liu and colleagues first described the Y3H system and its application to screening of cDNA libraries for target discovery [1]. An engineered glucocorticoid receptor (GR) protein was used as a DNA-bound docking platform to display a dexamethasone (DEX)-tethered FK506 hybrid ligand to target proteins that could activate reporter gene expression upon binding. FKBP12, a known target of FK506, was identified, indicating that Y3H could be a valuable tool for the identification of novel small molecule-protein interactions, at least in principle. Despite this first promising report on Y3H and the broad uses of Y2H for mapping protein-protein interactions, only very few studies have subsequently made use of Y3H for the characterization of small molecule-protein interactions [11–14]. Only one other study made use of Y3H in screening cDNA libraries for target identification [14], reporting the successful identification ("rediscovery") of dihydrofolate reductase (DHFR) as a target of methotrexate (MTX) when using MTX as the "bait" tethered to DEX. Whether Y3H is suitable for the detection of weaker and novel interactions (FK506 and MTX display affinities for FKBP12 and DHFR in the nanomolar to picomolar range, respectively) and is amenable to hybrid ligands incorporating different types of organic small molecules, e.g., enzyme inhibitors, remained unclear.

In the present study, we set out to explore in more detail the potential and limitations of Y3H for the identification of novel small molecule protein targets, with emphasis on the characterization of the target spectrum of ATP-competitive active site kinase inhibitors. More

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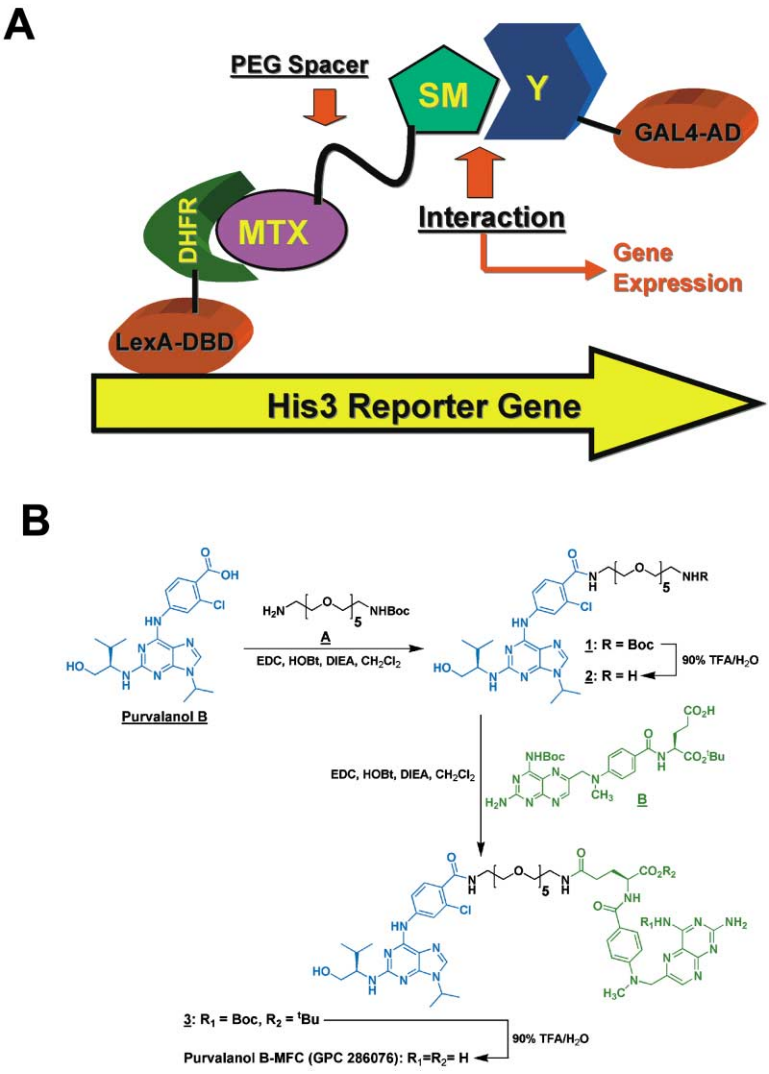


Figure 1. Y3H Assay System

(A), Y3H system components; (B), illustration of the general MFC synthesis strategy using purvalanol B as an example. Details of chemical synthesis are described in Experimental Procedures (see Supplemental Data).

than 500 putative kinases are encoded by the human genome, and they play a central role in the regulation of multiple cellular processes and diseases [15]. Two hundred forty-four kinases map to disease loci or cancer amplicons [15]. Thus, methods that would support the identification of the target spectrum of kinase inhibitors could have an impact on drug discovery across diverse therapeutic areas. Here, we describe the successful implementation of versatile screening approaches with a Y3H system that employs MTX-based hybrid ligands and the identification of novel candidate targets for various widely used cyclin-dependent kinase inhibitors with antitumor activity.

Results

The Y3H Assay System

Figure 1A highlights the three major components of the Y3H system used in this study. (1) A hybrid ligand, here named methotrexate fusion compound (MFC), which is composed of a methotrexate "anchor" moiety coupled to a small molecule test compound (SM) of interest via a polyethylene glycol spacer (or linker). (2) A DNA binding hybrid protein consisting of a LexA DNA binding domain (LexA-DBD) fused to dihydrofolate reductase (DHFR), as described previously [11–13]. (3) A second hybrid protein consisting of a transcriptional activation domain (AD) derived from the yeast transcription factor GAL4 fused to a polypeptide (Y) potentially capable of binding to the test compound of interest. Compound-mediated recruitment of the AD-fusion protein to the proximity of the promoter region of an integrated reporter gene induces reporter gene expression. In this study, we relied mostly on the use of a reporter gene encoding HIS3, an auxotrophic marker widely used in various Y2H systems. Expression of HIS3 enables yeast cells to grow in the absence of histidine in the culture medium, allowing for easy and sensitive detection of a hybrid ligand-induced interaction event.

Figure 1B shows an example of the synthesis of a kinase inhibitor MFC used in this study, GPC286076 (purvalanol B-MFC). We found that use of a PEG-spacer yielded MFCs with good water solubility properties. This facilitates an even diffusion of compound through agar

and more uniform and robust growth of affected yeast clones. PEG-spacers containing three or more ethyleneglycol subunits were found necessary for good performance in Y3H (data not shown). Test experiments using the DEX-derived MFC, GPC285937 (Figure 2A), showed strong reporter expression (HIS3 or an alternative reporter, LacZ) in the presence of a GR-AD fusion protein (data not shown), consistent with previous studies in which MTX-DEX hybrid ligands incorporating different chemical spacers were employed [11–13].

Synthesis of Kinase Inhibitor Hybrid Ligands

Our interest in cell cycle control led us to select well-characterized CDK inhibitors to explore the use of ATP-competitive kinase inhibitors in Y3H and to evaluate the potential of Y3H for identifying novel small molecule-protein interactions. Purines are widely studied CDK kinase inhibitors with antiproliferative activities [16–18]. The purine purvalanol B is a nanomolar inhibitor of CDK1, CDK2, and CDK5 (Figure 2A; [19]). Recent protein binding studies revealed additional kinase targets for this inhibitor, including casein kinase 1 (CK1), ERK1, and ERK2 [20, 21]. Purvalanol B was reported to inhibit these latter targets with IC_{50} values in the micromolar range. (R)-Roscovitine (also known as CYC202), a related purine analog [17, 22], exhibits antitumor activity in vivo and is currently in phase I/II clinical trials as an anticancer agent [23, 24]. Compared to purvalanol B, it is a moderate inhibitor of CDK1, CDK2, and CDK5 (Figure 2A) [25, 26]. No other targets for roscovitine have been reported to our knowledge. Indenopyrazoles are a structurally different class of kinase inhibitors, with an overlapping but distinct CDK inhibition profile. Various indenopyrazoles have been reported to be potent, nanomolar inhibitors of CDK1, CDK2, and CDK4 [27–29]. Similarly, IP-1 (Figure 2A) is a potent inhibitor of CDK1, CDK2, and CDK4, and it displays potent antiproliferative activity against tumor cells in vitro, with IC_{50} values in the low nanomolar range (data not shown).

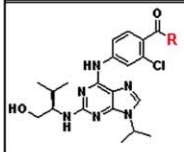
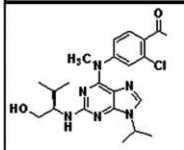
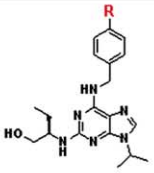
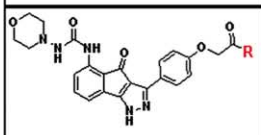
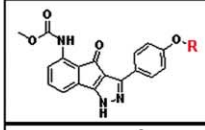
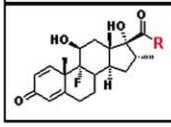
For the design of the purvalanol B-MFC, GPC286076 (Figure 2A), a suitable attachment site for the spacer was derived from the CDK2-purvalanol B crystal structure, which suggested that attachment to the carboxylic acid of the 6-anilino substituent of the inhibitor would not interfere with its interaction with the kinase [16, 20, 30]. As a control, we synthesized a CDK-inactive N6-methylated purvalanol B (Me-Purvalanol B)-MFC, GPC286207 (Figure 2A). Replacement of the N6 hydrogen, which forms a key hydrogen bond with Leu83 of CDK2, with a methyl group was shown to greatly reduce the CDK-inhibitory activity of purvalanol B as well as its ability to bind to other kinases [20]. The chemical structures of the roscovitine-MFC (GPC286077) and the indenopyrazole IP-1-MFC (GPC286026) are also shown in Figure 2A. The rationale for selecting the PEG-spacer attachment site for these two inhibitors was also derived from published human CDK2-inhibitor complex crystal structures and, in the case of IP-1, also structure-activity-relationship information on indenopyrazoles [22, 27–29]. IP-1* is a close structural variant of IP-1 but lacks CDK-inhibitory activity (Figure 2A). The IP-1*-MFC, GPC286060, was used as a Y3H control compound along with the hybrid ligand GPC285937 (a DEX-MFC) and MTX-5PEG-OCH_3, a "universal control" compound that contains methotrexate and PEG moieties but lacks a test compound (Figure 2A). All MFCs derived from the CDK inhibitors retained CDK inhibitory activity (data not shown).

Relative cellular uptake and binding of the MFC hybrid ligands to the LexA-DHFR hybrid protein in cells were indirectly determined by competition experiments. A "reference MFC" with known target binding properties was used to activate HIS3-reporter gene expression (see Experimental Procedures for details). The ability of a test MFC to compete via its MTX moiety with the reference MFC for binding to LexA-DHFR was assayed as a function of a decrease in HIS3 reporter expression (yeast cell growth) in response to increasing concentration of the test MFC. Similar assays have been described for measuring relative uptake of FK506-analog (AP1867) hybrid ligands by mammalian cells [31]. As shown in Figure 2B, MFCs incorporating either purvalanol B or roscovitine (GPC286076 and GPC286077) competed nearly equally well, as did the control compounds MTX-5PEG-OCH_3 and GPC285937. IP-1 and IP-1* MFCs (GPC286026 and GPC286060) showed a competition profile comparable to that of the other MFCs in a different set of experiments (data not shown). In summary, no significant differences were observed for these compounds, facilitating comparative interaction analysis, as described below.

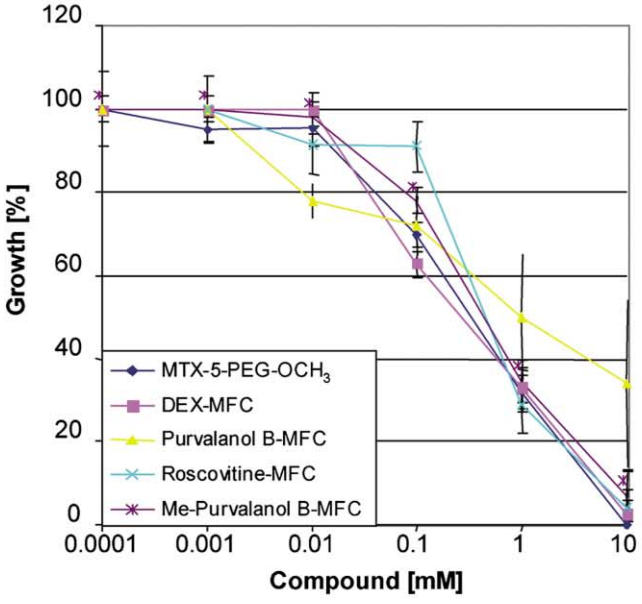
Identification of Novel Targets for the Purine Analog Purvalanol B

Figure 3A outlines the cDNA library screening strategy pursued for target identification with the purvalanol B-MFC. As shown, all candidate targets identified in cDNA library screens were routinely subjected to a follow-up analysis. This consisted of retransformation of yeast strains with the individually identified cDNA clones and subsequent interrogation of a robotically generated recombinant yeast cell array with test MFC and control compounds (we refer to this as yeast halo-analysis). In addition, kinase clones that were otherwise independently cloned or identified in Y3H screens with compounds other than purvalanol B were used to generate recombinant yeast strains that were included in the replica spotting of arrays. As discussed below, analysis of such an extended array led to the identification of additional candidate purvalanol B binders and facilitated a more thorough comparative analysis using a diverse set of MFCs derived from other kinase inhibitors. Interrogation of replica arrays was performed at a high and low dose of MFCs. Spotting of a high amount of compound translated into an estimated concentration of compound in the yeast halo-analysis that is comparable to that used in the purvalanol B cDNA library screens. Spotting of a low amount served the purpose of annotating targets that yielded signals at significantly lower concentration of compound (10-fold lower). Figure 3B shows a representative result obtained by interrogating a yeast cell array representing a panel of kinases with compounds at the high dose. The left panel in Figure 3B summarizes array results obtained with candidate targets identified in cDNA library screens with purvalanol

A

Structure	Parent		MFC
	R =	IC_{50} (μM)	R =
	OH **(Purvalanol B)**	CDK1/B: 0.036 CDK2/E: <0.003 CDK4/D: >10 CDK5/p35: 0.004 Published data[16-21] CDK1/B: 0.006 CDK2/E: 0.006 CDK5/p35: 0.006 ERK1: 3.3 ERK2: 1.0	NH-$(PEG)_5$-MTX, **(GPC 286076)**
	OH **(Me-Purvalanol B)**	CDK1/B: 1.5 CDK2/E: 3.8 CDK4/D: >10	NH-$(PEG)_5$-MTX **(GPC 286207)**
	H **(Roscovitine)**	CDK2/E: 0.6 CDK4/D: >10 Published data[22,25,26] CDK1/B: 0.65 CDK2/E: 0.7 CDK4/D: >100 CDK5/p35: 0.16 ERK1: 34 ERK2: 14	C(O)NH-$(PEG)_5$-MTX **(GPC 286077)**
	OH **(IP-1)**	CDK1/B: 0.01 CDK2/E: 0.005 CDK4/D: 0.009	NH-$(PEG)_5$-MTX **(GPC 286026)**
	CH_3 **(IP-1*)**	CDK1/B: 80 CDK2/E: 100 CDK4/D: 200	$CH_2C(O)$NH-$(PEG)_5$-MTX **(GPC 286060)**
	CH_2OH **(Dexamethasone)**	nd	NH-$(PEG)_5$-MTX **(GPC 285937)**

B



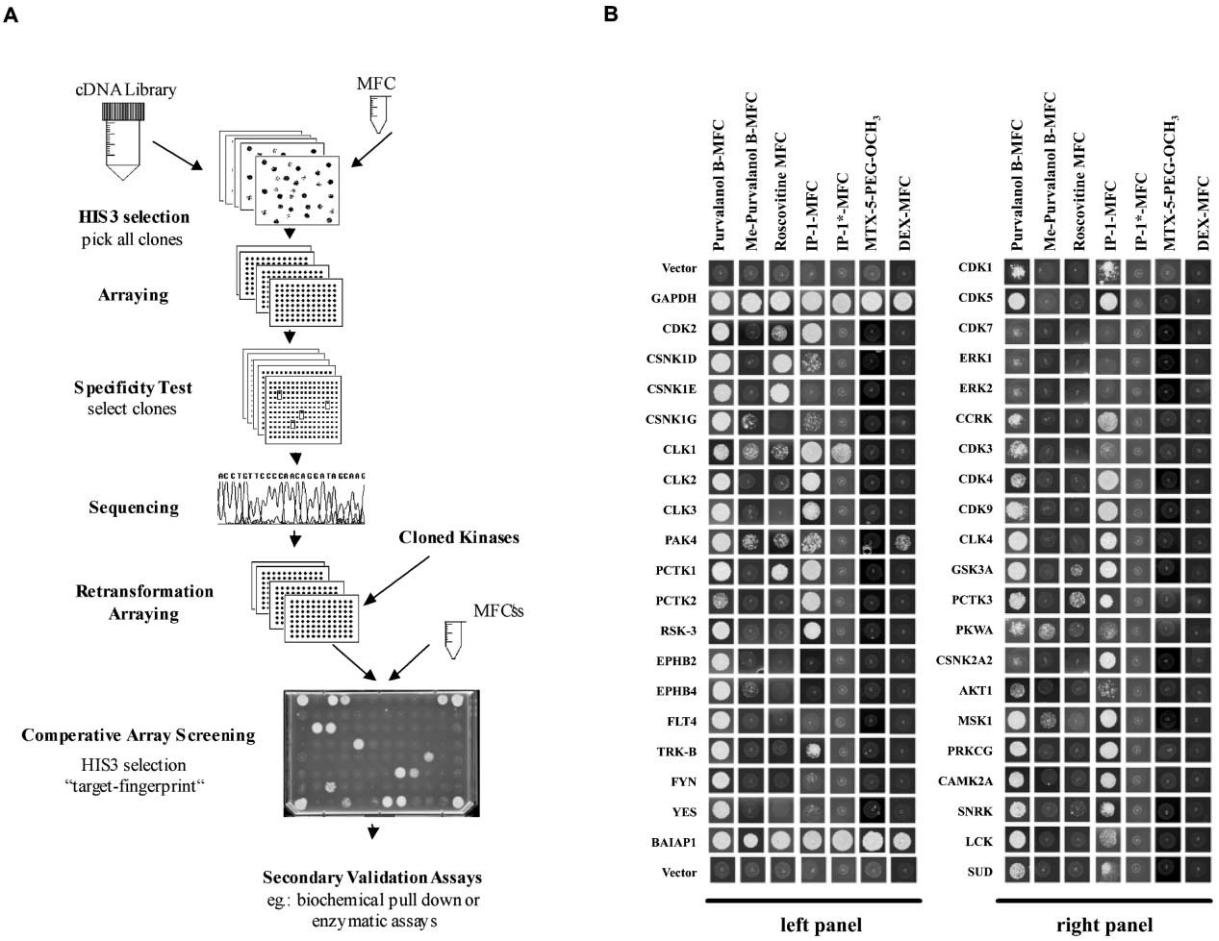


Figure 3. Y3H Identification of Kinase Inhibitor Targets

(A) Y3H cDNA library screening workflow as utilized with purvalanol B-MFC. Screening of a cDNA library involved the following: (1) transformation of LexA-DHFR-expressing yeast cells with a cDNA library of choice, (2) selection for transcriptional expression of the HIS3 auxotrophic marker in the presence of an MFC (reflected by yeast cell growth in absence of histidine), (3) selection/picking of positive colonies, (4) robotic arraying of these yeast cells and reconfirmation of compound dependence of HIS3-reporter expression, (5) plasmid isolation and sequencing, (6) retransformation of yeast expressing LexA-DHFR with purified plasmids, (7) robotic arraying of the transformed yeast cells, and (8) reconfirmation of specific HIS3-reporter activation by the test MFC using a series of genetic or compound-based counterscreens. The genetic counterscreens involved tests assaying for dependency of a seemingly positive interaction event on the presence of both the LexA-DHFR and AD-fusion proteins (the concept of genetic counterscreening is described in [40]). The compound-based counterscreens involved tests assaying the specificity of an interaction by comparing HIS3-reporter activation in response to test MFC versus control MFCs. All of these steps were found to be necessary for the identification of candidates with a high likelihood of encoding bona fide small molecule binding proteins. Replica yeast cell arrays of 96-well format (generally more sensitive than random cDNA library screens, see below) were also used to simultaneously interrogate an additional set of cloned kinases with the MFCs.

(B) Array-based interaction analysis (halo-analysis) of a series of kinases that were either identified in purvalanol B cDNA library screens (left panel) or otherwise independently cloned (right panel). Arrays were interrogated with the indicated MFCs (high dose) and the MTX-PEG "universal control." Images from each 96-well format array screen (one compound per array) were clustered to yield the composite image shown here.

B-MFC. The right panel summarizes array results obtained with the extended list of kinases. Overall scoring of the array results obtained at both high and low dose of MFCs is summarized in Figure 4.

Analysis of the array images shown in Figure 3B, summarized in more detail in Figure 4, revealed numerous interaction events for purvalanol B-MFC. Noteworthy is that all candidate targets identified in the cDNA library screens were kinases (Figure 3B, left panel). These included a subset of the known targets for purvalanol B,

Figure 2. Synthesis and Characterization of Kinase Inhibitor Hybrid Ligands

(A) Structures and enzyme inhibitory properties of parent compounds and MFCs.

(B) Comparable cellular uptake/functionality of MFCs. Competitive inhibition of "reference"-MFC-induced HIS3 reporter activation (reflected by a decrease in yeast growth) in response to increasing concentration of test MFCs (n = 3 independent experiments).

	Name	Group	Y3H Array Analysis Relative Signal Intensity: 3/strong, 2/medium, 1/weak [H]: positive signal only at high dose Purvalanol B-MFC	Me-Purvalanol B-MFC	Roscovitine-MFC	IP-1-MFC	IP-1*-MFC	MTX-5-PEG-OCH_3	DEX-MFC
Targets identified in cDNA screens with Purvalanol B-MFC	CDK2	CMGC	3	-	2[H]	3	-	-	-
	(CSNK1D)	CK	3	-	3	1[H]	-	-	-
	(CSNK1E)	CK	3	-	3[H]	-	-	-	-
	(CSNK1G)	CK	3	1[H]	-	1[H]	-	-	-
	CLK1	CMGC	2	1[H]	1 [H]	3	2	-	-
	CLK2	CMGC	3	-	-	3	-	-	-
	CLK3	CMGC	3	-	-	2	-	-	-
	PAK4	CMGC	3	1[H]	1[H]	1[H]	-	-	-
	PCTK1	CMGC	3	-	2	3	-	-	-
	PCTK2	CMGC	1[H]	-	-	3	-	-	-
	RSK-3	AGC	3	-	-	3	-	-	-
	EPHB2	TK	3[H]	-	-	-	-	-	-
	EPHB4	TK	3	-	-	-	-	-	-
	FLT4	TK	3[H]	-	-	-	-	-	-
	TRK-B	TK	3	-	-	2[H]	-	-	-
	FYN	TK	3	-	-	1[H]	-	-	-
	YES	TK	3	-	-	1[H]	-	-	-
	BAIAP1	other	3	3	3	3	3	3	3
Additional arrayed kinases	CDK1	CMGC	2[H]	-	-	2	-	-	-
	CDK5	CK	3[H]	-	-	3	-	-	nd
	CDK7	CMGC	-	-	-	-	-	-	-
	ERK1	CMGC	-	-	-	-	-	-	-
	ERK2	CMGC	-	-	-	-	-	-	-
	CCRK	CMGC	2[H]	-	-	3	-	-	-
	CDK3	CMGC	2[H]	-	-	1[H]	-	-	-
	CDK4	CMGC	1 [H]	-	-	3	-	-	-
	CDK9	CMGC	2[H]	-	-	3	-	-	-
	CLK4	CMGC	3	-	-	3	-	-	-
	GSK3A	CMGC	3	-	1[H]	3	-	-	-
	PCTK3	CMGC	2	-	1[H]	2	-	-	-
	PKWA	CMGC	1	1[H]	-	1	-	-	-
	CSNK2A2	CK	(1[H])	-	-	3	-	-	-
	AKT1	AGC	1[H]	-	-	1[H]	-	-	-
	MSK1	AGC	3[H]	-	-	3[H]	-	-	-
	PRKCG	AGC	3[H]	-	-	3[H]	-	-	-
	CAMK2A	CAMK	2[H]	-	-	2[H]	-	-	-
	SNRK	CAMK	2[H]	-	-	2[H]	-	-	-
	LCK	TK	3	-	-	2[H]	-	-	-
	SUD	other	2[H]	-	-	2[H]	-	-	nd

Figure 4. Summary of Higher-Resolution Y3H Kinase-Array Analysis

Experiments were performed as shown in Figure 3B. In addition, MFCs were used at both high dose (spotting of 1 μl of a 5 mM solution) and low dose (spotting of 1 μl of a 0.5 mM solution). Instances in which a signal was detected only at the high dose of compound are highlighted by inclusion of an [H]. Signal intensity was scored as either strong (3), medium (2), or weak (1). BAIAP1 is an example of a "false positive" that produced a signal with all compounds. Genetic counterscreens revealed that none of the positively marked interactions could be detected in the absence of either one of the hybrid proteins. Generally used acronyms for the various kinases are shown. Gray shaded boxes indicate previously known purvalanol B targets. Full name and ENSMBLE IDs for each gene are provided in the Supplemental Data, appendix 1. This includes annotations with respect to the nature of the cDNA clones/type of fusion protein expressed in the recombinant yeast strains (FL, full-length; KD, partial ORF including the kinase domain).

i.e., CDK2 and casein kinase 1 (CK1 or CSNK1). A previous study that reported CK1 binding properties of purvalanol B did not resolve which subtypes of CK1 could interact with this purine analog [20]. Here, we identified three distinct forms of CSNK1, namely CSNK1D, CSNK1E, and CSNK1G (all encoded by distinct genes). Besides these known targets, several novel candidate purvalanol B binders were identified, including CDC/CDK-like proteins (CLK1, CLK2, CLK3, PCTK1, PCTK2), other types of serine/threonine kinases (PAK4, RSK3), nonreceptor tyrosine kinases (FYN, YES), and, interestingly, receptor tyrosine kinases (EPHB2, EPHB4, FLT4, TRK-B; in each case the cytoplasmic domain, which encodes the RTKs kinase domain, was encoded by the selected cDNA). The extended kinase array analysis (Figure 3B, right panel) revealed an additional 18 positive interaction events, which included two other known targets for purvalanol B, CDK1, and CDK5 (Figures 3B and 4). Interestingly, a weak but significant signal was observed consistently also with CDK4, which is inhibited by purvalanol B in vitro at micromolar IC_{50} (see Figure 2A). The 15 additional novel interaction events (excluding CDK4) included several additional CDC/CDK-related kinases (CDK3, CDK9, CCRK, CLK4, PCTK3), other types of serine/threonine kinases (GSK3A, PKWA, CSNK2A2, AKT1, MSK1, PRKCG, CAMK2A, SNRK), one additional nonreceptor tyrosine kinase (LCK), and one nonprotein kinase (SUD). Collectively, a total of 35 kinase interaction events were observed with purvalanol B-MFC (Figure 4), 31 of which were found to involve novel candidate kinase targets. These results indicate that the target spectrum of purvalanol B is significantly broader than one may have anticipated, although it remains to be determined what the affinity of purvalanol B is for any of these targets.

Purvalanol B-MFC did not promote halo-growth of yeast cells when either the DBD- or AD-fusion proteins were absent (see Figure 4 legend, genetic counterscreens). Also, none of the kinases scored positively with the control compounds MTX-PEG_5-OCH_3 ("universal control") and GPC 285937 (DEX control). These control tests indicate that all the putative purvalanol B-MFC Y3H interaction events are likely to reflect a specific and direct interaction of target with purvalanol B. Furthermore, most of the kinases did not score positively with the MFC of the methylated, CDK-inactive form of purvalanol B (Me-purvalanol B, GPC286207). Those kinases that did score positively, i.e., CSNK1, CLK1, PAK4, and PKWA, appeared to have a significantly lower apparent affinity for Me-purvalanol B as compared to purvalanol B, as suggested by the significantly lower halo-signal intensity observed at the high dose of compound and

the complete lack of signal at the lower dose of compound (see Figure 4 for overall scores). In summary, the results presented in Figure 4 reveal a broad spectrum of candidate targets for purvalanol B and indicate that overlapping structural requirements dictate the interaction of the CDK-active form of purvalanol B with its known and newly identified candidate interactors.

The target spectrum outlined in Figure 4 included a number of known targets of purvalanol B but not all (i.e., CDK7, ERK1, ERK2). Also, for a few of the known targets, the presumed "interaction strength" (as reflected by the extent of yeast growth/Y3H halo-signal) did not correlate well with the known potency of purvalanol B toward these targets (CDK1, CDK5). This is not surprising, given that a number of factors may govern detection of an interaction event. These include but are not limited to the following factors. (1) Variability in AD-fusion protein expression, folding, and nuclear translocation (e.g., CDK1 expression could barely be detected, in contrast to CDK2 and CDK4; data not shown). (2) Affinity for a small molecule may be influenced or dependent on accessory/regulatory factors (e.g., p25/p35; the CDK5 cyclin is primarily expressed in brain and is required for activation of CDK5 [32–34]. Yeast cyclins may not complement p25/p35 function. Active CDK7 requires three components, CDK7, cyclinH, and MAT). (3) Affinity of the AD-fusion protein for the small molecule may be close to the presumed detection limits of Y3H (see below, e.g., ERK1 and ERK2 are inhibited by purvalanol B with IC_{50}s in the micromolar range). (4) Positioning of the PEG-spacer may affect interaction of a small molecule with different target proteins differentially. (5) When performing a cDNA library screen, presence and relative abundance of a particular cDNA in a given cDNA library and the redundancy at which a screen is performed will also influence the extent to which targets can be identified.

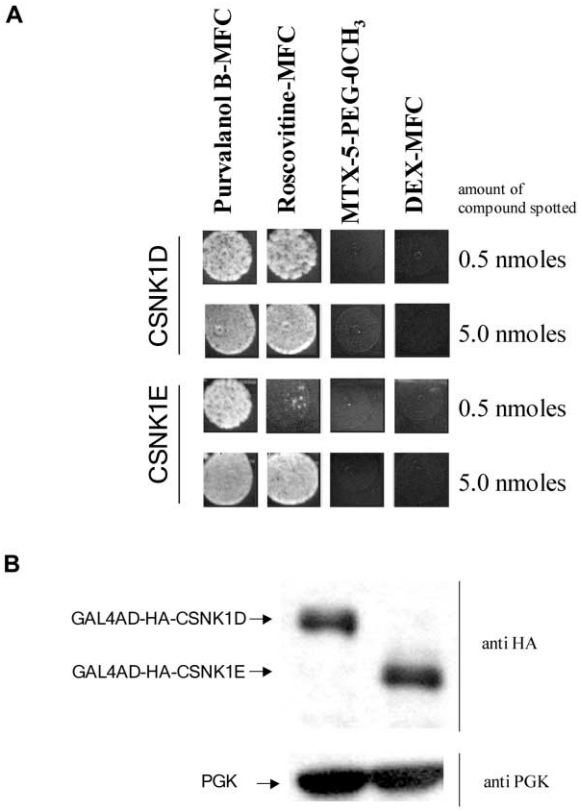


Figure 5. Differential Interaction of Purine Analogs with CK1 Subtypes

(A) Yeast halo-analysis at high and low dose of MFC.

(B) Western blot analysis (blotting for anti-HA-tag) of CK1 subtype AD-fusion proteins expressed in yeast. PGK (phospho-glycerate-kinase) was included in the analysis to control for sample loading.

Comparative Array-Based Y3H Screening with Kinase Inhibitors

As described in the previous section, replica yeast cell array screening can provide a useful approach to target profiling and comparative analysis of small molecules. Features distinguishing even closely related compounds can be revealed by this approach. This is further highlighted by the comparative analysis performed with MFCs derived from the purine analogs purvalanol B and roscovitine, the indenopyrazole IP1, and derivatives of these compounds (Figures 3B and 4). Replica arrays including the 38 selected kinases were interrogated with the respective MFCs. Roscovitine-MFC scored positively with its known target CDK2. Interestingly, it also scored positively with CSNK1D and CSNK1E but, in contrast to purvalanol B, not with CSNK1G. Figure 5A shows that the differential behavior of these closely related purine analogs becomes even more pronounced when scores at low dose are considered. Purvalanol B-MFC scored positively with all three CK1 isotypes, whereas roscovitine-MFC only scored with CSNK1D. Given that both the purvalanol B and roscovitine MFCs displayed a comparable "cellular uptake"/DHFR binding competition profile (Figure 2B), these results suggest that purvalanol B has higher affinity for CSNK1E and G than roscovitine, at least in the context of Y3H. In contrast, drawing conclusions with respect to potential differences in relative binding affinities of a given compound for one or the other target is more speculative, given the variables associated with expression, folding, and translocation of fusion proteins in yeast. However, if one controls for protein expression levels, it may be possible to tentatively rank closely related targets according to observed "interaction strength." For instance, as shown in Figure 5B, both CSNK1D and CSNK1E seem to achieve similar expression levels in yeast. However, at low dose of compound, roscovitine-MFC scored with CSNK1D but not CSNK1E, suggesting that CSNK1D could be a higher-affinity target. More studies will be necessary to evaluate the extent to which such correlations can be made with any level of confidence. Like purvalanol B-MFC, roscovitine-MFC also scored with PCTK1 and comparatively weakly with CLK1, PAK4, GSK3a, PCTK3, and PKWA. Hence, roscovitine-MFC appears to have a more restricted Y3H-target profile than purvalanol B-MFC. Comparatively, the indenopyrazole IP-1-MFC recognized a broader spectrum of targets. It scored positively with its known targets CDK1, CDK2, and CDK4. In addition, the IP-1-MFC interaction profile extended to other CDC/CDK-related kinases and other

types of serine threonine kinases (Figures 3B and 4). These targets have not been described previously for any indenopyrazole. The CDK-inactive IP-1*-MFC did not score positively with any of the kinases, except weakly with CLK1, indicating that overlapping structural requirements dictate interaction of the CDK-active form of IP-1 with its known targets and most of the newly identified kinase targets. Thus, as for MFCs of purvalanol B and roscovitine, IP1-MFC appears to have a more extended target profile than one may have anticipated, especially considering the putative associations with kinases other than cell cycle kinases.

Biochemical Analysis and Validation of Y3H Interactions

To further scrutinize the extent to which positive Y3H scores are likely to reflect bona fide small molecule-protein interactions, we evaluated the ability of purvalanol B to interact in vitro with targets identified by Y3H. A representative sample of interactions was evaluated that included known and novel candidate interactors. The analysis included the following: (1) in vitro enzyme assays with purified kinases and (2) affinity chromatography assays to detect binding of cellular proteins to immobilized purvalanol B (but not Me-purvalanol B). PEGylated purvalanol B (intermediate **A**, R = H, Figure 2A) was directly coupled to solid phase, as previously described [20], incubated with cell extracts, and bound proteins were eluted and subjected to analysis. For the detection of endogenous proteins, cellular extracts derived from Jurkat cells were used. The identity of bound proteins was determined by either LC-MS analysis (liquid chromatography-mass spectrometry) or immunoblot analysis using commercially available "antitarget" antibodies. In a complementary approach, extracts derived from transiently transfected 293 cells were used to detect binding of myc-tagged kinase/kinase domain-encoding polypeptides by immunoblot analysis using an anti-myc-tag antibody.

Figure 6 shows examples of typical immunoblot experiments. Specific binding to immobilized purvalanol B was detected for a number of endogenous or exogenous kinases, including the novel candidate targets CDK9, PCTK1, CLK3, GSK3a, CSNK1D, MSK1, EPHB2, and FLT4 (the approach was also validated by including analysis of known targets, such as CDK1, CDK2, CDK5, CDK7, ERK1, and ERK2). No binding to immobilized Me-purvalanol B or PEG-spacer alone was detected. Expression of some targets was barely or not at all detectable (AKT1, CLK1, PKWA, SNRK) and hence failed to be reconfirmed by this approach. As shown in Figure 7, several additional Y3H-identified targets were detected by affinity chromatography/LC-MS analysis of Jurkat cell extracts (i.e., detection of endogenous proteins), including the novel candidate targets CDK3, CDK9, CCRK, PCTK2, PCTK3, CSNK1D, CSNK1E, CSNK2A2, CAMK2G, PAK4, RSK-3, FYN, YES, and LCK (known targets, e.g., CDK1, CDK5, ERK1, and ERK2, were also identified). No binding to immobilized Me-purvalanol B was detected for any of the kinases. As also shown in Figure 7, several novel interactions were confirmed using in vitro enzyme assays, including CDK3, RSK-3, FYN, YES, and LCK. Enzyme inhibition at 1 μM purvalanol B was greater than 50% in each case, suggesting that IC_{50} values for these kinases likely range below 1 μM. In summary, with respect to targets identified in cDNA library screens, 12 of 16 novel candidate targets scored positively in at least one secondary assay. In total, 22 novel interactions were confirmed by at least one secondary assay.

Discussion

The ease with which recombinant yeast cells can be generated and manipulated in the laboratory has greatly contributed to the success of the development and application of yeast two-hybrid systems to mapping of a wide range of different protein-protein interactions on a proteomic scale [7]. In the present study, we explored the potential of a related yeast-based molecular interaction system, Y3H [1], for the discovery of novel small molecule-protein interactions and evaluated various screening approaches with MTX-based hybrid ligands incorporating small molecule kinase inhibitors. Both cDNA library and focused screening of yeast cell arrays displaying selected polypeptide ORFs led to the identification of known targets as well as many novel candidate targets. As shown for the purine analog purvalanol B, many of the interactions involving putative novel kinase targets could be confirmed by either one or more secondary in vitro binding or enzyme inhibition assays, indicating that a significant proportion of interaction events observed with the Y3H assay system described herein do reflect, or are likely to reflect, bona fide small molecule-protein interactions. Based on these findings, we conclude that Y3H is a powerful method for scanning of the proteome for candidate small molecule kinase inhibitor targets.

Prior to this study, it was unclear whether expression of various kinases would interfere with yeast growth and thus be incompatible with Y3H. The results we obtained suggest that many different types of kinases can be expressed as fusion proteins in yeast in a form suitable for interaction with small molecule ATP-competitive active site kinase inhibitors. Interestingly, in several instances we noticed that in the presence of compound the steady-state levels of kinase fusion proteins increased (data not shown), an effect that is likely to facilitate the detection of certain interactions. Either full-length open reading frame (ORF) or partial ORF-encoding polypeptides encompassing kinase domains may be suitable or even required for productive interactions to occur in Y3H, depending on the type of kinase (e.g., not surprisingly, only the cytoplasmic domains of receptor tyrosine kinases were identified). Hence, high quality, complex cDNA libraries encoding multiple fusion proteins of candidate targets are an important factor in Y3H screening, which is reminiscent of the well-established observations that complex protein-domain-encoding cDNA libraries are a major contributor to successful Y2H screening for the detection of protein-protein interactions.

Judging from the range of interactions identified in this study, the sensitivity limit of Y3H appears comparable to

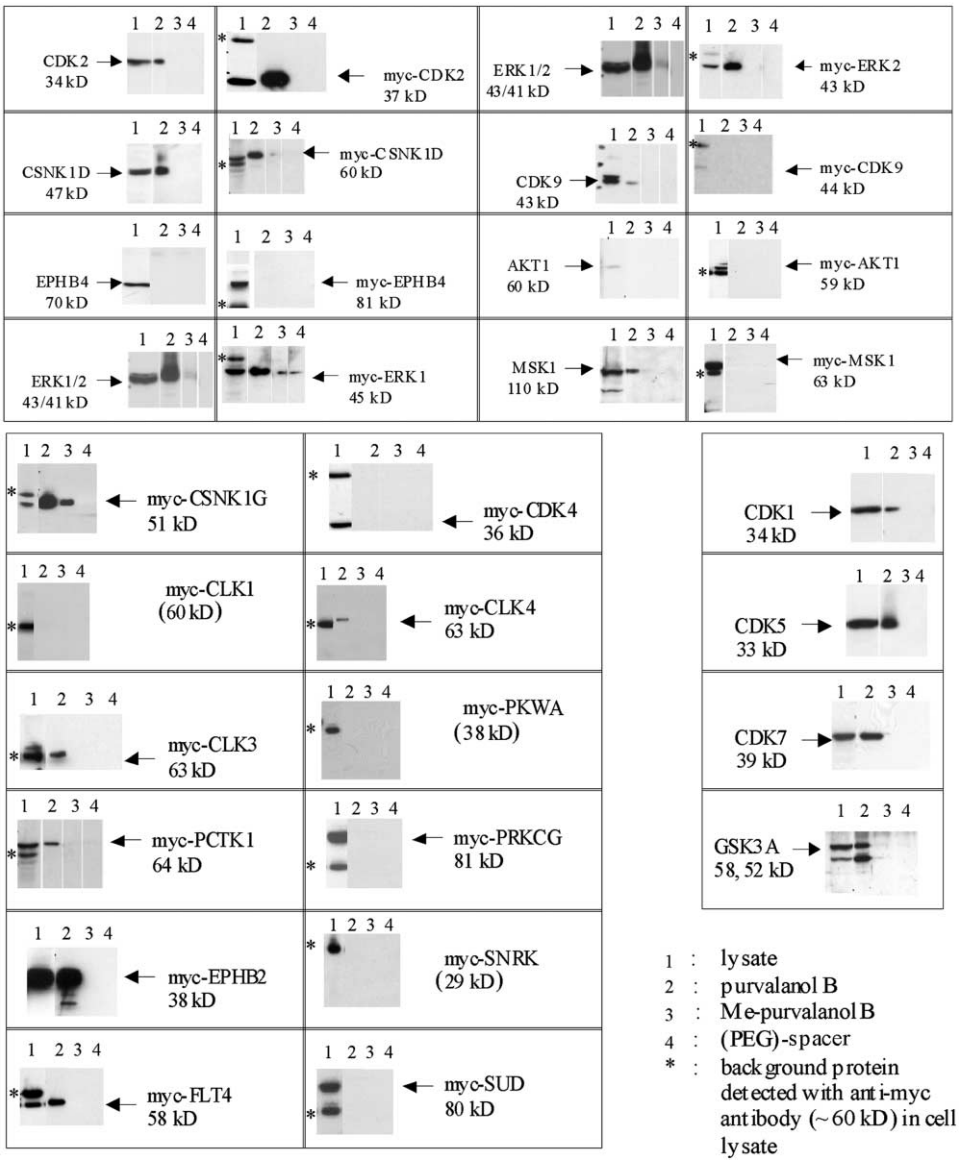


Figure 6. In Vitro Binding of Purvalanol B to Novel Candidate Targets

In vitro binding of cellular proteins from Jurkat cells (endogenous proteins) or 293 cells (exogenous, myc-tagged proteins) to bead-immobilized purvalanol B. Protein binding was detected by immunoblot analysis using antibodies directed against endogenous proteins or the myc-tag of transgene-encoded fusion proteins, as indicated. Immobilized, Me-purvalanol B and PEG-spacer alone served as controls, as indicated. Very weak or no significant protein expression was detected for AKT1, CLK1, PKWA, and SNRK.

that of Y2H [35] (low-micromolar affinity interaction). For instance, purvalanol B-MFC detected CDK1, CDK4 and CK1, while roscovitine MFC detected CDK2. Purvalanol B and roscovitine inhibit these enzymes in vitro with IC_{50} values in the higher nanomolar to low micromolar range. Thus, the sensitivity of Y3H ought to be adequate for detection of many molecular interactions of pharmacological interest. Y3H signal intensity may, however, not adequately reflect differences in relative affinity of a small molecule for different targets, given that factors other than ligand-protein affinity may affect readout, such as protein expression, folding, and nuclear translocation, or positioning of the PEG-spacer/linker on the test compound. Thus, Y3H analysis appears generally most suitable for a "yes or no" detection of an interaction, with a presumed limit for detection of low-micromolar affinity type of interactions. Thus, although the kinase inhibitor screens performed in this study revealed a number of interesting novel candidate targets, the pharmacological relevance of these interactions remains to be determined. Ranking such interactions by affinity criteria would provide an important next step and further facilitate the prioritization of targets for functional validation. For instance, the question of whether inhibition of certain CK1 subtypes by the purine analogs purvalanol B and roscovitine plays an important role in the antitumor activity of these compounds (and potentially other purine analogs) warrants further investigation, especially

	Name	Group	Y3H Array Analysis	Secondary Validation Assays				Y3H targets reconfirmed in at least one secondary assay	Name
				Affinity Chromatography			Enzyme Assay		
			Purvalanol B-MFC	MSIEndo	WB/Endo	WB/Exo	% Inhibition at 1μM Compound		
Targets identified in cDNA screens with Purvalanol B-MFC	CDK2	CMGC	3	+	+	+	99		CDK2
	(CSNK1D)	CK	3	+	+	+	nd		(CSNK1D)
	(CSNK1E)	CK	3	+	nd	nd	nd		(CSNK1E)
	(CSNK1G)	CK	3	-	nd	+	nd		(CSNK1G)
	CLK1	CMGC	2	-	nd	-	nd		CLK1
	CLK2	CMGC	3	-	nd	nd	nd		CLK2
	CLK3	CMGC	3	-	nd	+	nd		CLK3
	PAK4	CMGC	3	+	nd	nd	nd		PAK4
	PCTK1	CMGC	3	-	nd	+	nd		PCTK1
	PCTK2	CMGC	1[H]	+	nd	nd	nd		PCTK2
	RSK-3	AGC	3	+	nd	nd	61		RSK-3
	EPHB2	TK	3[H]	-	nd	+	nd		EPHB2
	EPHB4	TK	3	-	-	-	nd		EPHB4
	FLT4	TK	3[H]	-	nd	+	nd		FLT4
	TRK-B	TK	3	-	nd	nd	nd		TRK-B
	FYN	TK	3	+	nd	nd	98		FYN
	YES	TK	3	+	nd	nd	98		YES
Additional arrayed kinases	CDK1	CMGC	2[H]	+	+	nd	96		CDK1
	CDK5	CK	3[H]	+	+	nd	99		CDK5
	CDK7	CMGC	-	-	nd	+	99		CDK7
	ERK1	CMGC	-	+	+	+	28		ERK1
	ERK2	CMGC	-	+	+	+	72		ERK2
	CCRK	CMGC	2[H]	+	nd	nd	nd		CCRK
	CDK3	CMGC	2[H]	+	nd	nd	96		CDK3
	CDK4	CMGC	1 [H]	-	nd	-	nd		CDK4
	CDK9	CMGC	2[H]	+	+	-	nd		CDK9
	CLK4	CMGC	3	-	nd	+	nd		CLK4
	GSK3A	CMGC	3	-	+	nd	nd		GSK3A
	PCTK3	CMGC	2	+	nd	nd	nd		PCTK3
	PKWA	CMGC	1	-	nd	-	nd		PKWA
	CSNK2A2	CK	(1[H])	+	nd	nd	nd		CSNK2A2
	AKT1	AGC	1[H]	-	-	-	nd		AKT1
	MSK1	AGC	3[H]	-	+	-	nd		MSK1
	PRKCG	AGC	3[H]	-	nd	-	nd		PRKCG
	CAMK2A	CAMK	2[H]	+	nd	nd	1		CAMK2A
	SNRK	CAMK	2[H]	-	nd	-	nd		SNRK
	LCK	TK	3	+	nd	nd	57		LCK
	SUD	other	2[H]	-	nd	-	nd		SUD

Figure 7. Summary of Biochemical Analysis of Purvalanol B-Protein Interactions

Binding of proteins to immobilized purvalanol B but not Me-purvalanol B was evaluated by immunoblotting (as shown in Figure 6) or LC-MS (for endogenous Jurkat proteins). Enzyme assays were performed with purified enzymes, and percentage inhibition of kinase activity observed with 1 μM purvalanol B is shown (data provided by Upstate Inc.).

when considering that R-roscovitine (also known as CYC202) has entered phase I/II human oncology clinical trials as a CDK2 inhibitor [23, 24].

Scanning of focused gene/protein families (e.g., kinases) or selected ORF collections may be pursued using yeast cell array screening, as exemplified in this study by the kinase array analysis. The array screening approaches described in this study made use of 96-well array formats, and currently available robotics can support the analysis of hundreds to thousands of interaction events in a period of a few days. These screening formats have therefore the potential to enable rapid comparative screening of small molecules and the characterization of interactions of small molecules with targets for which standard or routine enzyme or biochemical assays are not readily available. Scanning of the proteome may be approached using cDNA-library screening. This screening format represents a more random approach to target discovery that may lead to valuable insights into the molecular basis of drug action. The Y3H system described herein may conceivably also be used to find novel ligands for targets. For instance, focused small molecule hybrid ligand libraries could possibly be used to screen for target binding properties by parallel array profiling of a selected group of candidate targets. Alternatively, a specific hybrid ligand-target assay could arguably be used to screen for compounds that would compete for binding to that target, although this type of assay would be more suitable in the context of mammalian cells, given the natural permeability barrier displayed by yeast cells to (nonhybrid) small molecules. A recent, elegant study reported the use of a Y3H-based assay for the identification of proteins with chemical bond-cleaving properties [39], providing an example of application to enzyme catalysis. The present study indicates that Y3H can be applied to the discovery of novel candidate drug targets, providing an alternative or complement to current approaches in small molecule mechanism of action studies.

Significance

The identification of protein targets of organic small molecules is a critical step toward an understanding of the molecular basis of drug efficacy and side effects. Unfortunately, the identification of such targets remains a current major bottleneck both for drug discovery and chemical genetic studies. We have developed a generic Y3H-based compound-protein binding assay and screening approaches that enable the identification of targets of small molecules kinase inhibitors, an important class of pharmaceutical agents. The systematic screening approaches described in this study should be applicable to mechanism of action studies

using other types of organic small molecules, and thus of broader relevance to drug discovery.

Experimental Procedures

MFC Synthesis

Details of the MFC synthesis (e.g., purvalanol B-MFC, GPC286076) are provided in the Supplemental Data (appendix 3).

Bacterial and Yeast Strains

E. coli strain DH10B (Life Technologies, UK) was used for all bacterial work in this study. The *Saccharomyces cerevisiae* strains used in this study were either L40 (Invitrogen, NL; *Mat*-α, *his3*-Δ*200, trp1-901, leu2-3,112*, *ade2*, *LYS2*::(4lexAopHIS3), *URA3*::(8lexAopLacZ), GAL4) or the derivative strain 6A (GPC Biotech; *MAT*a, *his3*-Δ*200, trp1-901, leu2-3,112, LYS2*::(4lexAopHIS3), *ade2*::(6lexAopURA3), ura3::(8lexAopLacZ), GAL4, gal80, can1, cyh2). Both strains enable the use of a LexA BD-based protein interaction system using the HIS3 and/or LacZ reporters and the use of commercial cDNA libraries fused to the GAL4p activation domain. Yeast cells were grown in synthetic dextrose (SD) media with appropriate auxotrophic supplements [36]. Standard genetic methods were followed.

Plasmids

The *E. coli* dihydrofolate reductase-encoding gene was amplified using the following primers: DHFR-1 (5′-GGGGGTCGACATGATCA GTCTGATTGCGGCGTTAGCG-3′) and DHFR-2 (5′-GGGGGCGGC CGCTTACCGCCGCTCCAGAATCTCAAAG-3′), using a genomic bacterial library (Clonetech, Palo Alto, CA) as a template. The PCR product was digested with SalI and NotI and subcloned into pBYK (GPC-Biotech), resulting in the vector pBYK-DHFR encoding a LexA (DBD)-DHFR fusion protein. pBYK is a variation of pBTM117c [40], in which a kanamycin-resistance gene replaces the β-lactamase gene.

cDNA Libraries

Strain 6A + pBYK-DHFR was transformed using the lithium acetate method [37] with any of the following pACT2-cDNA libraries purchased from Clontech, Palo Alto, CA.: human brain, liver, heart, placenta, and testis. Alternatively, oligo-dT primed, directionally cloned cDNA libraries (derived from human testis, HeLa and B-cell lymphoma line K562 mRNA) were generated via SalI-NotI adaptor-based cloning using a Superscript cDNA library construction kit following manufacturer's instructions (Invitrogen). The cDNAs were subcloned into the SalI-NotI linearazied yeast shuttle vector pUACH. pUACH is based on the plasmid pGAD425 [41] with the addition of the CYH2 expression cassette which confers sensitivity to cycloheximide [42]. All libraries encoded polypeptides fused to the carboxyl terminus of the GAL4 activation domain. The total number of independent yeast colonies reflected at least 1× the complexity of independent clones documented for the *E. coli* library, as specified by the manufacturer. The yeast libraries were harvested, quality control tested, and stored in small aliquots at −70°C.

cDNA Library Screening

MFC (final concentration 4–8 μM) was spread evenly onto screening Q-trays (Genetix, UK) with SD-agar lacking leu, trp, and his. A pretransformed yeast library was applied onto the plates with a density of approximately 5×10^7–1×10^8 cells per tray. For a complex mammalian cDNA library screen, five Q-trays were screened. Even distribution of compound and yeast cells on the medium was ensured by distribution of the liquid with sterile 5 mm glass beads. Resulting colonies were transferred into 96-well flat bottom MTP's (Nunc, FRG) containing SD medium lacking leu and trp and containing 1 M betaine and incubated at 30°C for 3 days. For array specificity testing, a 20 μl aliquot of yeast cells grown in a 96-well microtiter plate was spotted twice onto screening Q-trays using a robotic liquid handling system (MultiPROBE II, Packard Bioscience). One microliter of compound of interest (5 mM or 0.5 mM stock solutions) was spotted in the center of the cell spot. The trays were incubated for 2 days at 30°C, and compound-dependent growth was determined.

Array Screening

Plasmids encoding targets of interest were transformed into the strain L40 or strain 6A containing the pBYK-DHFR plasmid. Cotransformants were selected, the culture expanded and subsequently distributed in a 96-well MTP. A high and a low amount of MFC was applied (1 μl of a 5 mM solution or 1 μl of a 0.5 mM solution) onto several omni trays (Nunc, FRG) containing selective media. Onto each spot, a small aliquot of yeast cells (7 μl) was added. Growth of yeast was determined after an incubation of 3 days at 30°C. A genetic counterselection method was used to test the dependence of the reporter activation on the expression of both fusion proteins. The plasmid pBYK encodes for the dominant-negative selection marker CYH2, enabling active plasmid loss on media containing cyclohexamide. To test for DHFR-independent activation, all arrayed yeast colonies were tested for reporter activation in the presence of MFC on plates lacking leucine and histidine and containing 1 μg/ml cyclohexamide [40]. To test for AD-fusion protein independent activation, a strain expresssing only the pBYK-DHFR fusion protein was tested with MFC on media lacking tryptophane and histidine.

Plasmid Isolation

Plasmids were isolated from yeast cells using the QIAprep96 Turbo Miniprep Kit (Qiagen, NL). Cell material was directly taken from selective agar trays, resuspended in buffer 1 supplemented with zymolyase at a final concentration of ~0.4 mg/ml, and incubated at 37°C for 60 min. After this preincubation, the manufacturer's protocol was followed. One tenth of the eluted DNA was transformed into electro-competent *E. coli* cells using standard procedures. Ampicillin-resistant colonies were selected, amplified, the plasmids were isolated, and cDNA inserts were sequenced and identified using standard databases and blast algorithms.

Competition Experiments

The invariable component of all MFCs, the "anchor," is MTX. This shared feature was used to set up competition experiments in yeast cells in which competition for binding of a test MFC with a "reference" MFC to DHFR was determined. IP-1 was used as a reference MFC. A specific protein domain of human BAP28 (Q9H583), which was found to bind specifically to IP-1 in the Y3H assay, was used as an AD-fusion protein. Interaction induced a strong activation of the HIS3 reporter gene. Competitive binding to DHFR of a test MFC is reflected in a reduction of IP-1-MFC-induced reporter activation. A "growth" dose-response experiment was performed for each test MFC using serial dilution of the test compounds and a fixed amount of IP-1-MFC. For each data point, 2×10^5 yeast cells expressing the DBD-DHFR and the AD-BAP28 fusion proteins were incubated in standard liquid culture medium lacking histidine in a total volume of 150 μl, to which were added 1 μl 0.5 mM IP-1-MFC and 1 μl 0.4 mM MTX. To test for growth competition, either 1 μl of DMSO (negative control, 100% growth) or 1 μl of test MFC were added. Yeast cell growth was monitored and quantified by measuring the optical density of the culture at day three at a wavelength of 600 nm. A different reference MFC was used for IP-1 comparative analysis.

Enzymatic Assays

The appropriate kinases and cyclins were expressed in Sf9 insect cells. CDK/cyclin complexes were purified and assayed as described previously [28].

Preparation of Cell Extracts

cDNAs were cloned into pCMV-Tag3 vectors (Stratagene). Jurkat cells or 293 cells were propagated following standard protocols. 293 cells were transfected with pCMV-Tag3 vectors encoding myc-tagged proteins using Effectene Transfection Reagent (QIAGEN, 301425). Cells were propagated for 40 hr under normal growth conditions. Whole-cell extracts were prepared following a protocol adapted from Romero et al. [38] and either stored at −80°C or used directly for pull-down assays. Yeast extracts were generated by lysis of the cells with NaOH and 50% trichloroacetic acid. The proteins were precipitated with ice-cold acetone and resuspended in sample buffer.

Pull-Down

Modified purvalanol B and methylated purvalanol B (5 PEG-amine spacer) were coupled to ReactiGel agarose beads (Pierce, No 20259) as previously described [20]. The final concentration of compound on the resin ranged between 10 and 50 μmol/ml. Pull-downs were essentially performed as previously described [20]. Ten microliters of packed gel and 90 μl cell extract were used for immunoblotting experiments or 40 μl gel and 360 μl cell extract for LC-MS experiments.

LC-MS Analysis

Tryptic digestion was performed with sequencing grade trypsin (Roche, 1 418 025) following the manufacturer's instructions, and the resulting peptide mix was concentrated prior to LC-MS analysis. Peptides were separated on a PepMap C18 column on a nano-LC apparatus (Ultimate, Dionex) and submitted to MS/MS on an ESI-ion trap MS (Esquire 3000 plus, Bruker Daltonics). Proteins were identified using the Mascot database search engine (Matrix Science).

Immunoblot Analysis

Proteins bound to the compound resin were recovered with sample buffer (Roth, K929.1) heated at 95°C for 5 min, separated on SDS-PAGE, and submitted to immunoblotting analysis. Detection was performed using ECL reagent (Amersham, RPN2106), as suggested by the manufacturer. Antibodies used in this study are listed in the Supplemental Data (appendix 2).

Supplemental Data

Supplemental Data including experimental procedures for the synthesis of purvalanol B MFC (GPC286076), full name/ENSMBLE IDs for all kinase targets, and a description of all antibodies used in this study is available at http://www.chembiol.com/cgi/content/full/11/2/211/DC1.

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References

1. Licitra, E.J., and Liu, J.O. (1996). A three-hybrid system for detecting small ligand-protein receptor interactions. Proc. Natl. Acad. Sci. USA *93*, 12817–12821.
2. Tanaka, H., Ohshima, N., and Hidaka, H. (1999). Isolation of cDNAs encoding cellular drug-binding proteins using a novel expression cloning procedure: drug-western. Mol. Pharmacol. *55*, 356–363.
3. Sche, P.P., McKenzie, K.M., White, J.D., and Austin, D.J. (1999). Display cloning: functional identification of natural product receptors using cDNA-phage display. Chem. Biol. *6*, 707–716.
4. McPherson, M., Yang, Y., Hammond, P.W., and Kreider, B.L. (2002). Drug receptor identification from multiple tissues using cellular-derived mRNA display libraries. Chem. Biol. *9*, 691–698.
5. Fields, S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature *340*, 245–246.
6. Chien, C.T., Bartel, P., Sternglanz, R., and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA *88*, 9578–9582.
7. Phizicky, E., Bastiaens, P.I.H., Zhu, H., Snyder, M., and Fields, S. (2003). Protein analysis on a proteomic scale. Nature *422*, 208–215.
8. Schreiber, S.L. (1998). Chemical genetics resulting from a passion for synthetic organic chemistry. Bioorg. Med. Chem. *6*, 1127–1152.
9. Clackson, T. (2000). Regulated gene expression systems. Gene Ther. *7*, 120–125.
10. Pollock, R., and Clackson, T. (2002). Dimerizer-regulated gene expression. Curr. Opin. Biotechnol. *13*, 459–467.
11. Lin, H., Abida, W., Sauer, R., and Cornish, V.W. (2000). Dexamethasone-methotrexate: an efficient chemical induced of protein dimerization in vivo. J. Am. Chem. Soc. *122*, 4247–4248.
12. Abida, W.M., Crater, B.T., Althoff, E., Lin, H., and Cornish, V.W. (2002). Receptor-dependence of the transcription read-out in a small-molecule three-hybrid system. Chembiochem *3*, 887–895.
13. Baker, K., Sengupta, D., Salazar-Jimenez, G., and Cornish, V.W. (2003). An optimized dexamethasone-methotrexate yeast 3-hybrid system for high throughput screening of small molecule-protein interactions. Anal. Biochem. *315*, 134–137.
14. Henthorn, D.C., Jaxa-Chamiec, A.A., and Meldrum, E. (2002). A GAL4-based yeast three-hybrid system for the identification of small molecule-target protein interactions. Biochem. Pharmacol. *63*, 1619–1628.
15. Manning, G., Whyte, D.B., Martiniez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. Science *298*, 1912–1934.
16. Chang, Y.T., Gray, N.S., Rosania, G.R., Sutherlin, D.P., Kwon, S., Norman, T.C., Sarohia, R., Leost, M., Meijer, L., and Schultz, P.G. (1999). Synthesis and application of functionally diverse 2,6,9-trisubstituted purine library toward the development of functionally diverse CDK inhibitors. Chem. Biol. *6*, 361–375.
17. Knockaert, M., Greengard, P., and Meijer, L. (2002). Pharmacological inhibitors of cyclin-dependent kinases. Trends Pharmacol. Sci. *23*, 417–425.
18. Davies, T.G., Bentley, J., Arris, C.E., Boyle, F.T., Curtin, N.J., Endicott, J.A., Gibson, A.E., Golding, B.T., Griffin, R., Hardcastle, I.R., et al. (2002). Structure-based design of a potent purine-based cyclin-dependent kinase inhibitor. Nat. Struct. Biol. *9*, 745–749.
19. Gray, N.S., Wodicka, L., Thunnissen, A.M., Norman, T.C., Kwon, S., Espinoza, F.H., Morgan, D.O., Barnes, G., LeClerc, S., Meijer, L., et al. (1998). Exploiting chemical libraries, structure, and genomics in the search for new kinase inhibitors. Science *281*, 533–538.
20. Knockaert, M., Gray, N., Damiens, E., Chang, Y.-T., Grellier, P., Grant, K., Fergusson, D., Mottram, J., Soete, M., Dubremetz, J.-F., et al. (2000). Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immmobilised inhibitors. Chem. Biol. *7*, 411–422.
21. Knockaert, M., Lenormand, P., Gray, N., Schultz, P., Pouyssegur, J., and Meijer, L. (2002). p42/p44 MAPKs are intracellular targets of the CDK inhibitor purvalanol. Oncogene *21*, 6413–6424.
22. De Azevedo, W.F., Leclerc, S., Meijer, L., Havlicek, L., Strand, M., and Kin, S.-H. (1997). Inhibition of cyclin-dependent kinases by purine analogs. Eur. J. Biochem. *243*, 518–526.
23. Meijer, L., and Raymond, E. (2003). Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. Acc. Chem. Res. *36*, 417–425.
24. Fisher, P.M., and Gianella-Borradori, A. (2003). CDK inhibitors in clinical development for the treatment of cancer. Expert. Opin. Investig. Drugs *12*, 955–970.
25. Meijer, L., Borgne, A., Mulner, O., Chong, J.P., Blow, J.J., Inagaki, N., Inagaki, M., Delcros, J.G., and Moulinoux, J.P. (1997). Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur. J. Biochem. *243*, 527–536.
26. McClue, S.J., Blake, D., Clarke, R., Cowan, A., Cummings, L., Fisher, P.M., MacKenzie, M., Melville, J., Stewart, K., Wang, S., et al. (2002). Int. J. Cancer *102*, 463–468.
27. Nugiel, D.A., Etzkorn, A.M., Vidwans, A., Benfield, P.A., Boisclair, M., Burton, C.R., Cox, S., Czerniak, P.M., Doleniak, D., and Seitz, S.P. (2001). Indenpyrazoles as novel cyclin dependent kinase (CDK) inhibitors. J. Med. Chem. *44*, 1334–1336.
28. Nugiel, D.A., Vidwans, A., Etzkorn, A.M., Rossi, K.A., Benfield, P.A., Burton, C.R., Cox, S., Doleniak, D., and Seitz, S.P. (2002). Synthesis and evaluation of indenopyrazoles as cyclin-depen-

dent kinase inhibitors. 2. Probing the indeno ring substituent pattern. J. Med. Chem. *45*, 5224–5232.
29. Yue, E.W., Higley, A., Dimeo, S.V., Carini, D.J., Nugiel, D.A., Benware, C., Benfield, P.A., Burton, C.R., Cox, S., Grafstrom, R.H., et al. (2002). Synthesis and evaluation of indenopyrazoles as cyclin-dependent kinase inhibitors. 3. Structure activity relationships at C3[1]. J. Med. Chem. *24*, 5233–5248.
30. Gray, N.S., Detivaud, L., Dierig, C., and Meijer, L. (1999). ATP-site directed inhibitors of cuclin-dependent kinases. Curr. Med. Chem. *6*, 859–875.
31. Koide, K., Finkelstein, J.M., Ball, Z., and Verdine, G.L. (2001). A synthetic library of cell-permeable molecules. J. Am. Chem. Soc. *123*, 398–408.
32. Sadler, K.C. (2002). Attention, neurons, this CDK could save your life. Trends Cell Biol. *12*, 214.
33. Monaco, E.A., III, and Vallano, M.L. (2003). Cyclin-dependent kinase inhibitors: cancer killer to neuronal guardians. Curr. Med. Chem. *10*, 367–379.
34. Samuels, B.A., and Tsai, L.H. (2003). Cdk5 is a dynamo at the synapse. Nat. Cell Biol. *5*, 689–690.
35. Estojak, J., Brent, R., and Golemis, E.A. (1995). Correlation of two-hybrid affinity data with in vitro measurements. Mol. Cell. Biol. *15*, 5820–5829.
36. Kaiser, C., Michaelis, S., and Mitchell, A. (1994). Methods in Yeast Genetics. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
37. Gietz, R.D., and Woods, R.A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. *350*, 87–96.
38. Romero, F., Dargemont, C., Pozo, F., Reeves, W.H., Camonis, J., Gisselbrecht, S., and Fischer, S. (1996). p95vav associates with the nuclear protein Ku-70. Mol. Cell. Biol. *16*, 37–44.
39. Baker, K., Bleczinski, C., Lin, H., Salazar-Jimenez, G., Sengupta, D., Krane, S., and Cornish, V.W. (2002). Chemical complementation: a reaction-independent genetic assay for enzyme catalysis. Proc. Natl. Acad. Sci. USA *99*, 16537–16542.
40. Wanker, E.E., Rovira, C., Scherzinger, E., Hasenbank, R., Wälter, S., Tait, D., Colicelli, J., and And Lehrach, H. (1997). HIP-I: A huntingtin interacting protein isolated by the yeast-two hybrid system. Hum. Mol. Genet. *6*, 487–495.
41. Han, L., and Colicelli, J. (1995). A human protein selected for interference with Ras function interacts directly with Ras and competes with Raf1. Mol. Cell. Biol. *15*, 1318–1323.
42. Kaeufer, N.F., Fried, H.M., Schwindiger, W.F., Jasin, M., and Warener, J.R. (1983). Cycloheximide resistance in yeast: the gene and its protein. Nucleic Acids Res. *11*, 3123–3135.