JX401, a p38α inhibitor, containing a 4-benzylpiperidine motif, identified via a novel screening system in yeast

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Running title: Isolation of p38α inhibitor via in vivo screening in yeast

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Abstract

In vivo screening of compounds for potential pharmacological activity is more advantageous than in vitro screening. In vivo screens eliminate the isolation of compounds that cannot cross biological membranes, are cytotoxic, or are not specific to the target. However, animal-based or even cell-based systems are usually expensive, time consuming and laborious. Here we describe the identification of inhibitors of the MAP kinase p38α via a high throughput screen using yeast cells. p38α is hyperactive in inflammatory diseases and various indications suggest that its inhibition would reverse inflammation. However, there are currently no p38α inhibitors in clinical use. As the human p38α imposes severe growth retardation when expressed in yeast, we screened a library of 40,000 randomly selected small molecules for compounds that would restore a normal growth rate. We identified two compounds. Both share a structural motif of 4-benzylpiperidine and both were shown to be efficient and selective p38α inhibitors in vitro. They were also active in mammalian cells, as manifested by their ability to reversibly inhibit myoblast differentiation. Thus, the yeast screen identified efficient and specific p38α inhibitors that are capable of crossing biological membranes, are not toxic and function in mammalian cells. The rapid and cost efficient HTS used here could be applied for isolation of inhibitors of various targets.
Introduction

The development of a novel drug from the basic research stages to the clinical application is a long, complex and expensive process. The first step in this expedition is the identification of a small molecular weight molecule (a "lead compound") with a distinct activity (usually inhibitory) on a selected target.

One approach to the development of lead compounds is a structure-based rationale drug design. This approach is applied in cases where the 3-dimensional structure and the mechanism of action of the target are known. However, the approach is limited in many aspects (Blundell, 1996; Blundell et al., 2002; Davies et al., 2002; Noble et al., 2004). Another approach is based on the screening of combinatorial chemistry libraries comprised of hundreds of thousands of small molecules (Broach and Thorner, 1996; Hogan, 1996). In the vast majority of cases, High-Throughput Screening (HTS) of these libraries is carried out in vitro and is designed to address one property (e.g., inhibitory activity) of the compound. There are, however, additional requirements from a "lead-compound": The compound should be able to cross biological membranes, should not be cytotoxic, should be highly specific to the target and should be potent in vivo. Many compounds identified via in vitro HTS as efficient inhibitors of the target are destined to fail to fulfill some of these requirements.

In contrast to in vitro screening, in vivo screening can detect compounds possessing most or all of the required properties. However, the main drawbacks of in vivo screens, in animals or in cell cultures, are the expense and time requirements. In this study we describe the development of a yeast-based in vivo screen, which is time, cost and labor efficient.

There has been a widespread recognition of the potential of cell-based assays in yeast (Broach and Thorner, 1996; Kirsch, 1993; Munder and Hinnen, 1999; Pausch, 1997; Silverman et al., 1998; Young et al., 2004) and several such assays were established. Some studies screened for inhibitors of endogenous yeast proteins, that are orthologs of mammalian targets (Bach et al., 2003; Lum et al., 2004). Other studies described the expression in yeast of mammalian proteins such as G protein-coupled receptors (GPCRs), Topoisomerase II, phosphatase 1B and a potassium channel (Montalibet and Kennedy, 2004; Pausch, 1997; van Hille and Hill, 1998; Zaks-Makhina et al., 2004; Zhang et al., 2005). The systematic expression of numerous mammalian proteins in yeast as a way for establishing HTS was also described (Tugendreich et al., 2004).
However, although the potential of yeast has been repeatedly noted, only a few studies described an actual application of the proposed screen for the isolation of novel lead compounds. Furthermore, some of these yeast screening systems are also too laborious to allow a simple large-scale HTS.

The in vivo yeast-based HTS that we describe could be readily applied for the screening of hundreds of thousands of molecules. We applied it for the isolation of p38α inhibitors. p38α is a member of the mitogen activated protein (MAP) kinase super-family that includes the p38, JNK, ERK and BMK sub-families (Bogoyevitch and Court, 2004). MAPKs function in signaling cascades that control complex cellular and multicellular programs such as embryogenesis, differentiation, proliferation and cell death (Kyriakis and Avruch, 2001; Lewis et al., 1998; Ono and Han, 2000). p38α was shown to be an essential gene in mice, required for the development of red blood cells and the placenta (Adams et al., 2000; Tamura et al., 2000). In humans p38α has been shown to be involved in various inflammatory diseases, such as rheumatoid arthritis and Crohn's disease, in congestive heart failure and in some cancers. Consequently a large number of studies strongly support the notion that p38α inhibitors could serve as anti-inflammatory drugs (Benhar et al., 2001; Bulavin and Fornace, 2004; Crawley et al., 1997; Engelberg, 2004; Esteva et al., 2004; Hommes et al., 2002; Kumar et al., 2003; Lee et al., 2000; New and Han, 1998; Olson and Hallahan, 2004; Redman et al., 2001). Although extensive efforts have been devoted to the development of drugs that inhibit p38α (Behr et al., 2003; Cuenda et al., 1995; English and Cobb, 2002; Goehring, 2002; Kumar et al., 1999; Redman et al., 2001; Schultz, 2003; Young et al., 1997), there are currently no p38α inhibitors in clinical use.

The p38 ortholog in yeast is a MAPK called High Osmolarity Glycerol-1 (Hog1) that is essential for growth under high osmotic pressure (Brewster et al., 1993; Gustin et al., 1998). Mutants in which Hog1 is hyperactive are lethal (Yaakov et al., 2003). Previous studies reported that expression of the murine p38α in a hog1Δ strain partially restored growth under osmotic stress while introduction of p38α into wild type strains imposed growth arrest (Han et al., 1994; Kumar et al., 1995). We introduced expression vectors carrying the human p38α to several yeast strains and observed a significant inhibition of the growth rate, but not an absolute arrest. We took advantage of this phenotype and developed a high-throughput screening system for identifying small molecules that restore normal growth rate to yeast cultures expressing p38α. We screened
a combinatorial library and identified two compounds that restore yeast growth. The compounds, termed JX401 and JX162, were found to share a common structural motif of 4-benzylpiperidine and to be efficient *bona fide* p38α inhibitors *in vitro*. The p38α inhibitory activity of 27 additional compounds containing 4-benzylpiperidine was also tested and revealed the basic structure-activity relationships of this family of compounds. Finally, we show that JX401 and some of the derivative compounds are capable of reversibly inhibiting p38α in mammalian cells, manifested by blocking differentiation of myoblasts. Thus, JX401, isolated via a yeast-based HTS, is one of a family of efficient p38α inhibitors that are active in mammalian cells.

**Materials & methods**

**Materials:** SB203580 and PD169316 were purchased from Calbiochem (La Jolla, California). The small chemical combinatorial library was purchased from Chemical Diversity (San Diego, California).

**Yeast expression plasmids:** For expression of the human p38α protein in yeast the p38α cDNA (coding sequence only) was cloned into the pAES426 plasmid. This expression vector is a derivative of the plasmid pADNS (Colicelli et al., 1989) and contains the constitutive ADH1 promoter, the CYC1 terminator, the URA3 gene and the 2µ element. *PTC1* and *ptc1*D58N were cloned in the Yeplac181 plasmid. This vector contains the LEU2 gene and the 2µ element. Yeplac containing *PTC1* or *ptc1*D58N were obtained from Dr. Irene Ota (Dept. of Chemistry and Biochemistry, University of Colorado, Boulder, CO).

**Yeast strains and growth conditions:** Strains used were SP1 (Toda et al., 1985), YPH102 (Sikorski and Hieter, 1989) and JBY13 (*hog1Δ*; Brewster et al., 1993). In all experiments the cultures were grown in SD media (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose) lacking uracil (for strains harboring p38α expressing plasmid), or lacking both uracil and leucine (for strains harboring both a p38α expressing plasmid and a phosphatase expressing plasmid). Screening of compound libraries was performed with JBY13 strain into which we introduced the pAES426-p38α plasmid.

**Westerns analyses:** Exposure of yeast culture to osmotic stress, preparation of lysates and analysis of protein expression in western blots were carried out exactly as described by Bell et al. (Bell et al., 2001). Anti p38α and anti phospho p38α antibodies were purchased from NEB.

**Kinase Assays:** Reactions were carried out in 96-well plates with conical bottoms in a final volume of 50 µl/well. 0.2 µg of purified recombinant p38α or p38γ proteins were
used. Activation of p38s by MKK6 was performed as previously described (Diskin et al., 2004). Purified active MKK6, phosphorylated by MEKK1 was purchased from Upstate (USA; cat #14-303). The kinase assays were initialized by the addition of 45 µl of reaction mixture to 5 µl of p38 enzyme. Final reaction conditions were 25 mM Hepes, pH 7.5, 20 mM MgCl₂, 20 mM 2-glycerolphosphate, 5 mM p-nitrophenyl phosphate, 0.1 mM Na₃VO₄, 1 mM dithiothreitol, 64 µg (35.1 µM) of GST-rat ATF2, 250 µM ATP, and 10µCi of [γ-³²P]ATP. The kinase reactions proceeded for 10 or 20 min (to test MKK6-treated or untreated kinases, respectively) and were terminated by the addition of 50 µl of 0.5 M EDTA, pH=8, (250 mM final) and placement on ice. Following reaction termination, aliquots of 85 µl from each well were spotted onto 3 x 3-cm Whatman 3MM paper squares and briefly air-dried. Each square was rinsed three times in 10% trichloroacetic acid and 3% sodium pyrophosphate (10 ml/square) for 1.5 h (each rinse) with gentle agitation, and then for 16 hours without agitation. The squares were then rinsed twice in 100% ethanol (4 ml/square), 20 min. each rinse, and air-dried. The radioactivity of each square was determined using a scintillation counter running a ³²P Cherenkov program. Experimental points were performed in triplicate and standard errors were about 15%.

Myoblast culture: L8 myoblasts (Yaffe and Saxel, 1977) were grown in growth medium (GM; 15% BCS in DMEM). For induction of differentiation, the medium was changed to differentiation medium (DM; no serum, 10 µg/ml insulin and transferrin in DMEM) when the culture became confluent. JX401 and its various derivatives were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM. Aliquots from these stocks were added directly to DM to obtain a final concentration of 10 µM. Control cells were incubated with the same volumes of DMSO without the inhibitors. The growth medium was replaced and fresh inhibitors were added every 24 hours. Preparation of lysates of L8 cells was performed as described (Gredinger et al., 1998). For monitoring myogenin induction 40 µg of lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti Myogenin monoclonal antibodies (F5D; Santa Cruz) and anti α tubulin (DM1A; Sigma ) at 1:1000 dilution.

Results

Expressing human p38α in yeast leads to growth inhibition

To test the effect of human p38α on yeast cells, we expressed the p38αwt in the wild type strains SP1 and YPH102 and in the hog1Δ strain JBY13. Upon introduction of
p38α expression vectors into these strains, a large number of colonies appeared, similar to the number that appeared in control cultures, which were introduced with empty expression vectors. However, the colonies harboring the p38α expressing-vectors grew more slowly and were significantly smaller than colonies formed by cells harboring an empty vector. We tested the growth rates of yeast expressing p38α in liquid cultures, and found their growth to be greatly retarded compared to control cultures (Figure 1).

To determine the reason for the p38α-mediated growth inhibition we examined the expression levels and phosphorylation status of p38α in yeast cells. Specific anti phospho-p38 antibodies revealed that the human p38α molecules were spontaneously phosphorylated in yeast (Fig. 1 inset). In the same cultures the yeast Hog1 molecules were phosphorylated only in cells exposed to osmotic stress (Fig. 1 inset). Thus, when p38α is expressed in yeast it is phosphorylated even in cultures not exposed to any stress, rendering this enzyme constitutively active. To further verify that p38α catalytic activity is responsible for the growth retardation we expressed in yeast a kinase dead version of p38α (p38K53A). Yeast cells expressing this p38α molecule grew at even better growth rate then wild type yeast cells (Fig. 1). Strikingly, we found that the human p38α is spontaneously phosphorylated even in pbs2Δ cells (PBS2 encodes the relevant MEK). This MEK-independent p38α phosphorylation is most probably a result of autophosphorylation activity acquired by the human p38α in yeast cells (Levin, V. and D. E., unpublished results). The unregulated hyperactivity of p38α explains the growth retardation observed in yeast cells expressing this enzyme, in view of the growth arrest demonstrated when a constitutively active Hog1 was introduced into yeast (Yaakov et al., 2003). The fact that growth retardation and spontaneous phosphorylation of p38α occurs in all strains tested show that the phenomenon is universal and not peculiar to a particular genetic background.

**Overexpressing yeast MAPK phosphatase rescues the growth retardation phenotype of p38α-expressing yeast**

As the growth retardation of cells expressing p38α was correlated with the phosphorylated, active state of p38α, we wondered whether over-expression of the yeast phosphatase Ptc1, known to de-phosphorylate Hog1 (Warmka et al., 2001), would restore normal growth rates. We generated a yeast strain expressing both p38α and Ptc1. This strain manifested a growth rate similar to that of the parental wild type (Fig. 2A). On the
other hand, yeast cells expressing p38α and a mutated Ptc1 that lacks phosphatase activity (Ptc1\textsuperscript{D58N}) grew similarly to yeast cells expressing p38α alone (Fig. 2A). This result verifies that growth can be rescued by phosphatase activity. As Ptc1 dephosphorylates Hog1 and not its MEK Pbs2 (Warmka et al., 2001), it probably dephosphorylates Hog1's ortholog (p38α) as well. Also, we found that p38α activity in yeast is independent of upstream MKKs (Levin, V. and D. E., unpublished results), strongly suggesting that the target of Ptc1 is p38α or its downstream substrates.

**Known inhibitors of p38α have minute effects on growth of p38α-expressing yeast**

The effect of the Ptc1 phosphatase on growth suggested to us that the growth retardation phenotype imposed by the p38α is reversible, and could therefore serve as a basis for screening for p38α suppressors. These suppressors could be proteins (e. g., phosphatases or scaffold proteins) or small molecules - pharmacological inhibitors - that are capable of entering the cell. As described in the ‘Introduction’, such pharmacological inhibitors may serve as potential anti-inflammatory drugs (Kumar et al., 2003; Lee et al., 2000; Redman et al., 2001). To validate this idea, we tested whether known p38α inhibitors could rescue the growth inhibition phenotype. We measured the growth rates of yeast expressing p38α in media supplemented with the p38α inhibitors SB203580 and PD169316. The presence of either PD169316 or SB203580 rescued cell growth very partially (improved cell growth by 5 – 10%; Fig. 2B). This small growth improvement was highly reproducible (PD169316 served in fact as a positive control in our screen so that its small positive effect on growth was reproduced hundreds of times). The presence of both compounds together in the growth medium did not improve the rescue above levels achieved by each compound alone (data not shown). It should be noted that the inhibitors rescued growth only partially whereas the phosphatase allowed growth at wild type rates (compare Fig. 2A to 2B). Furthermore, although both PD169316 and SB203580 rescued growth we could not measure a dose-response for neither of them: We found that 1µM rescued growth almost as efficiently as 200µM. It is plausible that the compounds are toxic to yeast cells at high concentrations, and that the marginal positive effect on cell growth reflects a balance between inhibition of p38α, that improves growth, and a toxic effect that suppresses growth. Indeed, high levels of SB203580 or PD169316 reduced growth rates of wild type yeast cultures (Fig. 2C).

**Using the growth-inhibition phenotype to screen for potential p38α inhibitors**
The partial growth rescue by p38α inhibitors and the complete rescue by Ptc1 suggested that the growth inhibition phenotype could be utilized to identify p38α blockers. The results with SB203580 suggest that toxic compounds (at least to yeast cells) will not be isolated. Thus, the basic idea is that compounds from combinatorial libraries could be systematically added to the yeast growth medium, and those that rescue growth could be easily identified by following the optical density of the culture. To allow the efficient screening of a large number of components we established a HTS in 96-well plates. To test the system we verified that the partial growth rescue of SB203580 and PD169316 and the complete growth rescue of Ptc1 are clearly observed in cultures grown in 96-well plates. To test the screening idea we plated yeast cells expressing p38α in wells of 96-well plates and in addition supplemented each well with 10µM of a compound taken from a diverse small molecule library containing 40,000 compounds. Plates were incubated at 30°C and after ~45 hours the optical density (at 600nm) of the culture in each well was measured (see Fig. 3A for an example of the O.D.600 readings of an assay plate and for a detailed description of the screen and the controls used). Of the 40,000 compounds screened, several hundreds manifested various degrees of growth rescue. Notably, growth rescue was considered as 20% improvement of growth or higher, i.e., compounds such as SB203580 and PD169316 would not be isolated. The positive compounds of the primary screen were tested again. One of the repeat plates is shown in Fig. 3B. This plate contains 2 compounds that improved the yeast growth significantly (200%). We named these compounds JX162 and JX401 and tested them in dose-response assays (Figs. 3C and 3D). JX162 had no effect at a concentration of 1µM and growth rescue was apparent only at 10µM (the same concentration used in the screen). Supplementing the medium with 100µM of JX162 led to toxicity and growth rate was below that of untreated cells expressing p38α (Fig. 3C). JX401 rescued growth at 1µM and was more effective as the concentration increased up to 100µM (Fig. 3D). Increasing the concentration to 200µM did not induce better growth, but did not have a negative effect either (data not shown). Also, JX401 had no effect on the growth of yeast cultures not expressing p38α (Fig. 3E) or expressing both p38α and Ptc1. Thus, unlike JX162 (Fig. 3C), and unlike PD169316 and SB203580 (Fig. 2C), JX401 showed a dose-response in rescuing yeast growth (Fig. 3D) and was not toxic when applied at high concentrations (Fig. 3E).

**JX162 and JX401 inhibit p38α in vitro**
JX162 and JX401 were tested for their ability to inhibit p38α activity in vitro. Purified recombinant p38α was activated by MKK6 and its kinase activity was assayed using GST-ATF2 as a substrate. When either JX162 or JX401 were added to the assay, they inhibited p38α most efficiently. JX401 was significantly more potent as a p38α inhibitor with an IC₅₀ of 32nM than JX162 that inhibited p38α with an IC₅₀ of 480nM. In the same experiment, the IC₅₀ of PD169316 was 15nM. In order to test the specificity of the inhibition of JX401 towards p38α we assessed its effect on the p38γ isoform. PD169316, which is a very specific inhibitor of p38α (Gallagher et al., 1997; Kummer et al., 1997), was used as a control. As shown in Fig. 4A JX401 did not inhibit p38γ activity even at a concentration of 10μM. PD169316, in contrast, manifested some inhibitory activity on p38γ. This demonstrates that PD169316 is less specific than JX401. To look into the mechanism of inhibition used by JX401 we tested whether it may compete with ATP or with the substrate GST-ATF2. In the presence of 1μM JX401 increasing the ATP concentrations up to 500 μM did not rescue p38α activity (Fig. 4B). p38α was also fully inhibited by JX401 (1 μM) when GST-ATF2 level were increased to 100 μM. It seems that JX401 is not a competitive inhibitor vs. either ATP or GST-ATF2. This finding is not surprising given that JX401 was isolated in vivo under conditions in which intracellular ATP concentrations are in the millimolar range. It is not clear yet if JX401 is a non-competitive or uncompetitive inhibitor.

**JX162 and JX401 have a similar structure and constitute a new family of inhibitors**

The structures of JX162 and JX401 are depicted in Figure 5. Both compounds, which were isolated independently out of 40,000 compounds, share a common motif of 4-benzylpiperidine. Only eight additional compounds containing 4-benzylpiperidine were present among the 40,000 compounds we screened. One of these was actually isolated as a compound with a low inhibitory activity in the yeast screen. Many more compounds (about 100) contain a modified 4-benzylpiperidines. Since none of these compounds was active, we concluded that the structure of 4-benzylpiperidine itself is essential for the inhibitory properties of JX162 and JX401. Finally, seven compounds out of the 40,000 compounds screened contained a phenyl ring with 2-methoxy and 4-thiomethyl substitutions (as in the phenyl ring of JX401). These compounds manifested no inhibitory activity. It seems therefore that the combination of a benzene linked to 4-benzylpiperidine is critical for p38α inhibition as each of these components alone is not active.
To check whether JX401 and JX162 had been previously identified as p38α inhibitors, we searched databases of chemicals published, patented or manufactured in combinatorial libraries. We found that JX162 was described in a patent by Goehring et al. from Scios Inc. as p38α inhibitor (Goehring, 2002). JX401 did not appear in any database, patent or scientific article. Since JX401 is a more potent inhibitor than JX162, is not toxic (whereas JX162 is; Figs. 3C & 3D) and was not previously identified, we characterized it further.

To reveal structural motifs, in addition to 4-benzylpiperidine, that are required for p38α inhibition, we obtained a battery of JX401 derivatives (some of those were described by Goehring et al. (Goehring, 2002). Twenty-seven of these compounds were tested in an in vitro kinase assay at a concentration of 1µM (see Table 1). Seven compounds did not inhibit p38α activity (shown as group C in Table 1). Ten compounds had a slight inhibitory activity (group B compounds in Table 1; 19 – 68% inhibition) and 10 compounds showed a very efficient inhibition of p38α in vitro (Group A in Table 1; 74% - 97.6% inhibition). The 27 compounds were further tested for their ability to rescue growth of yeast expressing p38α. Nine of the ten compounds of group A rescued growth to various degrees whereas none of the compounds of groups B and C rescued growth (Table 1).

In summary, there seems to be a correlation between a compound’s ability to rescue growth and its p38α inhibitory activity. Interestingly, the activity of JX401 is equal or even superior to that of the best compounds (Table 1). One compound, B2, manifests a higher inhibitory potency in vitro than JX401, but is less efficient in rescuing the yeast cultures. It is also toxic to mammalian cells (see below). Another compound, F7, is more efficient than JX401 in rescuing the yeast, but is a less efficient inhibitor in vitro.

**JX401 and its derivatives inhibit p38α activity in mammalian cells**

The obvious goal of the yeast screening system is to select for inhibitors that are directly relevant to mammalian systems (i.e., efficient, membrane permeable and non-toxic). To assess the activity of JX401 and its derivatives on mammalian cells, we examined their effect on differentiation of myoblasts to myotubes in culture. The differentiation of myoblasts in culture was shown to be absolutely dependent on p38α activity (Cuenda and Cohen, 1999; Wu et al., 2000; Zetser et al., 1999) and the p38 inhibitor SB203580 has been shown to inhibit myogenic differentiation (Cuenda and
Cohen, 1999; Puri et al., 2000; Wu et al., 2000; Zetser et al., 1999). We treated L8 myoblasts by supplementing the differentiation medium with 10µM of inhibitors of the JX401 family. Myoblasts were allowed to differentiate for 60 hours and differentiation was assessed by inspecting the appearance of myotubes under the microscope (data not shown) and by monitoring the expression levels of Myogenin (Fig. 6). We found that JX401 and 14 of its related compounds inhibited the differentiation, with efficiencies similar to those of SB203580 and PD169316 (Fig. 6A). Some of these compounds were toxic to myoblasts as judged by a large number of dying cells, floating in the plates (Table 1). JX401 and the 14 compounds also prevented the phosphorylation of Elk1, a substrate of p38 (not shown).

In order to check if the effect of the inhibitors is reversible, myoblasts were grown in the presence of the compounds for 36 hours, followed by their removal and an additional 36 hours growth in their absence. The effect of several compounds (JX401, D2, D4, G4, and G5) was reversible, as the cells recovered completely and differentiated, following the removal of these drugs (Fig. 6B). Note that compounds G5 and D4 belong to group A (Table 1) and compounds G4 and D2 belong to group B. None of the group C compounds was found to be an efficient non-toxic p38α inhibitor in myoblasts. Compound B2, one of the most efficient inhibitors in the in vitro and the yeast assays was toxic to myoblasts. Another compound from group A, F8, efficiently inhibited myoblast differentiation, but was not reversible (Fig. 6B). Interestingly, compound F8 did not rescue yeast growth. Overall, we found that JX401, which was discovered in the yeast screen, inhibited p38α in myoblasts. Some of JX401 closely related compounds were also active in the myoblast system. In general, there is a good correlation between the effect of a compound on yeast cultures and its effect on myoblasts, but there are some exceptions, such as compound B2 that was not toxic to yeast cells but was cytotoxic to myoblasts.

Discussion

The potential use of engineered yeast systems in screening for “hit compounds” for drug development has been proposed continuously in the last 20 years (Broach and Thorner, 1996; Kirsch, 1993; Munder and Hinnen, 1999; Pausch, 1997; Silverman et al., 1998; Young et al., 2004). However, just a handful reports described the actual isolation of novel inhibitors of human enzymes using yeast-based systems (Bach et al., 2003; Montalibet and Kennedy, 2004; Zaks-Makhina et al., 2004). Further, in some of these
cases the screening system was too laborious to allow a simple large scale HTS (Bach et al., 2003). Here we show the feasibility of using a yeast-based in vivo screening assay for the identification of kinase inhibitors. JX401, the p38α inhibitor isolated by our assay, was found to be highly specific, as it does not inhibit p38γ, and to be active and non-toxic in mammalian cells. Comparison of JX401 to 27 similar molecules, some described previously to be efficient p38α inhibitors (Goehring, 2002), suggests that JX401 is one of the most potent compounds of the family (Table 1). As we have shown in this paper, our yeast-based screen revealed a compound that possesses many of the hoped-for properties of a lead compound, thus providing a proof of concept for the long-standing idea of using yeast for isolation of hit compounds. By screening just 40,000 compounds we found two “hits”. We propose that the screening for p38α inhibitors in yeast is far from being exhausted and should be continued with libraries composed of hundreds of thousands of compounds. Although having many advantages, the yeast screening system has some disadvantages. For example, the yeast cell wall might not be permeable to some of the compounds in the library and as a result some potential inhibitors may be missed in the yeast screen.

JX401 contains 4-benzyl-piperidine, a motif found also in the p38α inhibitors reported by Goehring et al. (Goehring, 2002). It seems that this motif is essential for p38α inhibition since compounds containing various modifications of the motif or substitutions at its various positions were not identified by the screen. Other elements of JX401 can be modified with different effects on activity. JX401 contains a methoxy and a methyl thio substituents in ortho and para positions on the phenyl methanone, bound to the 4-benzylpiperidine. G8 that contains two methoxy substituents on meta positions and F8 that possesses just a single ortho methoxy substituent are significantly less active than JX401. Compound F8 is also toxic to myoblasts. A methyl substituent instead of a methoxy (C8) reduces the activity of the compound, as does a phenyl group without a substitution (D6). Halogens can in some cases serve as contributing substitutions, but in other cases they reduce activity: G5, one of the best inhibitors, contains a chlorine atom at the ortho position. A fluorine atom at this position (D4) renders the compound less potent, and an iodine substituent (B8) renders the compound inactive. Also, a compound containing a chlorine and a methoxy (D2) is toxic to myoblasts. The position of the atom on the phenyl ring is also very important for the compound potency. It appears that a substituent in position 2 contributes to the activity while substituents in positions 3 and 4
are less helpful, as seen by comparing compounds G5 to D2, D4 to F6 and G7, and E3 to B5. Taking all these observations together it seems that a phenyl methanone is an important moiety of the 4-benzylpiperidine. Several substitutions on this phenyl group improve activity, including halogens and methyl ether at particular positions. The results clearly point to the importance of the thiomethyl group and predict that other thioalkyl substitutions at this and other positions on the phenyl ring may result in even more potent p38α inhibitors.

Further development and improvement of JX401 are certainly required in order to bring it to the pre-clinical and clinical stages. Yet, our yeast-based screen that identified JX401 destined it to possess important useful properties that facilitate these further developments. In fact, given the potency of JX401 in vitro, in the yeast assay and in myoblasts in culture, it would be of great interest to test JX401 itself in an animal model of a p38α-related disease.

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Legends for Figures

Fig. 1. The human p38α is constitutively phosphorylated in yeast cells and imposes severe growth retardation. p38α was expressed as a HA-tagged protein. Shown are growth curves of yeast cultures harboring an empty expression vector (diamonds), p38α-expressing vector (triangles) or kinase dead p38α (squares). Experiment was repeated 3 times in triplicates. Bars show standard errors. Inset) A Western blot analysis of yeast lysates prepared from cells exposed or not to osmotic shock (1M NaCl for 10 min.). The upper panel shows a blot probed with anti phospho-p38α antibodies (these antibodies cross react with phospho-Hog1). The lower panel shows a blot probed with anti-HA antibodies. NS - a non-specific band that appears both in yeast expressing or not expressing human p38α.

Fig. 2. Expression of the phosphatase Ptc1, or exposing cultures to p38α inhibitors rescue growth retardation of p38α-expressing yeast. A) Growth curves of yeast cultures harboring either an empty vector (diamonds), a p38α-expressing vector (squares), a p38α expressing vector and a Ptc1-expressing vector (triangles), or p38α expressing vector and a Ptc1_D58N-expressing vector (X shapes). B) Growth curves of yeast cultures harboring either an empty vector (diamonds), or a p38α expressing vector, grown in medium supplemented with either DMSO (squares), 10 µM SB203580 (asterisks), or 10 µM PD169316 (circles). Experiment was repeated 3 times in triplicates. Bars show standard errors. C) SB203580 is toxic to yeast cultures. Cultures of yeast cells harboring an empty vector were exposed to either DMSO (diamonds) or to SB203580 (10 µM; squares).

Fig. 3. The screening system and isolation of JX162 and JX401. A) An example of the results obtained from a single assay plate. The figure shows the optical density measurements (the Y-axis) at a wavelength of 600 nm of all wells of a 96-well assay plate. The X-axis shows the location of wells in the plate by row (letters) and column (number). Locations of only some wells in each row are marked, but results obtained from all wells are shown. From the far left, two wells contained p38α-expressing cultures in medium supplemented with DMSO only. The next two wells contained cells harboring an empty vector and medium supplemented with DMSO, the next two wells contained cells expressing both p38α and Ptc1 in medium supplemented with DMSO and next to them there is one well that contained p38α-expressing yeast cells in medium supplemented with
10 µM of PD169316. All other wells contained p38α-expressing yeast cells and a single compound from the combinatorial library at a concentration of 10 µM. As some of the compounds may absorb light at a wavelength of 600 nm, the OD$_{600}$ of each well was measured at time 0 (immediately after addition of cells; white bars). Plates were then incubated at 30°C and OD$_{600}$ was read again 35-45 hours later (black bars). In the particular plate shown none of the compounds tested improved the growth of p38α-expressing cultures except PD169316. B) A “repeat plate”. Following the screen of the entire library, all compounds that showed some ability to rescue p38α-expressing cells were re-tested. In the plate shown two of the compounds, 63 & 66, rescued p38α-expressing cells very significantly. The compounds were termed JX401 and JX162 respectively. C and D) Growth curves of yeast cultures harboring either an empty vector (asterisk) or a p38α-expressing vector grown in media supplemented with different concentrations of either JX162 (C) or JX401 (D). E) Growth curves of wild type yeast cells exposed to DMSO (squares) or to JX401 (10µM, diamonds).

Fig. 4. JX401 has no effect on p38γ catalysis and is not ATP competitive. A) Catalytic activity of recombinant dually phosphorylated p38γ was assessed in the presence of the indicated concentrations of JX401 or PD169316. As controls we measured the activity of non-phosphorylated p38γ (most right bar), of activated p38γ exposed to vehicle only (second from right) and of activated p38α treated with JX401 or PD169316 (three bars on the left). Results shown are averages of two experiments, each performed in triplicates. B) Catalytic activity of p38α was measured in the presence of increasing ATP concentrations in the absence (diamonds) or presence (squares) of JX401 (1 µM). Results are shown as averages and standard errors (vertical bars) of three experiments.

Fig. 5. Chemical structures of JX401 and JX162.

Fig. 6. JX401 and some of its derivatives reversibly inhibit the differentiation of myoblasts. Expression of myogenin, the early-induced activator of the myogenic program, was monitored by a Western blot analysis. A) Myoblasts cultures were incubated in differentiation media supplemented with DMSO only (-, the left lane), but in the presence of DMSO or in differentiation medium supplemented with 10 µM of the indicated inhibitors. Lysates were prepared 60 hours following initiation of treatment. B) The
inhibitory effect of JX401 and its derivatives is reversible. Cells were incubated for 36 hours in the presence of the indicated compounds in duplicate. The media were then replaced with media supplemented (in one plate of each duplicate) with the inhibitor (10 µM) or with a regular differentiation media (in the other plate). Cultures were incubated for another 36 hours, lysed, and the levels of myogenin and tubulin were monitored by Western blots.
Table 1: Various properties of JX401 and its derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC50 (nM)</th>
<th>Effects on growth</th>
<th>% of myoblast activity</th>
<th>Toxicity to growth rate of yeast in vitro</th>
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<tr>
<td>JX-162</td>
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<td>1.49</td>
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<td>481.60</td>
<td>N.D.†</td>
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<td>4.47</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>B2</td>
<td></td>
<td>1.60</td>
<td>2.40</td>
<td>21.90</td>
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</tr>
<tr>
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<td></td>
<td>1.09</td>
<td>6.10</td>
<td>35.70</td>
<td>++ Toxic</td>
</tr>
<tr>
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<td></td>
<td>1.77</td>
<td>8.00</td>
<td>71.00</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>22.40</td>
<td>240.20</td>
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</tr>
<tr>
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<td>26.70</td>
<td>394.40</td>
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<td></td>
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<td>N.A.† Toxic</td>
</tr>
<tr>
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</tr>
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<td>Group C</td>
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</tr>
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<td>N.A.† Toxic</td>
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<td>N.A.† Toxic</td>
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<tr>
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<td>216.10</td>
<td>N.D.†</td>
<td>+ Not toxic</td>
</tr>
</tbody>
</table>

† Determined by appearance of myotubes under the microscope or expression levels of myogenin.
‡ Determined by appearance of intact/floating cells under the microscope.
§ Not determined.

---

*a* Did not cause cell death, but cells were unable to differentiate after removal of the drug.

*b* Could not be assessed due to a massive cell death.
**Fig. 1**

- **Strain:** WT + vector, WT + p38α
- **NaCl:** - or +
- **Phospho-Hog1:** - or +
- **Phospho-p38α:** - or +
- **p38α:** - or +

**Graph:**
- **O.D. 600**
- **Time (hours):** 0 to 45
- **Graph Legend:**
  - Vector
  - p38αKD
  - p38α

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Fig. 2A
Fig. 2B

- vector
- p38α+DMSO
- p38α+PD169316
- p38α+SB203580
Fig. 2C

- **Vector+DMSO**
- **Vector+SB203580**

**O.D.600** vs. **Time (hours)**

0.000 0.100 0.200 0.300 0.400 0.500 0.600 0.700 0.800 0.900 1.000

0.0 5.0 10.0 15.0 20.0 25.0 30.0
Fig. 3A

![Graph showing O.D.600 for different wells after 0 and 40 hours](image-url)
Fig. 3B

The figure shows a bar graph with the x-axis labeled as "Wells of 96 well plate" and the y-axis labeled as "O.D.600". The graph compares the O.D.600 at 0 hours and 40 hours for different wells. The wells are labeled from left to right as follows: p38 cells, control 3, 1, 11, 15, 19, 23, 27, 31, 35, 39, 43, 47, 51, 55, 59, 63, 67, 71, 75, 79, 83, 87.

The legend indicates two conditions: "0 hours" and "40 hours". The "0 hours" condition shows a clear increase in O.D.600 for wells 1, 15, 23, 27, 31, 35, 39, 43, 47, 51, 55, 59, 63, 67, 71, 75, 79, 83, 87, with well 1 having the highest O.D.600. The "40 hours" condition shows a general increase in O.D.600 across all wells, with wells 1, 15, 23, 27, 31, 35, 39, 43, 47, 51, 55, 59, 63, 67, 71, 75, 79, 83, 87 having higher O.D.600 values compared to the "0 hours" condition.
Fig. 3C
Fig. 3D
Fig. 3E
Fig. 4A

Isoform, inhibitor (concentration)

p38α

- p38α + DMSO
- p38α + JX401

p38γ

- p38γ + PD169316
- p38γ + PD169316
- p38γ + JX401
- p38γ + DMSO

CPM

0 20000 40000 60000 80000 100000 120000 140000 160000 180000 200000 220000 240000 260000 280000 300000 320000 340000 360000
Fig. 5
A.

**Inhibitor:**

- PD169316
- JX401

**Myogenin**

- 0-36h: -
- 37-72h: -

**Tubulin**

- 0-36h: +
- 37-72h: +

B.

**Inhibitor:**

- G4
- G5
- JX401
- C7
- D2
- D4
- F8
- SB203580

**Myogenin**

- 0-36h: -
- 37-72h: -

**Tubulin**

- 0-36h: +
- 37-72h: +

*Fig. 6*