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Pharmacological and Genetic Analysis of 90-kDa Heat Shock Isoprotein-Aryl Hydrocarbon Receptor Complexes

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ABSTRACT

The 90-kDa heat shock protein (Hsp90) is an abundant chaperone that regulates a diverse set of intracellular signaling proteins. Drugs that inhibit Hsp90 activity have been useful in the identification of novel Hsp90-dependent signaling pathways. One class of inhibitory compounds disrupts Hsp90-dependent processes by binding to the N-terminal ATPase/p23-binding domain of Hsp90, whereas a second inhibitor class binds within the C-terminal domain. We used signaling by aryl hydrocarbon receptor (AhR), an Hsp90-dependent transcription factor, as a functional probe to study the effects of Hsp90 inhibitors in yeast strains with deletion mutations of individual Hsp90 and p23 cochaperone genes. The more abundant and constitutively expressed Hsp90 isoform, Hsc82, functioned best in supporting AhR signaling. Deletion of the more inducible isoform, Hsp82, had no effect on signaling. AhR complexes containing Hsc82 were preferentially sensitive to the effects of low concentrations of the N-terminal inhibitors radicicol and herbimycin A. However, both Hsp90 isoforms were equally sensitive to the AhR-specific effects of novobiocin, which binds to the C terminus. Hsp90 inhibitors had no preferential effects on AhR signaling in strains that lacked p23, suggesting that the inhibitors exert their effects through a p23-independent mechanism. In contrast, overexpression of p23 buffered the effects of radicicol and herbimycin A, but not novobiocin, on AhR signaling. The data collectively suggest preferential use or function of the Hsc82 isoprotein in AhR signaling and provide new insight into the effects of three structurally unrelated Hsp90 inhibitors.

The 90-kDa heat shock proteins (Hsp90s) are a group of abundant proteins that act as classic chaperones by preventing aggregation and maintaining the native conformation of proteins in cells under various stress conditions (reviewed by Richter and Buchner, 2001). However, Hsp90 proteins also have a direct role in the regulation of intracellular signaling pathways in unstressed cells. Eukaryotic cells typically contain two Hsp90 isoforms that are expressed to different degrees. The yeast *HSC82* and human Hsp90β genes are, typically, more constitutive and moderately inducible in their expression, whereas yeast *HSP82* and human Hsp90α genes are generally expressed at lower basal levels and are strongly inducible under stress conditions. Hsp90 proteins are essential for viability in all eukaryotes. The *HSC82* and *HSP82* genes of *Saccharomyces cerevisiae* are individually dispensable but cannot be deleted simultaneously, suggesting that they have a redundant function in the maintenance of cell viability (Borkovich et al., 1989). Deletion of the Hsp84 gene in the mouse causes defects in placental development and produces an embryonic lethal phenotype (Voss et al., 2000).

Although Hsp90 isoproteins are structurally very similar, it is not known whether they have any specific functions or properties. There is distinct tissue specific regulation of Hsp90 isoprotein expression during vertebrate development (Krone et al., 1997). The expression levels of Hsp90 proteins are typically increased in cancers and may be needed to maintain the cancerous state (Ferrarini et al., 1992). Consequently, there is evidence suggesting that the levels and types of Hsp90 proteins expressed in cells are of biological importance.

Among the proteins known to require Hsp90 for proper function are some steroid hormone receptors, aryl hydrocarbon receptor (AhR), serine, threonine, and tyrosine kinases, and telomerase (reviewed by Richter and Buchner, 2001). The steroid hormone receptors are among the best-characterized Hsp90 client proteins and have served as useful model proteins for studying the Hsp90 chaperone complex. In addition to Hsp90, several cofactor proteins are needed for the proper regulation of steroid hormone receptor signaling pathways. The late steps in the steroid hormone receptor

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ABBREVIATIONS: Hsp90, 90-kDa heat shock protein; AhR, aryl hydrocarbon receptor; PAS, PER/aryl hydrocarbon receptor nuclear translocator/SIM; Hsf1, heat shock factor 1; Xap2, hepatitis B virus protein X associated protein 2; DMSO, dimethyl sulfoxide; AHRE, aryl hydrocarbon receptor response element; ARNT, aryl hydrocarbon receptor nuclear translocator.

maturation process involve the p23 cochaperone and an immunophilin protein.

AhR is a member of the basic helix-loop-helix/PAS family of transcription factors (Gu et al., 2000). In the presence of a ligand, AhR and its dimerization partner, the aryl hydrocarbon receptor nuclear translocator protein, form a heterodimeric transcription factor that binds AhR response elements (AHREs) on DNA. The AhR/aryl hydrocarbon receptor nuclear translocator protein complex influences the expression of a diverse set of genes that are involved in toxicity and other regulatory responses (Hankinson, 1995). Before ligand interaction, AhR exists in a complex with Hsp90 proteins and cochaperones. The helix-loop-helix and the PAS-B domains of AhR are in direct contact with Hsp90, and this interaction serves to regulate AhR function (Perdew and Bradfield, 1996). Maturation of AhR to a functional state is mediated through a process that is thought to involve the p23 cochaperone (Kazlauskas et al., 1999, 2001) and an immunophilin protein called the hepatitis B virus protein X associated protein 2 (Xap2) (Ma and Whitlock, 1997; Meyer and Perdew, 1999; Kazlauskas et al., 2000). Xap2 interacts with AhR through the PAS-B domain and reduces receptor degradation, influences nuclear translocation, and enhances signaling of AhR. Two proteins (Cpr7 and Cns1) from *S. cerevisiae* that display sequence similarity to Xap2 have also been shown to enhance AhR signaling (Miller, 2002). The p23 protein associates with the nucleotide-binding site of Hsp90. The interaction of p23 with Hsp90 is dependent upon the presence of ATP (Grenert et al., 1997; Chadli et al., 2000). Previous studies conducted in our laboratory demonstrated that p23 was needed for efficient AhR signaling (Cox and Miller, 2002).

The use of Hsp90 inhibitors has greatly facilitated the study of the Hsp90 chaperone complex and its role in intracellular receptor signaling. Several Hsp90 client proteins were discovered to be oncoproteins that function in malignant cells. Consequently, benzoquinone ansamycin antibiotics (geldanamycin, macbecin I, macbecin II, and herbimycin A) and their derivatives have been used in clinical trials as a result of their anti-tumor properties (Isaacs et al., 2003). The benzoquinone ansamycin antibiotics and the structurally unrelated compound radicicol have overlapping binding sites in the ATPase/p23-binding region in the N terminus of Hsp90. These drugs inhibit Hsp90 dependent processes by disrupting both ATP and p23 binding (Schulte et al., 1998; Sharma et al., 1998; Roe et al., 1999). Treatment of eukaryotic cells, including yeast, with the ansamycins and radicicol resulted in reduced steroid hormone receptor ligand binding and increased steroid hormone receptor degradation (Smith et al., 1995; Whitesell and Cook, 1996). The ansamycin antibiotics also enhanced Hsp70 and Hsp90 expression in mammalian cells by inhibiting Hsp90-mediated repression of the heat shock factor 1 (Hsf1) signaling pathway (Kim et al., 1999; Bagatell et al., 2000). The mechanism by which the Hsp90 inhibitor novobiocin disrupts Hsp90 function is less clear. Novobiocin binds to a second ATP binding site within the C terminus of Hsp90 and interferes with phosphorylation (Marcu et al., 2000; Langer et al., 2002). Furthermore, novobiocin disrupts the interaction of both p23 and Hsc70 with the Hsp90 chaperone complex. Thus, novobiocin seems to have pleiotropic effects with regard to Hsp90 function.

In the report below, we describe the use of pharmacological and genetic approaches to study the roles of p23 and the individual Hsp90 isoforms in a synthetic AhR signaling pathway. In essence, AhR signaling was used as a reporter of Hsp90 chaperone function under various conditions in cells. These studies help to define the mechanisms of drugs that disrupt Hsp90 dependent processes and provide new evidence for specific functional differences among Hsp90 isoproteins.

Materials and Methods

Reagents. Reagent grade chemicals were purchased from Fisher Scientific (Springfield, NJ) and Sigma Chemical (St. Louis, MO) companies. All restriction enzymes used in this study were from New England Biolabs (Beverly, MA). The geldanamycin was purchased from Alexis Biochemicals (San Diego, CA). The herbimycin A, radicicol, and novobiocin were purchased from Sigma Chemical Co. (St. Louis, MO). The geldanamycin, herbimycin A, and radicicol were stored as 10 mM stock solutions in dimethyl sulfoxide (DMSO) at −20°C. The novobiocin (sodium salt) was stored as a 100 mM stock solution in water at 4°C. The β-naphthoflavone used as an AhR ligand in the lacZ assays was purchased from Acros Organics through Fisher Scientific and was of 99% purity.

Yeast Strains and Plasmids. The genotypes and references for all strains used in this study are listed in Table 1. The *hsc82* and *hsp82* deletion strains (BY4742/*hsc82* and BY4742/*hsp82*) were purchased from Research Genetics (Huntsville, AL). The pLXRE5-Z reporter plasmid was transformed into the *hsc82* and *hsp82* deletion strains (Cox and Miller, 2002). An Hpa I fragment from the pIGAHRC plasmid containing an AHR/ARNT expression construct under control of the GAL 1, 10 promoter (AHR-GAL 1,10-ARNT) (Fig. 2A) was then integrated at chromosome III by a two-step recombination method as described previously (Miller, 1999). Integration at chromosome III was confirmed by Southern blot analysis

TABLE 1
Yeast strains used in this study

Strain	Genotype	Accession Number	Reference
BY4742	α Δ*leu2* Δ*his3* Δ*lys2* Δ*met3* Δ*ura3*	95400	
YMC1	α Δ*leu2* Δ*his3* Δ*lys2* Δ*met3* Δ*ura3* Chr.III::AHR-GAL1 10-ARNT pLXRE5-Z (*LEU2*)		Cox and Miller, 2002
YMC2	α Δ*leu2* Δ*his3* Δ*lys2* Δ*met3* Δ*ura3* *sba1*::Kan^r Chr.III::AHR-GAL1 10-ARNT pLXRE5-Z (*LEU2*)		Cox and Miller, 2002
BY4742/hsp82	α Δ*leu2* Δ*his3* Δ*lys2* Δ*met3* Δ*ura3* *hsp82*::Kan^r	11052	
YMCp82	α Δ*leu2* Δ*his3* Δ*lys2* Δ*met3* Δ*ura3* *hsp82*::Kan^r Chr.III::AHR-GAL1 10-ARNT pLXRE5-Z (*LEU2*)		This study
BY4742/hsc82	α Δ*leu2* Δ*his3* Δ*lys2* Δ*met3* Δ*ura3* hsc82::Kan^r	10771	
YMCc82	α Δ*leu2* Δ*his3* Δ*lys2* Δ*met3* Δ*ura3* hsc82::Kan^r Chr.III::AHR-GAL1 10-ARNT pLXRE5-Z (*LEU2*)		This study

Δ, deleted gene.

(data not shown). These strains were named YMCc82 and YMCp82, respectively. The YMC1 and YMC2 strains were described previously (Cox and Miller, 2002). Briefly, YMC1 and YMC2 are the parental and *sba1* deletion strains, respectively, and both contain the AHR-GAL 1, 10-ARNT expression construct integrated into chromosome III and the pLXRE5-Z reporter plasmid. All plasmid transformations in yeast were performed by the lithium acetate method. The 2μyeast p23 (Sba1) expression vector, pGal-Sba1, expressed Sba1 with an N-terminal tag of six consecutive histidines (His_6) from a galactose inducible promoter (Fang et al., 1998). The N-terminal His_6 tag had no effect on the function of Sba1.

LacZ Assays. The lacZ (β-galactosidase) assay that was used as a quantitative indicator of AhR signaling status has been described previously (Kippert, 1995). The original assay protocol was modified to fit a 96-well plate format. Saturated yeast cultures grown overnight in synthetic minimal glucose medium were diluted between 1:50 and 1:200 in 96-well plates containing synthetic minimal galactose medium lacking the appropriate amino acids and/or nucleosides to select for plasmid maintenance. β-Naphthoflavone (an AhR ligand) was added to a final concentration of 1000 nM with a final DMSO vehicle concentration of 1%. All other ligand concentrations used were serial dilutions from the 1000 nM treatment and were performed in triplicate. In some experiments, the medium also contained the indicated concentrations of radicicol, herbimycin A, and novobiocin. The dose responses of the Hsp90 inhibitors (radicicol, herbimycin A, and novobiocin) in the presence of the galactose-regulated lacZ reporter plasmid (pD16) were done in a similar manner except the ligand concentration was held constant at 1 μM and the inhibitor concentrations were serial dilutions from the highest inhibitor concentration used. The DMSO vehicle concentration never exceeded 1% in all experiments shown. The 96-well plates were incubated at 30°C for 16 to 18 h before performing the lacZ assays. Controls performed in parallel with the experimental treatments included cells dosed with 1% DMSO or medium alone. The lacZ and cell density absorbance readings were done at 405 nm and were within the linear range of the spectrophotometric plate reader. The lacZ activity units were calculated from the raw absorbance readings as described previously (Miller, 1999). The lacZ signaling data presented were from at least three independent clones and are representative of at least three independent experiments that produced similar results. Statistical significance was determined using the Student's t test and p values ≤ 0.05 were considered statistically significant. In cases in which three or more independent groups were present, pair-wise comparisons were performed using one-way analysis of variance followed by Bonferroni's multiple comparisons test, and p values ≤ 0.05 were considered statistically significant.

Hsp82 Immunoblot Analysis. Yeast whole cell lysates were prepared from saturated cultures of the indicated yeast strains in extraction buffer (0.3 M NH_4SO_4, 5% glycerol, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail) using the glass bead disruption method. Protein concentrations were determined using a protein assay (Bio-Rad, Hercules, CA). Total cellular proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). Nonspecific binding sites on the membrane were blocked with a solution of phosphate-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk. Immunoblotting for the Hsc82 and Hsp82 proteins was performed using a polyclonal rabbit anti-yeast Hsp90 serum (Ab4–2; from Susan Lindquist, Whitehead Institute, Cambridge, MA) as the primary antiserum and a horseradish peroxidase-conjugated goat anti-rabbit IgG serum (Bio-Rad) as the secondary antiserum. The primary and secondary antisera were used at 1:20,000 and 1:100,000 dilutions, respectively, in blocking buffer. Chemiluminescent detection was done using the SuperSignal West chemiluminescent detection/optimization kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Results

Assessing the General Toxic Effects of the Hsp90 Specific Inhibitors in Yeast. Several of the Hsp90 inhibitors disrupt p23/ATP binding to Hsp90. Thus, we reasoned that examining p23 and Hsp90 deletion strains that were treated with the Hsp90 inhibitors could determine the individual importance of p23 and each Hsp90 isoform in the regulation of Hsp90-dependent AhR signaling (Fig. 1). We chose to compare the effects of three different Hsp90 inhibitors on AhR signaling in the parental strain (YMC1), the p23 (*sba1*) deletion strain (YMC2), the *hsp82* deletion strain

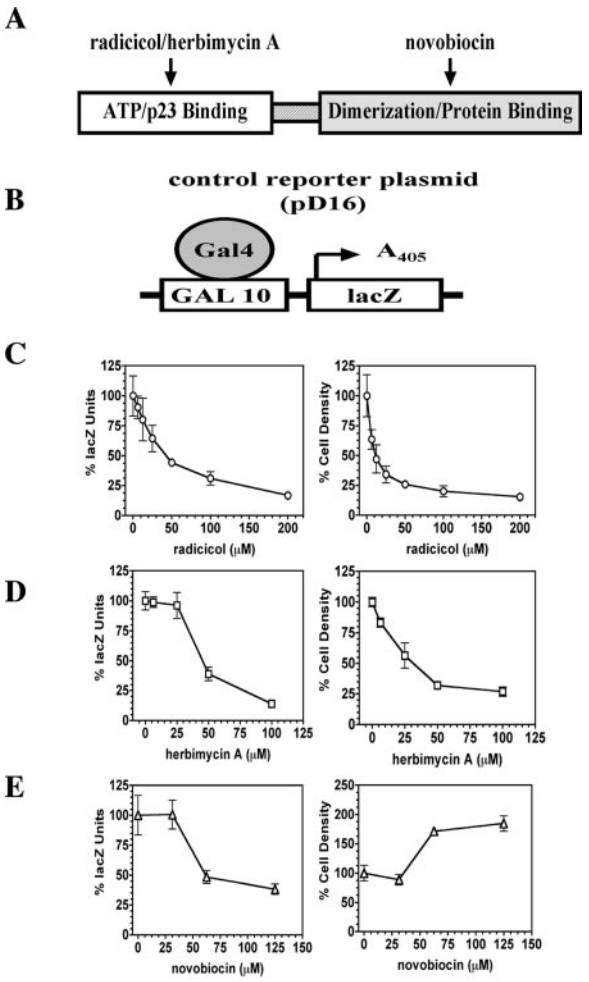

Fig. 1. Characterizing the toxic effects of the Hsp90-specific inhibitors in yeast. A, a diagram of the Hsp90 protein. General locations of radicicol, herbimycin A, and novobiocin binding sites are shown. The radicicol and herbimycin A binding sites are within the N-terminal ATP binding site, which is also the site of p23 binding. The novobiocin binding site is within the C-terminal dimerization and protein-protein interaction domain. B, the relevant features of the pD16 reporter plasmid are shown. pD16 expresses a lacZ reporter gene from a galactose inducible promoter (GAL 10). Drug dependent effects on lacZ expression from the pD16 plasmid were considered to represent general toxic effects on transcription, translation, protein stability, or the galactose regulatory pathway. C–E, left, the BY4742 strain carrying a galactose-regulated lacZ reporter vector (pD16) was treated with a range of concentrations of radicicol (C), herbimycin A (D), or novobiocin (E) and assayed for expression of the β -galactosidase reporter gene. The lacZ data were plotted as percentages with the level of lacZ signaling in the presence of untreated set to 100%. C–E, right, the cell densities were plotted as percentages, with the cell density of the untreated cells set to 100%. The apparent enhanced cell density in the novobiocin treated cells was actually caused by novobiocin precipitation at higher concentrations.

(YMCp82), and the *hsc82* deletion strain (YMCc82; see Table 1 for strains and their genotypes). Although the Hsp90 inhibitors have been used extensively in vitro to inhibit Hsp90-dependent processes, their effects in the BY4742 yeast strain have not been characterized. Therefore, we first assessed the effects of a range of inhibitor concentrations (herbimycin A, radicicol, and novobiocin) on yeast cell (BY4742) growth and on the level of lacZ expression from a galactose-regulated lacZ control plasmid (pD16; Fig. 1B). Drug-dependent effects on lacZ activity from yeast carrying the pD16 plasmid represented general toxic effects on transcription, translation, protein stability, or the galactose regulatory response. In addition, cell growth was monitored as an indicator of drug toxicity. As can be seen from Fig. 1, C to E, higher doses of all the inhibitors produced significant effects on lacZ expression from the pD16 plasmid. Radicicol could be used up to a concentration of 10 μM without producing significant changes in lacZ activity (Fig. 1C, left). However, radicicol had a strong negative effect on cell growth at 10 μM (Fig. 1C, right). Thus, 5 μM radicicol was the highest concentration used to assess effects on Hsp90 in subsequent experiments. Herbimycin A did not produce significant effects on lacZ signaling or growth when used at a concentration at or below 25 μM (Fig. 1D, left and right). We also tested a second ansamycin antibiotic, geldanamycin, for general effects. Geldanamycin did not produce any significant effects on galactose regulated lacZ expression or on cell growth up to a concentration of 500 μM (the highest concentration tested; data not shown). However, herbimycin A was a more potent inhibitor of AhR signaling and was the ansamycin antibiotic used in all experiments shown. Novobiocin did not produce significant general toxic effects up to a concentration of 33 μM (Fig. 1E, left). The high cell density readings at novobiocin concentrations above 33 μM were artifacts caused by the formation of a precipitate (Fig. 1E, right). The cell density was taken into account in the calculation of the arbitrary lacZ units. Thus, the precipitate that formed prevented the accurate calculation of lacZ units at novobiocin concentrations above 33 μM. These experiments were also performed in all strains used in this study. We observed only slight variations in the sensitivity to the general effects of the inhibitors between each strain that came from the BY4742 genetic background. These small differences cannot be attributed to the AhR-specific effects observed in the experiments described below. Thus, when the inhibitors were used within the dose ranges described above, the effects on AhR signaling were specific and independent of general toxic effects on gene expression or cell growth.

Hsp90 Isoprotein-AhR Complexes Display Differences in AhR Signaling. The study of AhR regulation by Hsp90 and its associated cochaperones is complicated by the fact that, similar to vertebrate cells, *S. cerevisiae* has two Hsp90 isoforms (Hsp82 and Hsc82). Thus, we assessed the ability of Hsp82 and Hsc82 to support normal AhR signaling individually. We constructed a functional human AhR signaling pathway (Fig. 2A) in the *hsp82* and *hsc82* deletion strains (named YMCp82 and YMCc82, respectively) and assessed the ability to support normal AhR signaling compared with AhR signaling in the parent strain (YMC1). Deletion of *hsp82* had no effect on the level of AhR signaling (Fig. 2B). However, deletion of *hsc82* resulted in a significant reduction in signaling across all ligand concentrations tested (Fig. 2B). Both the apparent potency and efficacy of ligand-induced signaling were strongly reduced in the *hsc82* (YMCc82) mutant strain. It was possible that the differences in AhR signaling observed between the YMCp82 and YMCc82 strains were caused by differences in Hsp82 and Hsc82 protein levels rather than functional differences. Thus, we assessed the Hsp82 and Hsc82 protein levels in the wild-type strain (YMC1) and the Hsp90 deletion strains (YMCp82 and YMCc82) by immunoblot analysis. The Hsc82 protein level in the YMCp82 strain was similar to the combined Hsp82 and Hsc82 levels in the parent strain (Fig. 2C). The Hsp82 protein level in the YMCc82 strain was approximately 50% lower than in the parent strain. Overexpression of *HSP82* in the YMCc82 strain to ~150% that of the total Hsp90 levels in the parent strain restored signaling to near wild-type levels (~80%) (Fig. 2, B and C). Thus, Hsp82 may better support AhR signaling because of its relative abundance, by having a preferential action on AhR, or both. Our results indicate that the predominant isoform expressed in the unstressed yeast cell is Hsc82. The levels of Hsp82, when serving as the sole Hsp90 family member in the cell, are likely to be induced to compensate for the loss of *HSC82*.

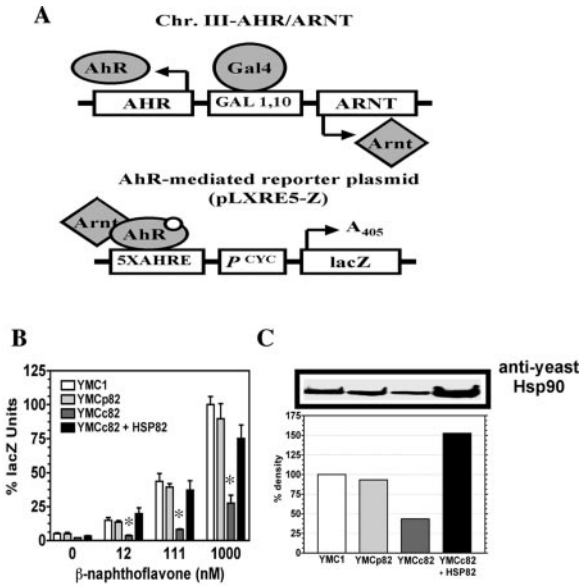

Fig. 2. Differences in AhR-mediated signaling in the *hsc82* and *hsp82* deletion strains. A, the relevant features of the β-galactosidase (lacZ) reporter plasmid (pLXRE5-Z) and the AHR-GAL 1, 10 promoter-ARNT expression construct integrated in chromosome III are shown. Expression of the AHR and ARNT genes is induced by galactose. Expression of the lacZ gene from the pLXRE5-Z reporter plasmid is mediated by five upstream AhR responsive elements (AHREs). Thus, cells grown in medium with galactose and an AhR ligand (β-naphthoflavone) expressed AhR/ARNT mediated transcriptional activation of the lacZ gene (YMCp82; *hsp82*/*HSC82*) (light gray bars), *hsc82* deletion strain (YMCc82; *HSP82*/*hsc82*) (dark gray bars), and the *hsc82* deletion strain (YMCc82; *HSP82*/*hsc82*) overexpressing *HSP82* (black bars) were assayed for AhR-mediated expression of the lacZ reporter gene in the presence of the indicated concentrations of β-naphthoflavone. The response of YMCc82 was significantly different from that of YMC1, YMCp82, and YMCp82 overexpressing *HSP82* at all ligand concentrations shown ($p < 0.01$, indicated by asterisks). C, yeast Hsp90 immunoblot analysis of cell lysates from the YMC1 strain (*HSP82*/*HSC82*), the YMCp82 strain (*hsp82*/*HSC82*), the YMCc82 strain (*HSP82*/*hsc82*), and the YMCc82 strain (*HSP82*/*hsc82*) overexpressing *HSP82*. The bands in the immunoblot (top) were measured by densitometry and the data were normalized to the combined amount of Hsp82 and Hsc82 in the wild-type YMC1 strain (bottom).

Yeast Hsp90 Isoprotein-AhR Complexes Display Differences in Their Sensitivities to the Hsp90 Inhibitors. Given the differences in AhR signaling in the YMCp82 and YMCc82 strains, we questioned whether the sensitivity of the two strains to the effects of the Hsp90 inhibitors would be different. We assayed the wild-type strain (YMC1), the *hsp82* deletion strain (YMCp82), and the *hsc82* deletion strain (YMCc82) for ligand-dependent AhR signaling in the presence of the indicated concentrations of radicicol, herbimycin A, and novobiocin (Figs. 3, A to C). The data presented for each yeast strain were normalized to the lacZ signaling in the absence of inhibitor for each strain. This method normalized the initial differences in AhR signaling between each strain (Fig. 2B) and allowed for comparison of the sensitivities of each strain to the effects of the inhibitors. AhR signaling in the wild-type (YMC1) and *hsp82*-deleted strains was reduced by 60 to 80% in the presence of the inhibitors (5 μM radicicol, 25 μM herbimycin A, and 33 μM novobiocin) (Figs. 3, A and B, respectively). The reductions in AhR signaling in the wild-type and *hsp82*-deleted strains were not significantly different. However, AhR signaling in the *hsc82*-deleted strain treated with 5 μM radicicol and 25 μM herbimycin A was unaffected or only mildly reduced (Fig. 3C). Thus, the strain expressing Hsp82 protein as the only source of Hsp90 was significantly less sensitive to the AhR-specific effects of radicicol and herbimycin A.

Treatment of cells with the Hsp90 inhibitors was shown to enhance Hsp90 protein levels by inhibiting Hsp90-dependent repression of the Hsf1 signaling pathway. Thus, the differences in sensitivity to the effects of the Hsp90 inhibitors observed in the *hsc82* and *hsp82* deletion strains could have been caused by changes in the levels of Hsp90. We analyzed the Hsp90 levels in the wild-type and deletion strains that were grown in the presence or absence of 5 μM radicicol. The cells used to prepare the protein lysates were grown under the same conditions as the cells in the previous lacZ assays, except for the presence or absence of 5 μM radicicol. Figure 4 shows that yeast Hsp90 levels remained constant among strains despite the presence or absence of 5 μM radicicol. Thus, changes in levels of Hsp90 proteins do not seem to account for the differential effects of the inhibitors at the concentrations and conditions tested here.

We also tested a range of Hsp90 inhibitor concentrations to determine the threshold for reduction of AhR signaling. In cells activated with ligand, AhR signaling in the *hsc82*-deleted strain was significantly resistant to radicicol concentrations between 0.08 and 5 μM (Fig. 5A) and at herbimycin A concentrations between 12.5 and 25 μM (data not shown). Doses of radicicol $\geq$0.8 μM had considerable effects on AhR signaling in cells that expressed only Hsc82. The reduction in AhR signaling in hsc82 deleted (YMCc82) cells treated with up to 33 μM novobiocin (71% decrease) was not significantly different from the reductions observed in the YMC1 (data not shown) and the hsp82 deleted (YMCp82) strains (Fig. 5B). In summary, the inhibitors that acted on the N-terminal region of Hsp90 displayed isoform specific effects, whereas the C-terminal inhibitor, novobiocin, inhibited each isoform equally.

High Levels of Yeast p23 (Sba1) Expression Buffer the Effects of the N-Terminal Binding Hsp90 Inhibitors. Our previous studies demonstrated that Sba1 influenced AhR signaling in yeast. Deletion of *sba1* resulted in a 40 to 60% reduction in AhR-mediated signaling, and overexpression of either human or yeast p23 restored AhR signaling to normal levels (Cox and Miller, 2002). Whether deletion of *sba1* (p23) affected the sensitivity of the AhR signaling pathway to the effects of the Hsp90 inhibitors was not known. Thus, we assayed the wild-type strain (YMC1) and *sba1* deletion strain (YMC2) for AhR signaling in the presence or absence of the indicated concentrations of radicicol, herbimycin A, and novobiocin (Fig. 6A). Again, the data presented for

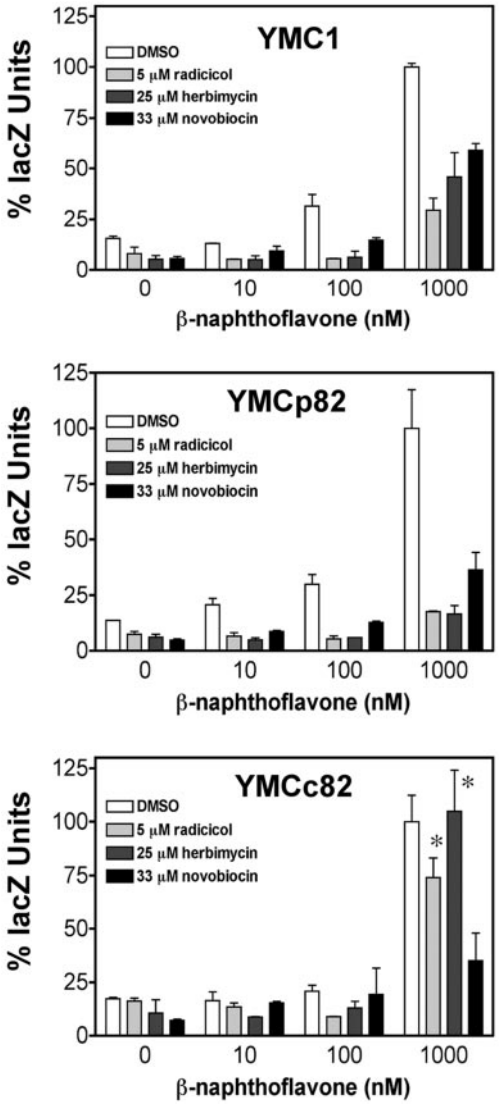

Fig. 3. Yeast Hsp90 isoprotein-AhR complexes display differences in sensitivity to the N-terminal binding Hsp90 inhibitors. The wild-type (YMC1), *hsp82* deletion strain (YMCp82), and *hsc82* deletion strain (YMCc82) were assayed for ligand-dependent, AhR-mediated expression of the AHRE-lacZ reporter gene in the presence of the indicated concentrations of Hsp90 inhibitors. Open bars represent the responses of cells to treatments with DMSO containing increasing amounts of ligand. The lighter, darker, and black bars reflect the responses to increasing ligand treatments in the presence of radicicol, herbimycin A, and novobiocin, respectively. All data from cells treated with inhibitors and ligand were normalized as percentages to the data obtained from cells treated with the highest dose of ligand alone (100% lacZ units). AhR signaling in the *hsc82* deletion strain (YMCc82) in the presence of radicicol and herbimycin A was significantly higher ($p < 0.05$, indicated by asterisks) than that in the wild-type (YMC1) and *hsp82* deletion strains (YMCp82). AhR signaling in the presence of novobiocin was not significantly different among the strains ($p > 0.05$).

each yeast strain were normalized to the lacZ signaling in the absence of inhibitor for each strain. The reductions in AhR signaling observed in the YMC1 and YMC2 strains treated with 5 μM radicicol, 25 μM herbimycin A, and 33 μM novobiocin were not statistically different. Thus, the wild-type and *sba1* deletion strains were equally sensitive to the effects of the inhibitors. Given that AhR signaling was reduced to near background levels in the presence of 5 μM radicicol and 25 μM herbimycin A, we questioned whether sensitivity differences between the YMC1 and YMC2 strains would become apparent in the presence of lower concentrations of the inhibitors that allowed for higher levels of AhR signaling. To this end, we assayed the YMC1 and YMC2 strains for AhR signaling in the presence of a range of inhibitor concentrations in an attempt to identify those concentrations at which AhR signaling might differ between the two strains. AhR signaling was not different between the YMC1 and YMC2 strains at other radicicol concentrations tested (0.08–5 μM). Similar experiments were also conducted in the presence of herbimycin A. For a given dose of herbimycin A, there was no difference in the level of AhR signaling in YMC1 and YMC2 (0.4–25 μM) with the exception of the 12.5 μM dose (data not

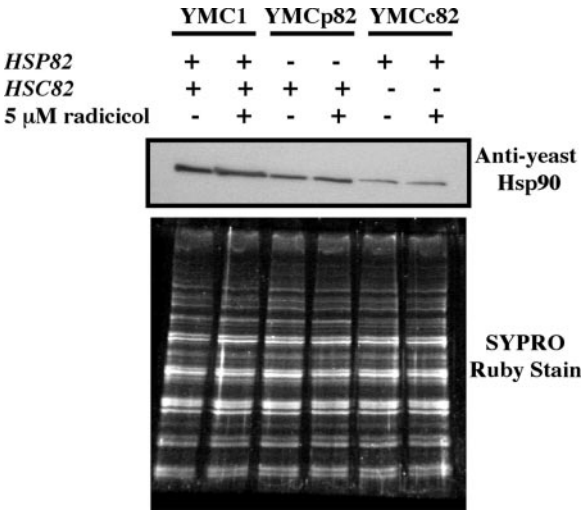

Fig. 4. A low concentration of radicicol does not increase Hsp90 levels. An Hsp90 immunoblot analysis of cell lysates from the YMC1 (*HSP82*/*HSC82*), YMCp82 (*hsp82*/*HSC82*), and YMCc82 (*HSP82*/*hsc82*) strains grown for 16 h in the presence or absence of 5 μM radicicol is shown. The bottom shows a stained replica of the blotted gel that contains equal amounts of the yeast cell lysates (2 μg protein/lane).

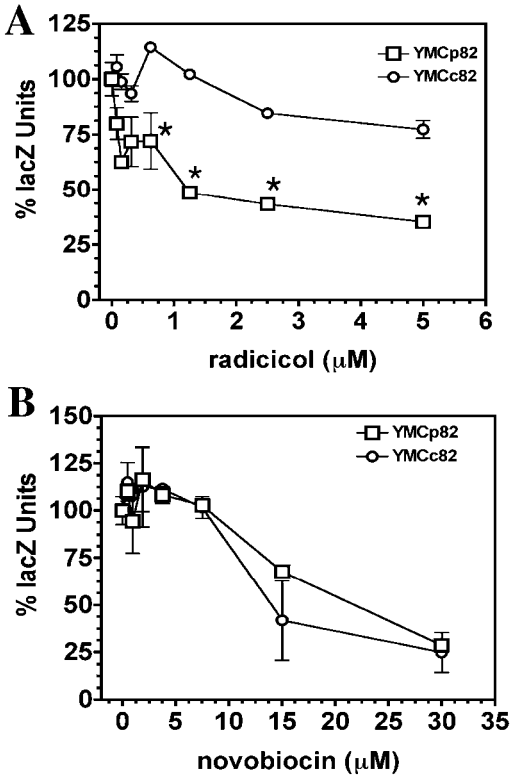

Fig. 5. Dose-dependent effects of inhibitors on Hsp90 isoprotein-AhR complexes. A, cells expressing either Hsc82 (YMCp82, square symbols) or Hsp82 (YMCc82, open symbols) were treated with 1 μM β-naphthoflavone ligand along with increasing amounts of radicicol. AhR signaling in cells treated with ligand was normalized as percentage of lacZ units. Radicicol treatments of 0.8 μM and greater selectively depressed AhR signaling in the YMCp82 strain ($p < 0.05$, indicated by asterisks). B, treatment with increasing amounts of novobiocin under conditions as described for Fig. 5A did not differentially affect AhR signaling in yeast expressing Hsc82 or Hsp82.

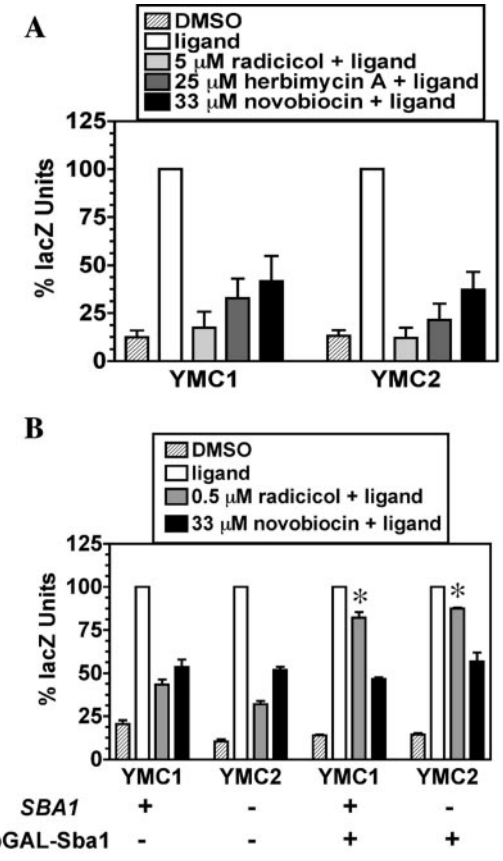

Fig. 6. Effects of p23 (Sba1) on the sensitivity of yeast to the effects of the Hsp90 inhibitors. A, the wild-type (YMC1) and *sba1* (p23) deletion (YMC2) strains were assayed for ligand-dependent, AhR-mediated expression of the AHRE-lacZ reporter gene in the presence of the indicated concentrations of Hsp90 inhibitors. AhR signaling was assayed as in Fig. 3. AhR signaling in YMC1 and YMC2 was not significantly different ($p > 0.05$) in the presence or absence of radicicol, herbimycin A, and novobiocin. B, the wild-type (YMC1) and *sba1* (YMC2) deletion strains in the presence or absence of a high-copy *SBA1* expression vector (pGAL-Sba1) were assayed for ligand-dependent, AhR-mediated expression of the AHRE-lacZ reporter gene in the presence of the indicated concentrations of Hsp90 inhibitors. AhR signaling in YMC1 and YMC2 overexpressing *SBA1* was significantly less sensitive to the effects of radicicol compared with that in the YMC1 and YMC2 strains alone ($p < 0.05$, indicated by asterisks).

shown). At 12.5 μM herbimycin A, AhR signaling in YMC1 was unaffected, and AhR signaling in YMC2 was reduced by ~50%. Thus, there was a narrow window in which the *sba1* deletion strain was more sensitive to the effects of herbimycin A.

The binding sites for radicicol and herbimycin A are within the p23 binding site on Hsp90 (Fig. 1A). We questioned whether p23 (*SBA1*) overexpression could buffer the inhibitory effects of radicicol and herbimycin A on AhR signaling. The wild-type strain (YMC1) and the *sba1* deletion strain (YMC2) with a high copy *SBA1* expression vector (pGal-Sba1) were assayed for AhR signaling in the presence or absence of 0.5 μM radicicol and 33 μM novobiocin. Figure 6B shows the wild-type strain (YMC1) and the *sba1* deletion strain (YMC2) that were overexpressing *SBA1* (p23) were significantly less sensitive to the effects of 0.5 μM radicicol (reduced by ~20% or ~60% with or without the *SBA1* vector, respectively). This result suggests that high levels of Sba1 (p23) buffered the effects of a low dose of radicicol. *SBA1* overexpression also buffered the effects of herbimycin A (data not shown). AhR signaling in the YMC1 and YMC2 strains treated with 33 μM novobiocin was reduced by 40 to 50% regardless of *SBA1* overexpression. Thus, overexpression of *SBA1* had no effect on the ability of novobiocin to inhibit AhR signaling in either strain.

Discussion

Hsp90 is a molecular chaperone that is required for the action of a diverse set of intracellular signaling proteins. Two closely related isoforms of Hsp90 are differentially expressed in all eukaryotes. It is generally assumed that, apart from differences in expression levels, the two isoforms are functionally indistinguishable. We demonstrated that the more abundant Hsc82 isoform in yeast was needed for normal AhR signaling (Fig. 2B). However, deletion of Hsp82 had no effect on AhR signaling (Fig. 2B). Our results from the analysis of Hsc82 and Hsp82 protein levels were consistent with the idea that Hsc82 is the predominant Hsp90 isoform under nonstress conditions in yeast. In the *hsp82* deletion strain, Hsc82 protein levels were similar to the combined levels of both isoforms in the wild-type strain (Fig. 2C). Either *HSC82* expression was up-regulated to make up for the loss of *HSP82*, through some compensatory mechanism, or Hsc82 typically constitutes ~90% of the total Hsp90 present in wild-type cells. On the other hand, the Hsp82 levels in the *hsc82* deletion strain were only ~50% that of the combined levels of both isoforms in the wild-type strain (Fig. 2C). Thus, induction of *HSP82* did not completely compensate for the loss of the *HSC82* gene. We assume that the *HSP82* gene is up-regulated because the levels of Hsp90 from strains expressing only one isoform equal ~150% when added. Therefore, the differences in AhR signaling in the two deletion strains may result from differences in Hsc82 and Hsp82 protein levels, may be caused by functional differences between the two isoforms, or both. Yeast can tolerate a 95% reduction in Hsp90 protein levels under nonstress conditions without compromising viability (Picard et al., 1990; Xu and Lindquist, 1993). If Hsc82 and Hsp82 are functionally indistinguishable, our results suggest that only a 50% reduction in Hsp90 levels compromised AhR signaling. Alternatively, if there are subtle functional differences in the AhR-Hsc82 isoform complexes, overexpression of the Hsp82 isoform largely corrects the deficit.

A recent study by Piper et al. (2003) demonstrated that yeast expressing human Hsp90β as the sole source of Hsp90 are hypersensitive to the effects of the Hsp90 inhibitors with respect to cell viability. In contrast, we found that yeast expressing only the Hsp82 isoform as the sole source of Hsp90 was hyposensitive to the AhR-specific effects of the Hsp90 inhibitors (Fig. 3). An alternative interpretation of the data in Fig. 3 is that the Hsc82 isoform is hypersensitive to the effects of the N-terminal Hsp90 inhibitors. Thus, use of the inhibitors identified Hsp90 isoform-specific effects on an Hsp90 client protein signaling pathway.

Hsp90 inhibitors were shown to enhance the levels of Hsp90 in cells by inhibiting the Hsp90-mediated repression of the Hsf1 signaling pathway (Kim et al., 1999; Bagatell et al., 2000). Thus, it was possible that the differences in sensitivity between the *hsc82* and *hsp82* deletion strains were caused by changes in Hsp90 levels. However, we showed that the Hsp90 levels were unchanged in cells treated with radicicol (Fig. 4). It is likely that the up-regulation of Hsp90 would become evident at higher concentrations of the inhibitors where general effects on transcription and cell growth are also apparent. If the effects observed were caused by differences in Hsp90 levels, one would expect the strain expressing the lowest levels of Hsp90 (YMCc82) to be the most sensitive to the effects of the inhibitors. However, the *hsc82* deletion strain (YMCc82) was the least sensitive to the effects of the inhibitors. Thus, we found differences in the sensitivity of yeast expressing different Hsp90 isoforms with respect to AhR signaling.

Yeast with a deletion of the p23 gene (*SBA1*) were reported to be hypersensitive to the effects of the ansamycin antibiotics with respect to glucocorticoid receptor signaling (Bohen, 1998). Furthermore, the wild-type strain was unaffected by the presence of the ansamycin antibiotics. Thus, the yeast p23 gene was named *SBA1* (sensitivity to benzoquinone ansamycin antibiotics). However, the wild-type strain used in those studies was an *sba1* deletion strain that overexpressed *SBA1* from the strong glyceraldehyde phosphate dehydrogenase promoter. Because the glyceraldehyde phosphate dehydrogenase promoter provides for high levels of protein expression, it is likely that this strain actually expressed high levels of the Sba1 protein. We are not able to make direct comparisons between our study and that of Bohen (1998) because we have assessed the action of Hsp90 inhibitors on a different receptor pathway. However, our results using AhR signaling as a reporter of Hsp90 function suggest that both the wild-type strain and the p23 deletion strain are equally sensitive to the effects of the hsp90 inhibitors (radicicol, herbimycin A, and novobiocin) (Fig. 6A). In our case, the wild-type strain expressed a normal level of Sba1 from the endogenous chromosomal locus. Thus, the mechanism by which the Hsp90 inhibitors affect AhR signaling is largely independent of p23 (Sba1). The reductions in AhR signaling observed in the absence of p23 may reflect the loss of ATP binding to Hsp90 proteins or other mechanisms. Although the mechanisms by which radicicol and herbimycin A inhibit Hsp90-dependent signaling pathways seem to be independent of endogenous p23 (Sba1), it is possible that high levels of p23 can form stable Hsp90-client protein complexes that are unaffected by the inhibitors (Fig. 6B). Overexpression of

SBA1 did not change the sensitivity of the cells to the effects of novobiocin, which is consistent with the idea that novobiocin acts through a different mechanism(s) with respect to Hsp90-dependent processes.

The results presented here provide new insight into the mechanisms by which three structurally unrelated Hsp90 inhibitors (radicicol, herbimycin A, and novobiocin) disrupt Hsp90-dependent signaling pathways. Our data suggest that the specific Hsp90 isoform composition and levels of p23 protein in a cell can influence the ability of a client protein to function properly and thus can alter the apparent potency and efficacy of a given concentration of ligand. The Hsp90 inhibitors have been exploited for their antitumor properties, which results from their inhibition of the Hsp90-dependent client proteins involved in tumorigenesis (Isaacs et al., 2003). Our findings may be relevant to cancer therapies that are based on inhibition of Hsp90-client protein regulation. If the inhibitors differentially affect the Hsp90 isoforms in human cells, then Hsp90 isoform levels should be taken into account when developing these drugs for use in specific tumor tissues. Furthermore, we would predict that the N-terminal Hsp90 inhibitors may be less effective in tumors that express p23 at high levels and may be adversely toxic in normal tissues that express low levels of p23.

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