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JX401, A p38α Inhibitor Containing a 4-Benzylpiperidine Motif, Identified via a Novel Screening System 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 mitogen-activated protein 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. Because 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 high-throughput screening used here could be applied for isolation of inhibitors of various targets.

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 in which the three-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 composed 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 (Kirsch, 1993; Broach and Thorner, 1996; Pausch, 1997; Silverman et al., 1998;

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ABBREVIATIONS: HTS, high-throughput screening; MAPK, mitogen-activated protein kinase; Hog1, High Osmolarity Glycerol-1; GST, glutathione transferase; DMSO, dimethyl sulfoxide; MEK, mitogen-activated protein kinase kinase; OD, optical density; ATF, activating transcription factor; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)1*H*-imidazole; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole.

Munder and Hinnen, 1999; 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, topoisomerase II, phosphatase 1B, and a potassium channel (Pausch, 1997; van Hille and Hill, 1998; Montalibet and Kennedy, 2004; 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 previously (Tugendreich et al., 2001). 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 kinase (MAPK) superfamily that includes the p38, c-Jun NH_2-terminal kinase, extracellular signal-regulated kinase, and big mitogen-activated protein kinase 1 subfamilies (Bogoyevitch and Court, 2004). MAPKs function in signaling cascades that control complex cellular and multicellular programs such as embryogenesis, differentiation, proliferation, and cell death (Lewis et al., 1998; Ono and Han, 2000; Kyriakis and Avruch, 2001). 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. Therefore, a large number of studies strongly support the notion that p38α inhibitors could serve as anti-inflammatory drugs (Crawley et al., 1997; New and Han, 1998; Lee et al., 2000; Benhar et al., 2001; Redman et al., 2001; Hommes et al., 2002; Kumar et al., 2003; Bulavin and Fornace, 2004; Engelberg, 2004; Esteva et al., 2004; Olson and Hallahan, 2004). Although extensive efforts have been devoted to the development of drugs that inhibit p38α (Cuenda et al., 1995; Young et al., 1997; Kumar et al., 1999; Redman et al., 2001; English and Cobb, 2002; Goehring et al., 2002; Behr et al., 2003; Schultz, 2003), 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 *hog1*Δ strain partially restored growth under osmotic stress, whereas 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 the 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 and Methods

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

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 (Department 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 (*hog1*Δ; Brewster et al., 1993). In all experiments the cultures were grown in SD media (0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 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.

Western 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. (2001). Anti-p38α and anti-phospho p38α antibodies were purchased from New England Biolabs (Beverly, MA).

Kinase Assays. Reactions were carried out in 96-well plates with U-shaped bottoms in a final volume of 50 μl/well. Purified recombinant p38α or p38γ proteins (0.2 μg) were used. Activation of p38s by MKK6 was performed as described previously (Diskin et al., 2004). Purified active MKK6, phosphorylated by MEKK1, was purchased from Upstate Biotechnology (Lake Placid, NY). 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 $MgCl_2$, 20 mM 2-glycerol-phosphate, 5 mM *p*-nitrophenyl phosphate, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 64 μg (35.1 μM) of GST-rat ATF2, 250 μM ATP, and 10 μCi of [γ-^{32}P]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. After reaction termination, aliquots of 85 μl from each well were spotted onto 3 $\times$ 3-cm Whatman 3-mm 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 h 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 ^{32}P-Cherenkov program. Experimental points were performed in triplicate, and standard errors were approximately 15%.

Myoblast Culture. L8 myoblasts (Yaffe and Saxel, 1977) were grown in growth medium (15% bovine calf serum in Dulbecco's modified Eagle's medium). For induction of differentiation, the medium was changed to differentiation medium (no serum, 10 μg/ml insulin, and transferrin in Dulbecco's modified Eagle's medium) 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 differentiation medium 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 h. Preparation of lysates of L8 cells was performed as described previously (Gredinger et al., 1998). For monitoring myogenin induction, 40 μg of lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with anti-myogenin monoclonal antibodies (F5D; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-α-tubulin (DM1A; Sigma, St. Louis, MO) 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 p38α^{wt} in the wild-type strains SP1 and YPH102 and in the *hog1*Δ 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 with control cultures (Fig. 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 (p38^{K53A}). Yeast cells expressing this p38α molecule grew at an even better growth rate than wild-type yeast cells (Fig. 1). It is noteworthy that we found that the human p38α is spontaneously phosphorylated even in *pbs2*Δ cells (*PBS2* encodes the relevant MEK). This MEK-independent p38α phosphorylation is most likely a result of autophosphorylation activity acquired by the human p38α in yeast cells (V. Levin, D. Engelberg, 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 shows that the phenomenon is universal and is not peculiar to a particular genetic background.

Overexpressing Yeast MAPK Phosphatase Rescues the Growth Retardation Phenotype of p38α-Expressing Yeast. Because the growth retardation of cells expressing p38α was correlated with the phosphorylated, active state of p38α, we wondered whether overexpression of the yeast phosphatase Ptc1, known to dephosphorylate 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^{D58N}) grew similarly to yeast cells expressing p38α alone (Fig. 2A). This result verifies that growth can be rescued by phosphatase activity. Because Ptc1 dephosphorylates Hog1 and not its MEK Pbs2 (Warmka et al., 2001), it probably dephosphorylates Hog1's ortholog (p38α) as well. In addition, we found that p38α activity in yeast is independent of upstream MKKs

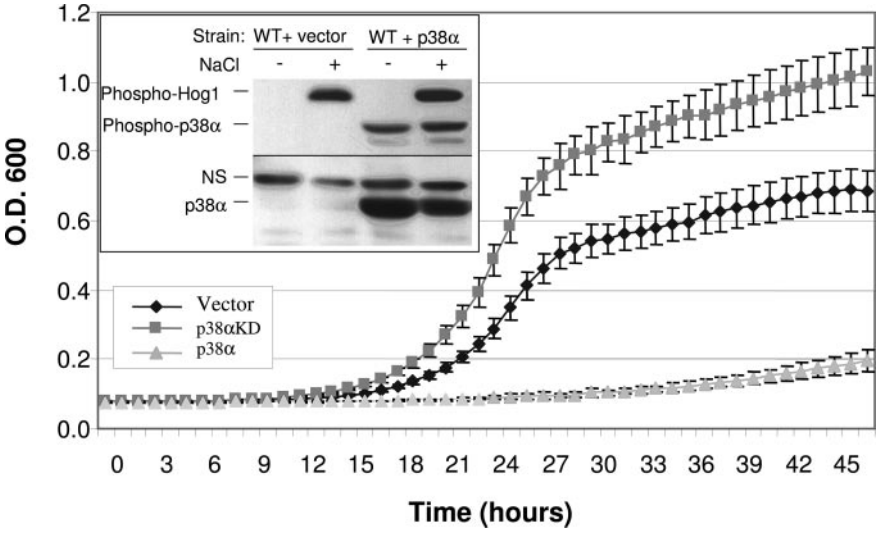


Fig. 1. The human p38α is constitutively phosphorylated in yeast cells and imposes severe growth retardation. p38α was expressed as a hemagglutinin-tagged protein. Shown are growth curves of yeast cultures harboring an empty expression vector (♦), p38α- expressing vector (▲), or kinase dead p38α (■). The experiment was repeated three times in triplicate. Bars show standard errors. Inset, a Western blot analysis of yeast lysates prepared from cells exposed or not to osmotic shock (1 M NaCl for 10 min). The top shows a blot probed with anti-phospho-p38α antibodies (these antibodies cross-react with phospho-Hog1). The bottom shows a blot probed with anti-hemagglutinin antibodies. NS, a nonspecific band that appears in yeast expressing and not expressing human p38α.

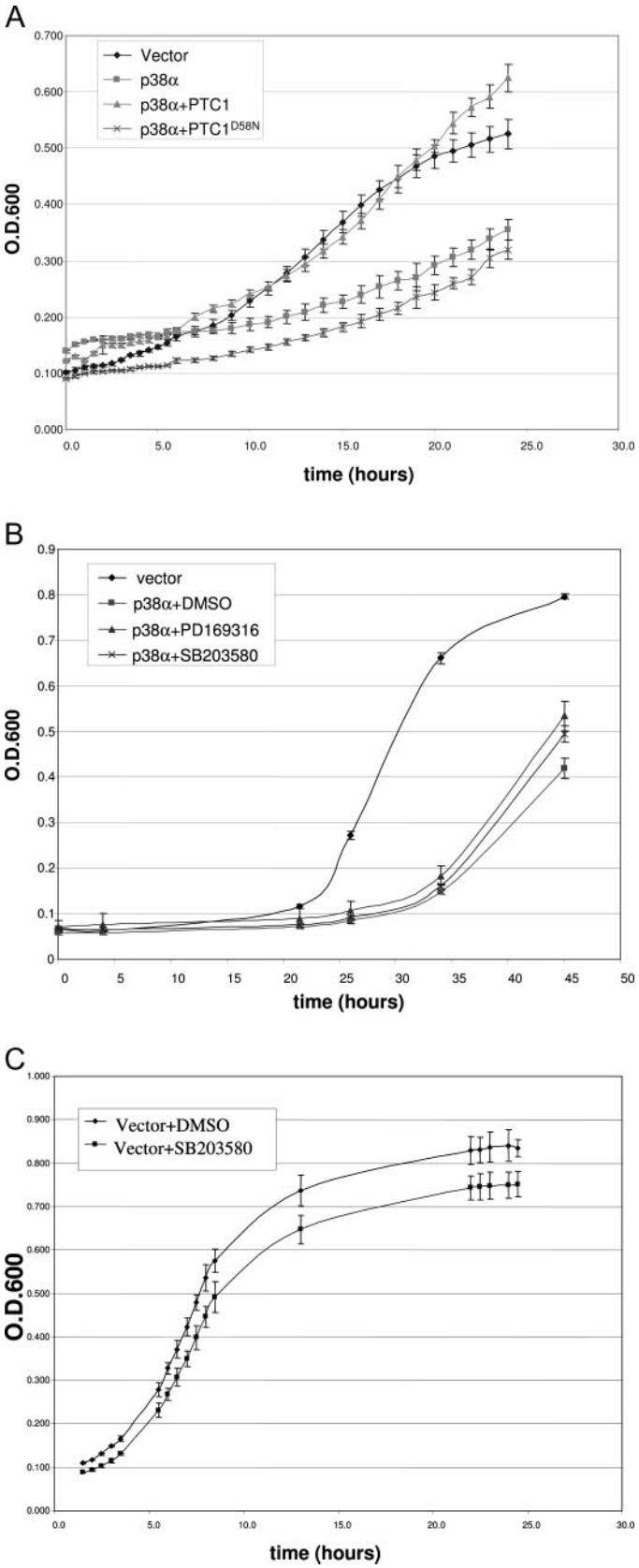


Fig. 2. Expression of the phosphatase Ptc1 or exposing cultures to p38α inhibitors rescue growth retardation of p38α-expressing yeast. A, growth curves of yeast cultures harboring either an empty vector (♦), a p38α-expressing vector (■), a p38α-expressing vector and a Ptc1-expressing vector (▲), or p38α-expressing vector and a $Ptc1^{D58N}$-expressing vector (×). B, growth curves of yeast cultures harboring either an empty vector (♦) or a p38α-expressing vector, grown in medium supplemented with either DMSO (■), 10 μM SB203580 (×), or 10 μM PD169316 (▲). The experiment was repeated three times in triplicate. Bars show standard errors. C, SB203580 is toxic to yeast cultures. Cultures of yeast cells harboring an empty vector were exposed to either DMSO (♦) or to SB203580 (10 μM; ■).

(V. Levin and D. Engelberg, 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 (Lee et al., 2000; Redman et al., 2001; Kumar et al., 2003). 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 beyond 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. 2, A and B). Furthermore, although both PD169316 and SB203580 rescued growth, we could not measure a dose-response for either of them; we found that 1 μM rescued growth almost as efficiently as 200 μM. 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 the inhibition of p38α, which 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 used 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 an 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

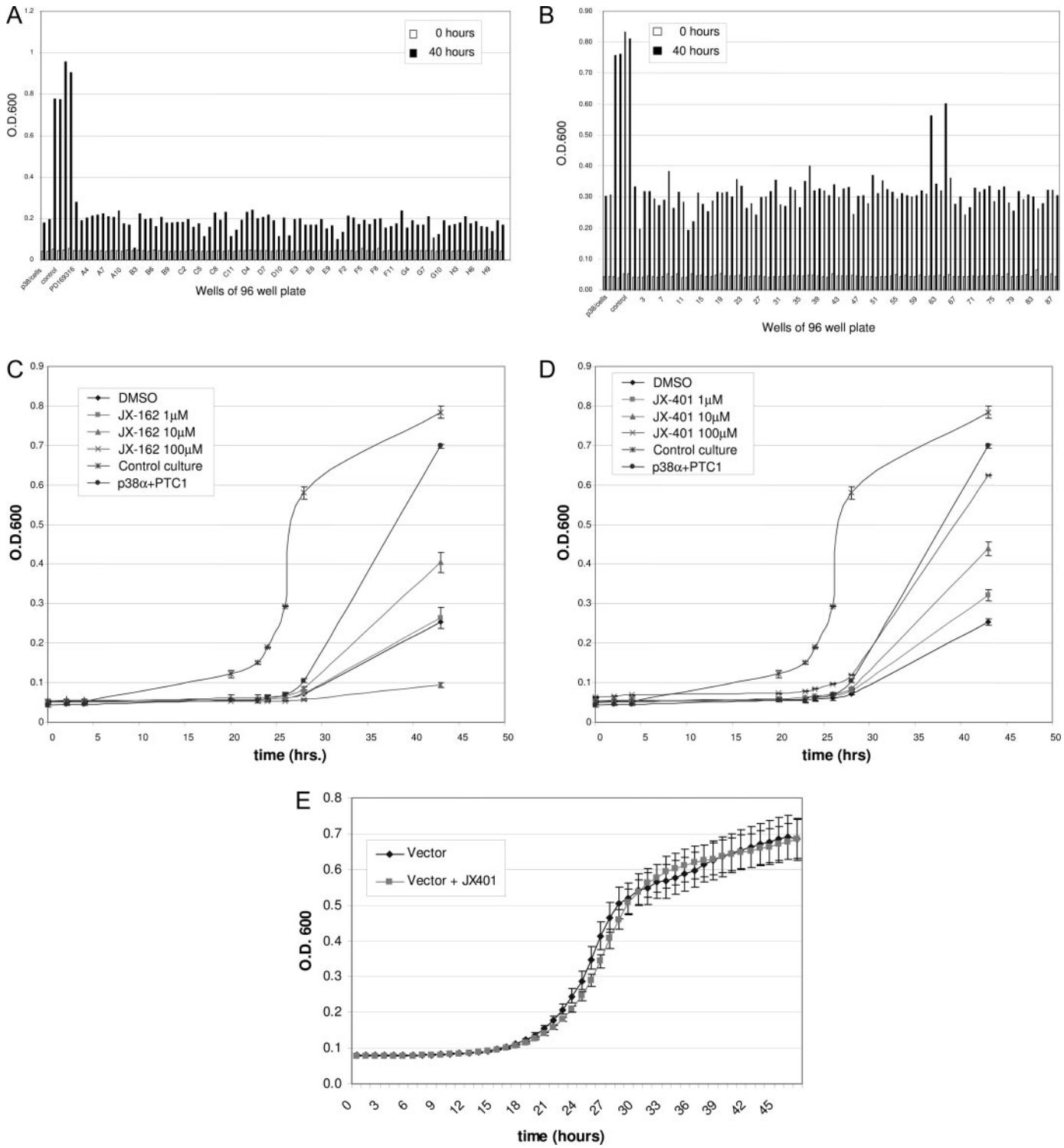


Fig. 3. The screening system and isolation of JX162 and JX401. A, an example of the results obtained from a single assay plate. The figure shows the optical density measurements (the *y*-axis) at a wavelength of 600 nm of all wells of a 96-well assay plate. The *x*-axis shows the location of wells in 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 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, and the OD_{600} of each well was measured at time 0 (immediately after addition of cells; □). Plates were then incubated at 30°C, and OD_{600} was read again 35 to 45 h later (■). In the particular plate shown, none of the compounds tested improved the growth of p38α-expressing cultures except for PD169316. B, a "repeat plate". After the screen of the entire library, all compounds that showed some ability to rescue p38α-expressing cells were retested. In the plate shown, two of the compounds, 63 and 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 (∗) 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 (■) or JX401 (10 μM; ♦).

grown in 96-well plates. To test the screening idea, we plated yeast cells expressing p38α in wells of 96-well plates and supplemented each well with 10 μM concentration of a compound taken from a diverse small-molecule library containing 40,000 compounds. Plates were incubated at 30°C, and after ~45 h, the optical density (at 600 nm) of the culture in each well was measured (Fig. 3A shows an example of the OD_{600} readings of an assay plate; for a detailed description of the screen and the controls used, see legend to Fig. 3A). Of the 40,000 compounds screened, several hundred manifested various degrees of growth rescue. It is noteworthy that growth rescue was considered to be 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 two compounds that improved the yeast growth significantly (200%). We named these compounds JX162 and JX401 and tested them in dose-response assays (Fig. 3, C and D). JX162 had no effect at a concentration of 1 μM, and growth rescue was apparent only at 10 μM (the same concentration used in the screen). Supplementing the medium with 100 μM JX162 led to toxicity, and growth rate was lower than that of untreated cells expressing p38α (Fig. 3C). JX401 rescued growth at 1 μM and was more effective as the concentration increased up to 100 μM (Fig. 3D). Increasing the concentration to 200 μM did not induce better growth but also did not have a negative effect (data not shown). In addition, 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 was added to the assay, it inhibited p38α most efficiently. JX401 was significantly more potent as a p38α inhibitor, with an IC_{50} value of 32 nM, than JX162, which inhibited p38α with an IC_{50} value of 480 nM. In the same experiment, the IC_{50} value of PD169316 was 15 nM. To test the specificity of the inhibition of JX401 toward 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 10 μM. 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 1 μM 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 versus 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 whether JX401 is a noncompetitive 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 Fig. 5. Both compounds, which were isolated independently from 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 (approximately 100) contain a modified 4-benzylpiperidine. Because none of these compounds was

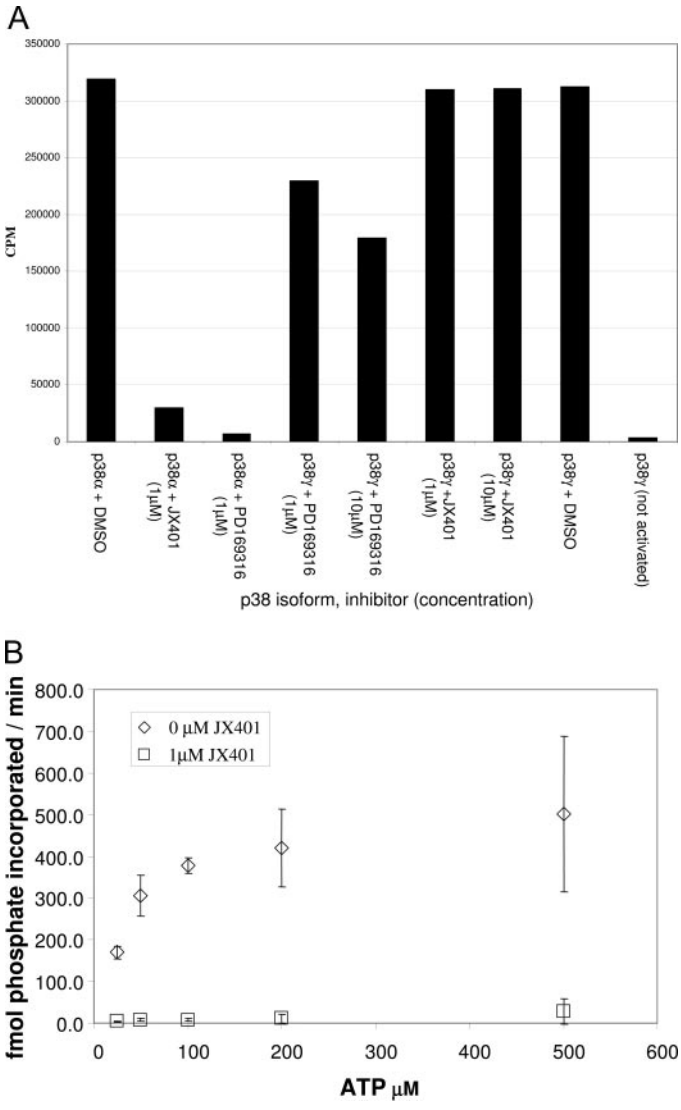


Fig. 4. JX401 has no effect on p38γ catalysis and is not ATP-competitive. A, catalytic activity of recombinant dually phosphorylated p38γ was assessed in the presence of the indicated concentrations of JX401 or PD169316. As controls, we measured the activity of nonphosphorylated p38γ (far right bar), 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 triplicate. B, catalytic activity of p38α was measured in the presence of increasing ATP concentrations in the absence (◇) or presence (□) of JX401 (1 μM). Results are shown as averages and standard errors (vertical bars) of three experiments.

active, we concluded that the structure of 4-benzylpiperidine itself is essential for the inhibitory properties of JX162 and JX401. Finally, 7 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, because each of these components alone is not active.

To check whether JX401 and JX162 had been identified previously 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 et al., 2002). JX401 did not appear in any database, patent, or scientific article. Because JX401 is a more potent inhibitor than JX162, is not toxic (whereas JX162 is; Fig. 3, C and D), 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 which were described in Goehring et al., 2002). Twenty-seven of these compounds were tested in an in vitro kinase assay at a concentration of 1 μM (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 the growth of yeast expressing p38α. Nine of the 10 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. It is interesting that 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.

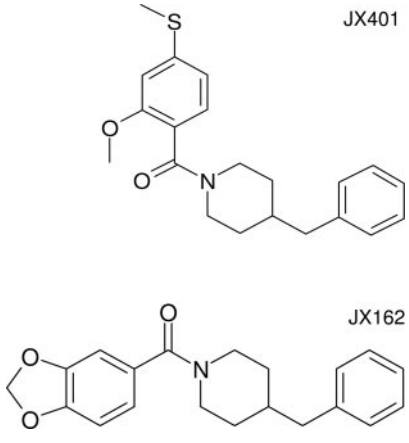


Fig. 5. Chemical structures of JX401 and JX162.

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 nontoxic). 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; Zetser et al., 1999; Wu et al., 2000), and the p38 inhibitor SB203580 has been shown to inhibit myogenic differentiation (Cuenda and Cohen, 1999; Zetser et al., 1999; Puri et al., 2000; Wu et al., 2000). We treated L8 myoblasts by supplementing the differentiation medium with 10 μM concentration of inhibitors of the JX401 family. Myoblasts were allowed to differentiate for 60 h, 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 (data not shown).

To check whether the effect of the inhibitors is reversible, myoblasts were grown in the presence of the compounds for 36 h, followed by their removal and an additional 36 h growth in their absence. The effect of several compounds (JX401, D2, D4, G4, and G5) was reversible, because the cells recovered completely and differentiated after 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 nontoxic 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). It is interesting that 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 the 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 (Kirsch, 1993; Broach and Thorner, 1996; Pausch, 1997; Silverman et al., 1998; Munder and Hinnen, 1999; 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). Furthermore, in some of these

TABLE 1
Various properties of JX401 and its derivatives

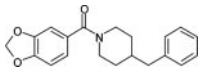
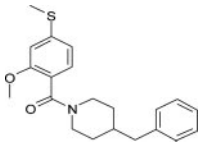
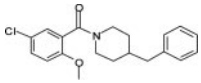
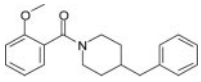
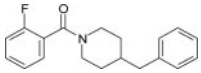
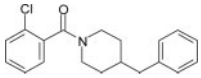
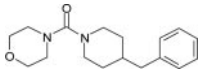
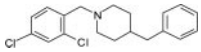
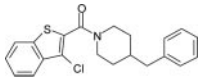
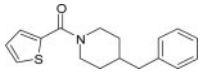
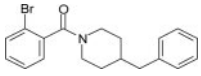
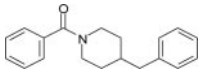
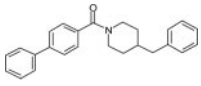
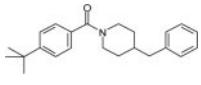
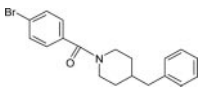
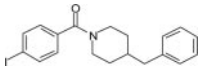
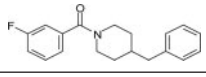
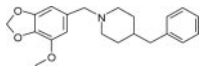
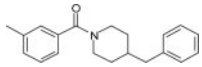
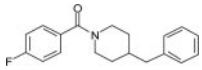
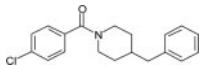
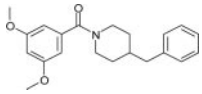
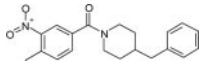
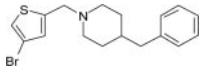
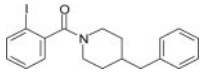
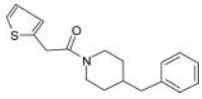
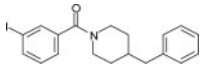
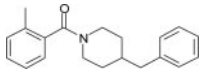
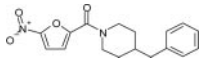
Compound	Structure	Effect on Growth (Growth Rate of Yeast Expressing p38α = 1)	p38α Activity In Vitro in the Presence of 1 μM Concentration of the Compound (No inhibition = 100% Activity)	IC_{50}	Efficiency in Blocking Myoblast Differentiation[a]	Toxicity to Mammalian Cells[b]
			%	*nM*		
JX-162		1.49	31.83	481.60	N.D.	N.D.
JX-401		1.76	4.47	32.20	++	Not toxic
Group A						
B2		1.60	2.40	21.90	++	Partially toxic
F8		1.09	6.10	35.70	++	Toxic[c]
D4		1.77	8.00	71.00	++	Not toxic
G5		1.86	9.70	63.60	++	Not toxic
C7		1.93	10.70	100.90	+	Not toxic
B7		1.24	13.30	125.30	++	Partially toxic
F7		1.99	15.60	147.40	+	Not toxic
C4		1.50	19.20	158.10	+	Partially toxic
E3		1.76	22.40	240.20	+	Not toxic
D6		1.71	26.70	394.40	−	Not toxic
Group B						
D3		0.69	32.00	N.D.	−	Not toxic
E5		0.38	32.70	N.D.	N.A.	Toxic
B5		0.23	40.50	N.D.	N.A.	Toxic
G4		0.64	45.10	N.D.	++	Partially toxic
F6		0.94	53.80	N.D.	−	Not toxic

TABLE 1—*continued*

Compound	Structure	Effect on Growth (Growth Rate of Yeast Expressing p38α = 1)	p38α Activity In Vitro in the Presence of 1 μM Concentration of the Compound (No inhibition = 100% Activity)	IC_{50}	Efficiency in Blocking Myoblast Differentiation[a]	Toxicity to Mammalian Cells[b]
			%	*nM*		
D7		0.38	63.80	N.D.	N.A.	Toxic
F4		0.58	64.60	N.D.	N.A.	Toxic
G7		0.53	72.60	N.D.	++	Partially toxic
D2		0.49	74.20	N.D.	++	Not toxic
G8		0.72	81.10	N.D.	–	Not toxic
Group C						
C2		0.34	104.30	N.D.	N.A.	Toxic
G2		0.74	122.00	N.D.	–	Not toxic
B8		0.87	154.60	N.D.	+	Partially toxic
E4		0.53	155.20	N.D.	–	Not toxic
D8		1.01	157.80	N.D.	N.A.	Toxic
C8		0.74	182.30	N.D.	–	Not toxic
C3		0.03	236.10	N.D.	+	Not toxic

N.D., not determined; N.A., could not be assessed because of a massive cell death; ++, efficient blocker; +, partial blocker; –, nonblocker.
[a] Determined by the appearance of myotubes under the microscope and/or by expression levels of myogenin.
[b] Determined by the appearance of intact/floating cells under the microscope.
[c] Did not cause cell death, but cells were unable to differentiate after removal of the drug.

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, because it does not inhibit p38γ, and to be active and nontoxic in mammalian cells. Comparison of JX401 with 27 similar molecules, some described previously to be efficient p38α inhibitors (Goehring et al., 2002), suggests that JX401 is one of the most potent compounds of the family (Table 1). As we have shown in this article, 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. (2002). It seems that this motif is essential for p38α inhibition because 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 substituent in ortho and para

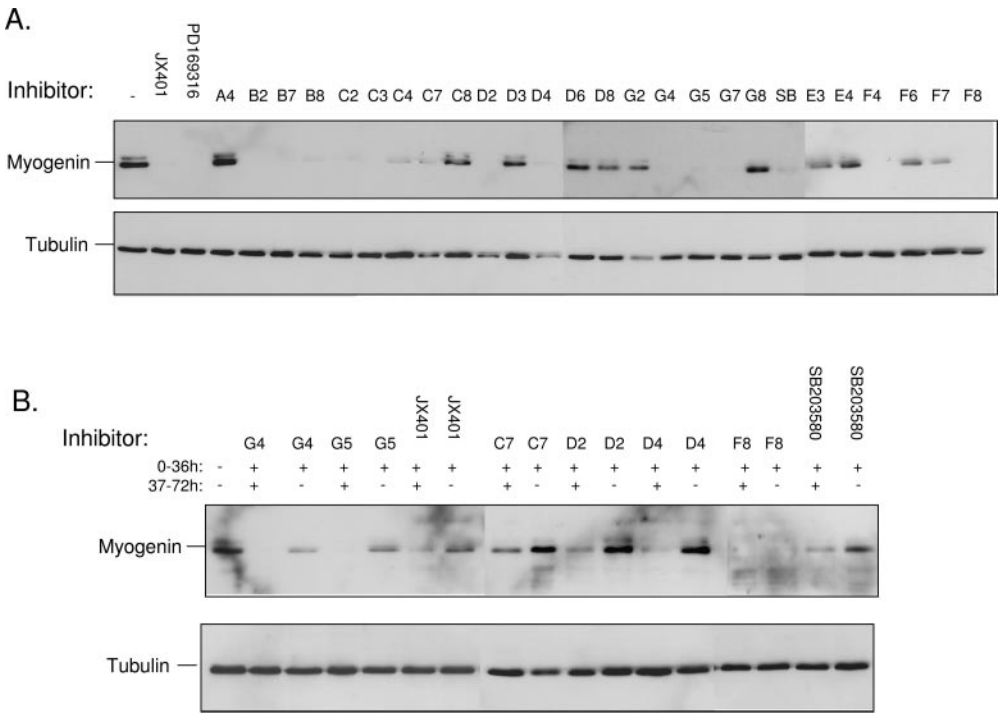


Fig. 6. JX401 and some of its derivatives reversibly inhibit the differentiation of myoblasts. Expression of myogenin, the early induced activator of the myogenic program, was monitored by a Western blot analysis. A, myoblast cultures were incubated in differentiation media supplemented with DMSO only (−, the left lane), or in differentiation medium supplemented with 10 μM concentrations of the indicated inhibitors. Lysates were prepared 60 h after the initiation of treatment. B, the inhibitory effect of JX401 and its derivatives is reversible. Cells were incubated for 36 h 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 h, lysed, and the levels of myogenin and tubulin were monitored by Western blots.

positions on the phenyl methanone, bound to the 4-benzylpiperidine. G8, which contains two methoxy substituents on meta positions, and F8, which 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. In addition, 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 seems that a substituent in position 2 contributes to the activity, whereas 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 of 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 to bring it to the preclinical 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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