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A simple yeast-based system for analyzing inhibitor resistance in the human cancer drug targets Hsp90α/β

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ABSTRACT

Heat shock protein 90 (Hsp90), a highly conserved molecular chaperone, is one of the most promising targets for cancer drug development. Whether any resistance to these Hsp90 inhibitor drugs could arise by Hsp90 mutation is still unknown. Yeast is readily engineered so that its essential Hsp90 function is provided by either isoform of the human cytosolic Hsp90, Hsp90α or Hsp90β. However, its high intrinsic resistance to most drugs poses a major obstacle to the use of such Hsp90α- or Hsp90β-expressing yeast cells as a model system to analyse whether drug resistance might arise by Hsp90 mutation. In order to overcome this problem, we have generated a strain that is both hypersensitive to Hsp90 inhibitors as it lacks multiple drug resistance genes, and in which different heterologous and mutant Hsp90s can be expressed by plasmid exchange. It is not rendered appreciably stress sensitive when made to express Hsp90α or Hsp90β as its sole form of Hsp90. Should there be any development of resistance to the Hsp90 drugs now in cancer clinic trials, this system can provide a rapid initial test of whether any single nucleotide polymorphism appearing within the coding regions of Hsp90α or Hsp90β could be a contributory factor in this resistance. We have used this strain to demonstrate that significant levels of resistance to the Hsp90 inhibitors radicicol and 17-allylamino-demethoxygeldanamycin (17-AAG) are generated as a result of the same single point mutation within the native Hsp90 of yeast (A107N), the human Hsp90α (A121N) and the human Hsp90β (A116N).

1. Introduction

Heat shock protein 90 (Hsp90) orchestrates a multi-stage chaperone cycle, essential for the final maturation, stabilisation and localization events of a diverse set of important proteins in eukaryotic cells. It is increasingly attracting attention as a promising target for cancer drug development, since many of the proteins responsible for the oncogenic phenotype of cancer cells are highly dependent upon Hsp90 for their activity. In cancer cells treated with highly-selective inhibitors of this chaperone, several oncogenic activities are inactivated and destabilized simultaneously, enabling such drugs to cause the combinatorial depletion of many cancer-causing pathways and a modulation of all of the hallmark traits of malignancy [1–3]. Fortuitously, Hsp90 inhibitors also show a high selectivity for cancer versus normal cells [4,5] and a therapeutic activity at doses that are well tolerated in cancer patients [6].

Inhibitors of Hsp90 include the natural antibiotics geldanamycin (GdA), monocillin 1 and radicicol/monorden (RAD). These, together with the most promising synthetic Hsp90 inhibitor drugs [7,8], bind within the highly-conserved ATP binding site of the Hsp90 N-terminal domain [9–13]. Cancer clinic trials of both derivatives of GdA [14], as well as of purine and 4,5-diaryisoxazole resorcinol Hsp90 inhibitors based on the interactions of RAD, are now well advanced [6,15,16].

Cancer chemotherapy is often compromised by the development of drug resistance. In cell cultures, a partial resistance to one of the GdA derivatives now in clinical trials, 17-allylamino-demethoxygeldanamycin (17-AAG), is able to develop through an altered expression of NAD(P)H:quinone oxidoreductase 1 [17,18]. However, whether any appreciable resistance to Hsp90 inhibitors could arise by mutation to tumour Hsp90 is still unknown. Part of the strong case for Hsp90 drug development is the prediction that the probability of such an occurrence might be relatively low. The amino acid residues that facilitate drug interactions within the nucleotide binding site of Hsp90 are generally highly conserved in Hsp90-family proteins from bacteria to man [19], such that mutational changes that compromise drug binding would mostly be expected to inactivate this essential chaperone. However, since

Abbreviations: RAD, radicicol; GdA, geldanamycin; 17-AAG, 17-allylamino-demethoxygeldanamycin; DMSO, dimethylsulphoxide; 5-FOA, 5-fluoroorotic acid.

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the cytosolic Hsp90s that are major targets of the cancer drug therapy (Hsp90α, Hsp90β) appear to be encoded by just three genes in the human genome [20], it needs to be investigated if any drug resistance could arise this way. It has already been shown that certain naturally occurring single nucleotide polymorphisms (SNPs) within the Hsp90α coding region probably act to compromise the activity of this isoform *in vivo* [21,22].

These two forms of human cytosolic Hsp90, Hsp90α and Hsp90β, are each readily expressed as the sole Hsp90 of yeast [23–26]. However, the extreme pleiotropic drug resistance of yeast cells poses a major obstacle to exploiting this as a model system to investigate whether Hsp90 might be rendered drug resistant by mutation. Though yeast is rendered more sensitive to RAD and GdA with loss of the Pdr5 ATP-binding cassette (ABC) transporter [24], it is often still difficult to demonstrate inhibitory effects even in strains of a *pdr5Δ* mutant background. During our recent analysis of a mutation causing partial RAD resistance in a fungal Hsp90 [27], the intrinsic resistance of the most resistant *pdr5Δ* strains made it almost impossible to measure their IC50 for RAD inhibition of growth. With GdA, this problem is even more acute. Yeast is so resistant to GdA, that PDR5+ and *pdr5Δ* cells have been reported as growing at 2 mg ml^{-1} [28] and 0.14 mg ml^{-1} [27] GdA, respectively. To overcome this problem, we have generated a robust strain that is at least an order of magnitude more sensitive than normal to Hsp90 inhibitors, a strain in which different heterologous and mutant Hsp90s can be expressed by plasmid exchange. It is not rendered appreciably stress sensitive when made to express the human Hsp90α or Hsp90β as its sole Hsp90. In this paper, we describe its use to identify a mutation causing significant levels of inhibitor resistance in these human Hsp90s. To the best of our knowledge, this is the first use of the yeast model system to identify a mutation that has the potential to cause drug resistance in a cancer drug target.

2. Materials and Methods

2.1. Yeast transformation and growth

All yeast transformation was by standard procedures [29] except that, in view of the enhanced drug sensitivity of the strains used [30], transformants were selected on YPD (2% (w/v) glucose, 2% bactopeptone, 1% yeast extract, 20 mg l^{-1} adenine, 1.5% agar) plates containing lower levels of antibiotic than normal (100 μg ml^{-1} G418 or hygromycin B [both purchased from Melford Laboratories Ltd., Ipswich, UK]). Correct gene integrations were confirmed by colony PCR [31]. For tests of stress sensitivity, serial dilutions of overnight YPD cultures were pronged onto YPD 1.5% agar plates, these plates then being grown under the stated conditions

2.2. Construction of yeast strain PP1-9p/c[pHSC82]

Initially, *LEU2* coding sequences were deleted in strain AD1-9 using a PCR-generated *kanMX4* cassette amplified from pUG6 [32], thereby generating PP1-9α (Table 2). Next, this *leu2Δ* strain was diploidised by transformation with a *LEU2* YEp vector containing the *HO* gene (YEpHO [33]). Curing of this vector, sporulation and tetrad dissection yielded PP1-9a. Subsequent deletion of *HSP82* or *HSC82* coding sequences in PP1-9α or PP1-9a respectively, using PCR-generated *hphMX4* cassettes amplified from pAG32 [29], was used to generate the haploid strains PP1-9α-hspΔ and PP1-9a-hscΔ. Subsequent mating of PP1-9α-hspΔ and PP1-9a-hscΔ generated the diploid PP1-9a/α-hsp/hsc (Table 2). This diploid was transformed to uracil prototrophy using plasmid pHSC82 [34]; then sporulated and tetrad dissected to yield the haploid strains PPa1-9p/c[pHSC82] and PPα1-9p/c[pHSC82] (Table 2), each carrying deletions in both chromosomal genes for Hsp90 but with their essential Hsp90 function now being provided by the *HSC82* gene of plasmid pHSC82.

2.3. Wild type and mutant genes for Hsp82, Hsp90α and Hsp90β

PCR templates for yeast *HSP82* gene amplification were the previously-described pRSETA-based vectors for *Escherichia coli* expression of wild-type and A107N mutant forms of yeast Hsp82 (pRSETA-p90) [35,27]. Templates for wild-type and A116N human Hsp90β gene amplification were also the pRSETA-Hu90β vectors from an earlier study [36]. Templates for Hsp90α gene amplification were either TOPO-XL (Invitrogen) or pRSETA clones of this gene (Topo-HuHsp90α, pRSETA-Hu90α, respectively). Topo-HuHsp90α was constructed by amplification of the Hsp90α open reading frame from cDNA (forward primer 5′CGGACGGGGATCCGCTAGC*CTGGAAGTTCTGTTCCAGGGGCCC*ATGCCTGAGGAAACCCAG [NheI restriction site underlined, PreScission cleavage site in italics] and reverse primer 5′TTAGACGTCTTAGTCTACTTCTTCCATGCG), followed by a cloning of this PCR product into vector TOPO-XL. Subsequent ligation of the Topo-HuHsp90α insert released by NheI/PstI digestion into pRSETA then yielded pRSETA-Hu90α, the latter also a vector for the expression of N-terminally His_6-tagged human Hsp90α in *E. coli*.

Single amino acid changes were generated in pRSETA-Hu90α using the QuickChange mutagenesis system (Stratagene) and the primers in Table 1A. All mutations were confirmed by dye-terminator sequencing.

Table 1
PCR primers.

Primer	Sequence
A. Primers for site-directed mutagenesis of the Hsp90α gene (mutagenised codon in bold)	
A121N-Hsp90αF	TCTGGGACCAAAGCGTTCATGGAA**AAT**TTGCAGGCTGGTGCAGATATCTCT
A121N-Hsp90αR	AGAGATATCTGCACCAGCCTGCAA**ATT**TTCCATGAACGCTTTGGTCCCAGA
B. Primers used in PCR amplification of Hsp90 genes for recombination cloning in yeast (homology to pHSCprom in italics; start and stop codons in bold).	
HR-Hsp82F	*ACAGAACCAATAGAAAAATAGAATCATTCTGAAAT***ATG**GCTAGTGAAACTTTTG
HR-Hsp82R	*CATAAATCATAAGAAATTCGCCCGGAATTAGCTTGG***CTA**ATCTACCTCTTCCATTTCGG
HR-HuHsp90αF	*ACAGAACCAATAGAAAAATAGAATCATTCTGAAAT***ATG**CCTGAGGAAACCCAGACC
HR-HuHsp90αR	*CATAAATCATTAAGAAATTCGCCCGGAATTAGCTTGG***TTA**GTCTACTTCTTCCATGCGTG
HR-HuHsp90βF	*ACAGAACCAATAGAAAAATAGAATCATTCTGAAAT***ATG**CCTGAGGAAGTGCACC
HR-HuHsp90βR	*CATAAATCATAAGAAATTCGCCCGGAATTAGCTTGG***CTA**ATCGACTTCTTCCATGCG
C. Primers used in "bridge" PCR amplification of chimeric Hsp90 genes for recombination cloning in yeast	
HuHsp90αLinkF	GATGATGAGGCTGAAGAA
HuHsp90αLinkR	TTCTTCAGCCTCATCATC
HuHsp90βLinkF	GATGATGAGGCAGAGGAA
HuHsp90βLinkR	TTCCTCTGCCTCATCATC

2.4. Construction of strains expressing mutant forms of Hsp82, Hsp90α and Hsp90β

To generate a set of isogenic yeasts that express, as their sole Hsp90, the mutant Hsp90s in Table 3, each mutant Hsp90 gene was initially PCR amplified from the relevant *E. coli* vector, using the primer pairs in Table 1B. Hsp82 genes were amplified using HR-Hsp82F/R (Table 1B) and either wild-type or mutagenised pRSETA-p90 as template; human Hsp90β genes were amplified using HR-HuHsp90βF/R and wild-type or mutagenised pRSETA-Hu90β as template; and human Hsp90α genes were amplified using HR-HuHsp90αF/R and mutagenised pRSETA-Hu90α as template. Each of the resultant PCR products contained a full-length Hsp90 gene (but lacking the N-terminal His_6 encoded in the *E. coli* plasmid), as well as 35–37 bp terminal homologies to sequences either side of the *Pst1* site on plasmid pHSCprom [34]. Next, strain PP1-9a-p/c[pHSC82] was transformed to leucine prototropy with each of these PCR products and *Pst1*-linearised pHSC82prom [34]. Finally, the transformants selected on the minus leucine plates were cured of their original pHSC82 vector by growth on plates containing 5-fluoroorotic acid (5-FOA) as previously described [34,37]

2.5. Construction of strains expressing Hsp90α/Hsp90β chimeras

Human Hsp90α/β and Hsp90β/α domain swops were constructed using a conserved 6-amino acid region (DDEAEE) in the charged linker of these Hsp90s as the cross-over point. Using HuHsp90α as template, the Hsp90α N-terminal domain (amino acids 1–237) coding sequence was PCR amplified using HR-HuHsp90αF/HuHsp90αLinkR (Table 1C); the Hsp90α C-terminal domain (amino acids 232–732) sequence amplified using HuHsp90αLinkF/HR-HuHsp90αR. Using pRSETA-Hu90β as template, the Hsp90β N-terminal domain (amino acids 1–232) was amplified with (HR-HuHsp90βF/HuHsp90βLinkR) (Table 1C); the Hsp90β C-terminal domain (amino acids 227–724) with HuHsp90βLinkF/HR-HuHsp90βR. Chimera genes were constructed by overlap PCR, using the relevant N- and C-template DNAs and either the corresponding forward and reverse PCR primers (HR-HuHsp90α/β, forward and reverse). The amplified chimerical genes were then cloned into pHSCprom in yeast, as described above.

2.6. Measurement of Hsp90 levels

Protein extracts were prepared and 20 μg samples of total cell protein western blotted as previously described [24,25]. Detection used a rabbit polyclonal antiserum raised in this laboratory against full-length, bacterially-expressed Hsp82 protein, an antiserum that recognizes the human Hsp90α and Hsp90β with similar efficiencies. Actin loading control detection used a mouse monoclonal antiserum raised against chicken gizzard actin (AbCam 2Q1055, 1:3000 dilution).

2.7. Analysis of inhibitor sensitivity

RAD and 17-AAG (both from Melford Laboratories Ltd., Ipswich, UK) were dissolved in dimethylsulphoxide (DMSO) to give 5 mg ml^{-1} stock solutions. Halo assays of drug inhibition on plates were as previously described [24]. For liquid growth at 28 °C, overnight synthetic defined (SD) complete medium [38] cultures were diluted in liquid SD to an optical density at 595 nm of 0.05, then transferred to 96 well microtitre plates (150 μl per well) containing increasing levels of RAD, 17-AAG or vehicle DMSO. After 42 h at 28 °C, the cells were resuspended by agitation and their final cell density monitored as optical density at 595 nm.

The data in Figs. 3b,c and 4a–c corresponds to that of eight replicate growths, expressed relative to the DMSO controls as a percentage of growth in the absence of inhibitor. Nonlinear regression dose-response curves; also IC50 values (the concentration of inhibitor leading to a growth half-way between minimal and maximal growth) were initially calculated for each of these eight replicate cultures using SigmaPlot; the mean and standard deviation then being plotted. *P* values were calculated using a two-sample *t*-test applied to the IC50 values for these replicate growths of cells expressing wild-type versus mutant chaperone, a $P > 0.05$ being deemed no evidence of a significant dose-response difference for the cells expressing the mutant Hsp90.

3. Results

3.1. A drug hypersensitive yeast that allows the expression of heterologous and mutant Hsp90s by plasmid exchange

The multiple ATP-binding cassette (ABC) transporter proteins of *Saccharomyces cerevisiae* are a major reason why this species exhibits such high pleiotropic drug resistance [39]. In an attempt to obtain an *S. cerevisiae* more sensitive to Hsp90 inhibitors, we investigated a set of strains which had been engineered – by sequential gene deletion – to lack several of these transporter proteins [30]. The multiple deletant AD1-9 (Table 2) lacks seven ABC transporters (Yor1, Snq2, Pdr5, Pdr10, Ycf1, Pdr11 and Pdr15); as well as two homologous transcription factors responsible for the activation of several of the genes for ABC transporters and for the major facilitator proteins involved in drug transport (Pdr1 and

Table 2
The yeast strains used in this study.

Strain	Genotype	Reference
2229-5C wild type	MATa *PDR1 ura3*	[30]
pdr1-3 strains		
US50-18C	MATα, *pdr1-3 ura3 his1*	[30]
AD1-8	MATα US50-18C *yor1ΔhisG snq2ΔhisG pdr5ΔhisG pdr10ΔhisG pdr11ΔhisG ycf1ΔhisG pdr15ΔhisG pdr3ΔhisG*	[30]
PDR5-6HIS	US50-18C *yor1ΔhisG snq2ΔhisG pdr5ΔPDR5-6HIS pdr10ΔhisG pdr11ΔhisG ycf1ΔhisG pdr15ΔhisG pdr3ΔhisG*	[30]
pdr1Δ strains		
AD1-9	MATα US50-18C *yor1ΔhisG snq2ΔhisG pdr5ΔhisG pdr10ΔhisG pdr11ΔhisG ycf1ΔhisG pdr15ΔhisG pdr1ΔhisG pdr3ΔhisG*	[30]
PP1-9α	MATα AD1-9 *leu2ΔkanMX4*	This study
PP1-9a	MATa AD1-9 *leu2ΔkanMX4*	This study
PP1-9α-hspΔ	MATα PP1-9α *hsp82Δkanhph*	This study
PP1-9a-hscΔ	MATa PP1-9a *hsc82Δkanhph*	This study
PP1-9a/α-hsp/hsc diploid	*HSP82/hsp82 hsc82/HSC82* diploid; generated by mating PP1-9α-hspΔ and PP1-9a-hspΔ	This study
PP19a-p/c[pHSC82]	MATa *hsc82Δkanhph hsp82Δkanhph [pHSC82]*; spore segregant of the PP1-9a/α-hsp/hsc diploid transformed with the *URA3* vector pHSC82.	This study
PP19α-p/c[pHSC82]	MATα *hsc82Δkanhph hsp82Δkanhph [pHSC82]*; spore segregant of the PP1-9a/α-hsp/hsc diploid transformed with the *URA3* vector pHSC82	This study

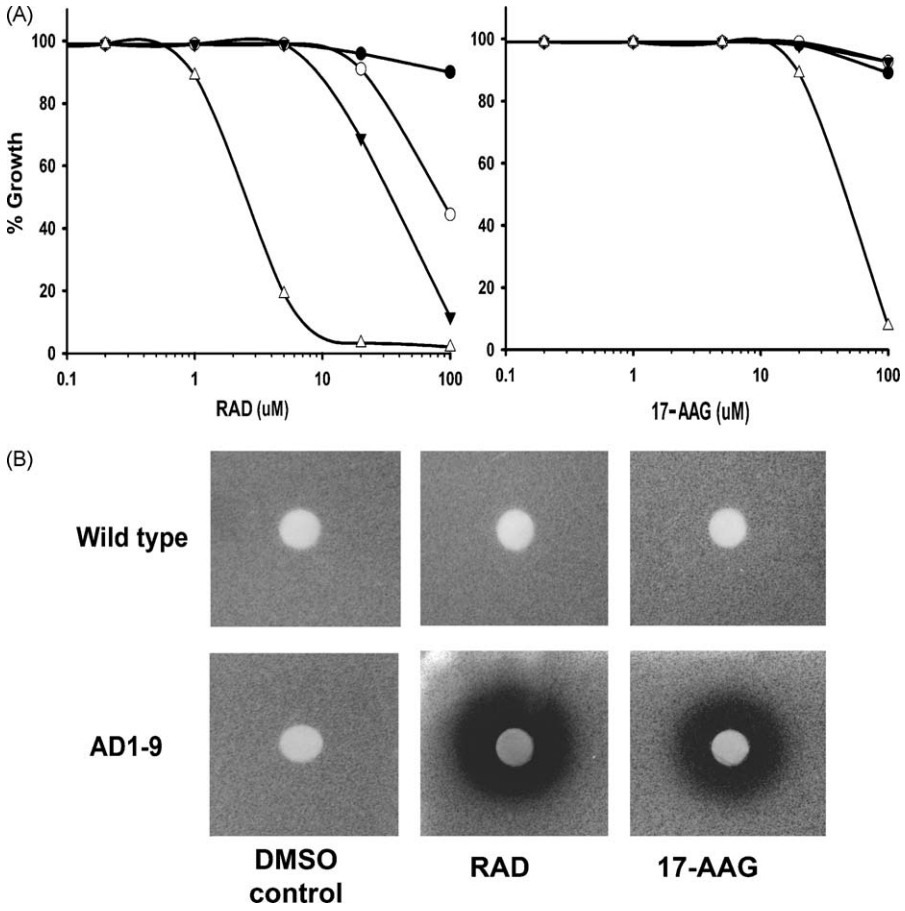

Fig. 1. a: final cell density of the 2229-5C wild-type (•), PDR5-6HIS (○), AD1-8 (▼), AD1-9 (△) grown in the presence of increasing concentrations of RAD or 17-AAG (OD 595 nm relative to DMSO controls, expressed as a percentage of growth in the absence of inhibitor); b: halo assay of the inhibition of wild-type and AD1-9 cells by RAD or 17-AAG. 2.5 μl DMSO, or 5 mg ml^{-1} RAD or 17-AAG in DMSO were added to paper disks placed on a lawn of cells and the plates incubated as previously described [24].

Pdr3)[30]. AD1-9 is sensitized to diverse compounds of different structure and modes of toxicity, its IC50 values for inhibition by ketoconazole and rhodamine 6G being, respectively, 160-fold and 135-fold lower than for the parental strain [30].

Another of the strains from this earlier study, AD1-8 (Table 2), is identical to AD1-9 except that – instead of lacking the *PDR1* gene – it contains a hyperactivated mutant allele of this *PDR1* (*pdr1-3*). This acts to increase the expression of those Pdr1 transcription targets that have not yet been deleted. Yet a third strain, PDR5-6HIS (Table 2), is essentially AD1-8, but possessing – rather than lacking – an important Pdr1 target, the gene for the Pdr5 plasma membrane ABC transporter. This – in view of the presence of the overactive *pdr1-3* allele in PDR5-6HIS – causes this Pdr5 ABC transporter to be highly overexpressed in PDR5-6HIS in the absence of Yor1, Snq2, Pdr10, Ycf1, Pdr11 and Pdr15 [30]. These strains were originally generated partly for such purpose of overexpressing a single, specific ABC transporter to high level, so as to provide a strain of particular use for either biochemical studies on this ABC transporter or for identifying small molecule inhibitors of this transporter.

Preliminary analysis revealed these strains to be considerably more RAD- and 17-AAG sensitive than the 2229-5C wild-type parent, AD1-9 displaying the greatest sensitivity (Fig. 1). The *pdr1-3* allele, present in AD1-8 but lacking in AD1-9, was clearly enhancing the RAD and 17-AAG resistance of AD1-8 (Fig. 1a), an indication that Pdr1-regulated genes other than the deleted *YOR1*, *SNQ2*, *PDR5*, *PDR10*, *YCF1*, *PDR11* and *PDR15* are contributing to resistances to these two Hsp90 inhibitors in yeast. The overexpression of *PDR5* in this AD1-8 genetic background was also enhancing resistance (compare AD1-8 with PDR5-6HIS; Fig. 1a), consistent with observations in other strains that the Pdr5 ABC transporter contributes to Hsp90 inhibitor resistance [24].

Fig. 2 shows the strategy employed in our earlier studies to generate isogenic yeasts that express as their sole Hsp90 – from a single copy gene on the centromeric *LEU2* vector pHSCprom – various heterologous and mutant Hsp90s [27]. The starting point is a *leu2Δ hsp82Δ hsc82Δ* strain that has the two chromosomal genes for Hsp90 (*HSP82*, *HSC82*) deleted, but which is still viable as it carries a copy of *HSC82* on the episomal *URA3* plasmid pHSC82. This is transformed to leucine prototrophy using *Pst*1-linearised pHSCprom, together with a PCR-amplified copy of the Hsp90 gene to be introduced into the yeast. The latter is conveniently amplified from either cDNAs or from vectors designed for the *E. coli* expression of this Hsp90 protein, using primers that possess short (35–37 nucleotide) regions of homology to the cut ends of the linearised pHSCprom. Gap repair (recombinational cloning), then curing of the original *URA3* plasmid by growth on 5-FOA, in turn yields a yeast that contains just the introduced Hsp90 gene on pHSCprom. Furthermore, as this introduced Hsp90 gene is now expressed from the promoter of the *HSC82* gene, the promoter that drives most of the Hsp90 expression in unstressed, wild-type *S. cerevisiae* [40], levels of its encoded Hsp90 in these cells are generally similar to the levels of Hsp90 normally found in yeast.

By strain manipulation (Section 2.1), we generated haploid *leu2Δ hsp82Δ hsc82Δ* derivatives of AD1-9 (both a and α mating types), viable as their essential Hsp90 function is carried on the episomal vector pHSC82 (PP1-9ap/c[pHSC82], PP1-9αp/c[pHSC82], respectively; Table 2). In these drug hypersensitive

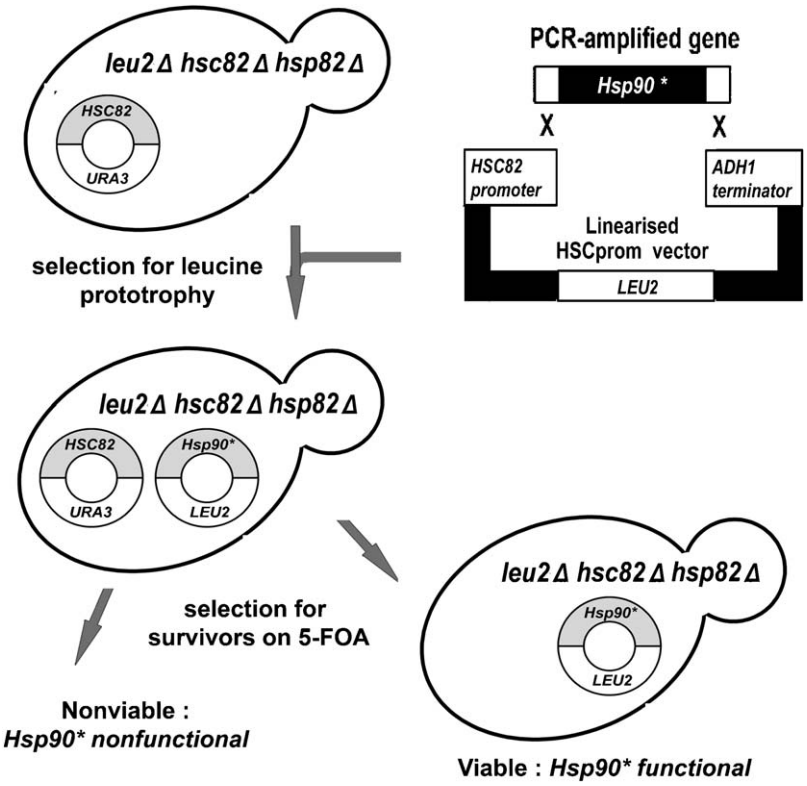

Fig. 2. The strategy used for generating yeast strains that express – as their sole Hsp90 – heterologous and mutant forms of Hsp90. The starting point is a strain that has deletions of the chromosomal Hsp90 genes, but that is viable since it carries the *HSC82* gene on a *URA3* plasmid. *LEU2* vectors for each expression are then generated by transforming this strain to leucine prototrophy a PCR-generated copy of the gene to be inserted (*Hsp90**) and the linearised pHSCprom vector, a process that requires homologous recombinations (X) between these two DNAs. Subsequent growth of the transformants on 5-FOA then cures the cells of their original *URA3* vector.

strains, we could then express different forms of Hsp90 by the strategy in Fig. 2.

3.2. Expression of different Hsp90s in this system

Starting with PP1-9a-p/c[pHSC82] (Table 2), we made strains that express – as their sole Hsp90 – the human Hsp90α or Hsp90β (Section 2.4). We also made strains that express functional chimeras of these Hsp90α and Hsp90β proteins; either Hsp90α/β (the N-terminus [1–237] of Hsp90α joined to the C-terminal region [227–724] of Hsp90β); or Hsp90β/α (the N-terminus [1–232] of Hsp90β joined to the C-terminal region [232–732] of Hsp90α) (Section 2.5)

These four strains were next analysed for Hsp90 expression level (Section 2.6), for their sensitivity to RAD and 17-AAG (Section 2.7) and for their sensitivity to stress. All exhibited similar levels of Hsp90 expression (Fig. 3a). Whether the cells were expressing either Hsp90α or Hsp90β had relatively small effects on their sensitivity to RAD (Fig. 3b). However, it affected their sensitivity to 17-AAG more considerably, the IC50 for 17-AAG inhibition of the Hsp90β-containing cells being some 5.8-fold lower than for the cells containing Hsp90α (Fig. 3c; Table 3). Quite unexpectedly, the expression of the Hsp90α/β and Hsp90β/α chimerical proteins increased cellular resistance to both inhibitors (resulting in an approximate two-fold enhancement in IC50 for RAD and 17-AAG inhibition as compared to the cells expressing Hsp90α [Fig. 3b,c]).

These strains were found not to be compromised in growth high temperature (Fig. 3d), or growth in the presence of a variety of stress agents (1 M sorbitol, 0.1 M lithium chloride, 50 $\mu g\,ml^{-1}$ calcofluor white (data not shown). They were therefore not being rendered appreciably stress sensitive by the possession of Hsp90α, Hsp90β, Hsp90α/β or Hsp90β/α as their sole Hsp90. In previous studies, yeast strains engineered to express either Hsp90α or Hsp90β were often found to display quite marked phenotypes of stress- and RAD-sensitivity [23–26]. It was therefore quite unexpected for us to find that PP1-9p/c cells expressing Hsp90α, Hsp90β, Hsp90α/β and Hsp90β/α were not displaying the same properties, despite this being a genetic background much more sensitive to Hsp90 inhibitors (Fig. 3); also that these cells were instead moderately sensitized to 17-AAG by Hsp90β expression (Fig. 3d). The stress- and RAD-sensitive phenotypes described as being associated with the expression of Hsp90α or Hsp90β in these earlier studies are therefore related to the use of certain strain genetic backgrounds and not an inevitable consequence of expressing these human Hsp90s in yeast.

3.3. A mutation generating significant levels of inhibitor resistance in Hsp90α and Hsp90β

Hsp90 is a dimeric chaperone protein, its two subunits being constitutively dimerised at their C-terminal domains, but undergoing an additional dimerization of their N-terminal domains in response to the binding of ATP [35,36,41]. ATP, but not ADP or drug, binding promotes the remodelling of a loop within these N-terminal domains (residues 94–123 in the yeast Hsp82), whereby this loop forms a "lid" over the bound nucleotide. This loop remodeling exposes small hydrophobic surfaces within the adjacent N-terminal domains which, in turn, self-associate to cause the dimerisation of these domains.

This "ATP-lid" closure creates an ATPase catalytic centre, essential for the ATPase-coupled chaperone cycle. The ease with which ATP-lid closure occurs in response to the binding of ATP can also be measured as the slow turnover, *in vitro* ATPase activity of the purified Hsp90 protein [35–37]. Mutations that strongly favour

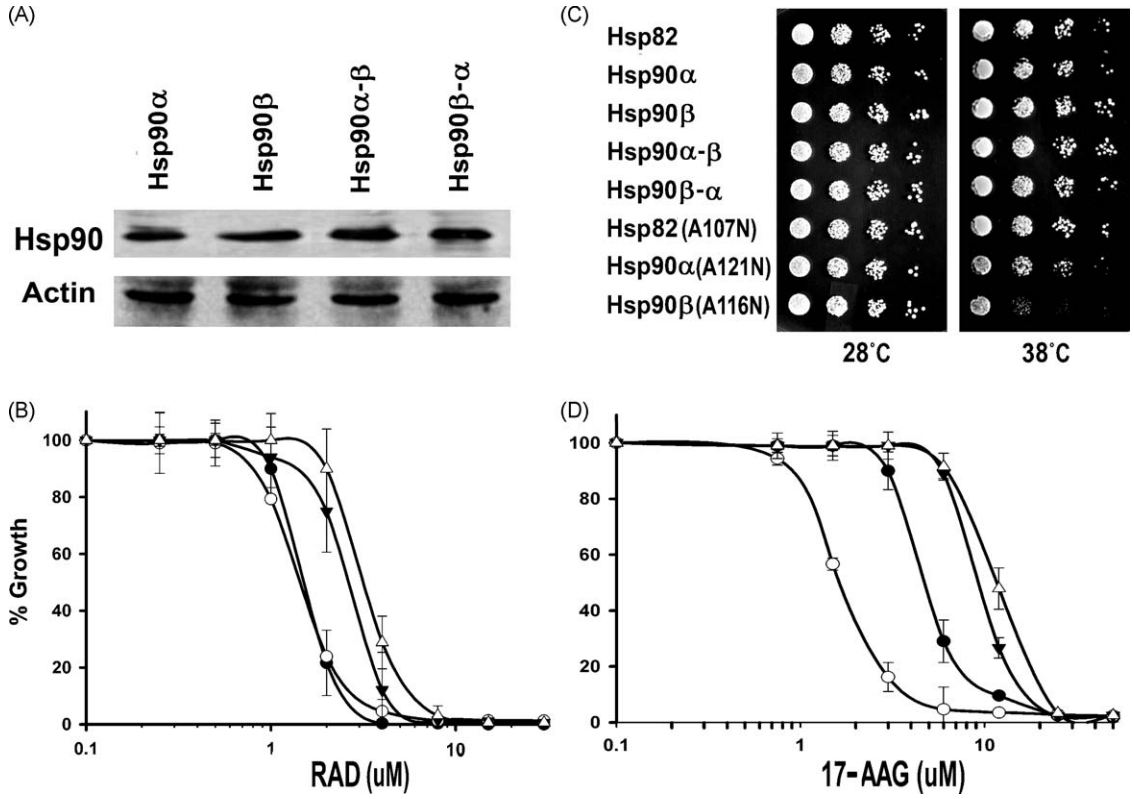

Fig. 3. a: western blot analysis of Hsp90 level in PP1-9a-p/c cells expressing either Hsp90α, Hsp90β, Hsp90α/β or Hsp90β/α; b,c: growth of these cells expressing Hsp90α (•), Hsp90β (○), Hsp90α/β (▼) or Hsp90β/α (△) in the presence of increasing RAD (b) or 17-AAG (c) (mean and standard deviation of 8 replicate growths); b: growth of PP1-9a-p/c cells expressing the indicated Hsp90s, an overnight culture serially diluted, then pinned onto YPD agar and photographed after 2d growth at 28 °C and 38 °C.

the ATP-lid closure/N-terminal domain dimerisation stimulate this activity. One such mutation is A107N in the native yeast Hsp82, an amino acid change that causes an additional hydrogen bond to form with Tyr47 as the chaperone adopts the closed ATP-lid conformation [36].

Since earlier *in vitro* studies had shown that A107N strongly promotes this conformational switching of the chaperone in response to the binding of ATP we considered this to be a good candidate for a mutation that might impact on drug resistance *in vivo*. We therefore expressed the A107N mutant form of the native Hsp82, as well as the corresponding Hsp90α(A121N) and Hsp90β(A116N) mutant forms of the human chaperone, in PP1-9a-p/c. These expressions of either Hsp82(A107N) Hsp90α(A121N) or Hsp90β(A116N) as the sole Hsp90 in yeast were fully compatible with cell viability, though Hsp90β(A116N) rendered the cells slightly temperature sensitive (Fig. 3d). The Hsp90 mutation in these cells containing Hsp82(A107N), Hsp90α(A120N) and Hsp90β(A116N) did not alter their levels of Hsp90, yet it generated significant increases in their resistances to RAD and 17-AAG relative to the cells expressing the corresponding wild-type form of each chaperone (Fig. 4; Table 3). Curiously, this A to N mutational change caused the most dramatic increase in RAD resistance when placed within the native Hsp82, whereas it caused the most marked increase in 17-AAG resistance when placed within Hsp90β (Fig. 4; Table 3).

Table 3
IC50 values for RAD and 17-AAG inhibition of cells expressing different Hsp90s.

Expressed Hsp90; IC50 (μM) and standard deviation		*P* mutant versus wild-type
RAD		
Hsp82	1.21 ± 0.15	<0.001
Hsp82 (A107N)	4.04 ± 0.17	
Hsp90α	3.62 ± 0.14	<0.001
Hsp90α (A121N)	5.98 ± 0.66	
Hsp90β	2.70 ± 0.28	<0.001
Hsp90β (A116N)	4.20 ± 0.36	
17-AAG		
Hsp82	5.32 ± 0.27	<0.001
Hsp82 (A107N)	17.37 ± 0.19	
Hsp90α	10.86 ± 0.43	<0.001
Hsp90α (A121N)	23.93 ± 2.65	
Hsp90β	1.87 ± 0.14	<0.001
Hsp90β (A116N)	7.04 ± 0.33	

4. Discussion

Cultured mammalian cells present a number of experimental difficulties when being considered for any analysis of how a mutational change in a cytosolic Hsp90 might impact on *in vivo* resistance to Hsp90 inhibitors. Invariably these cells will contain – in addition to this introduced mutant Hsp90 – variable amounts of the native Hsp90α and Hsp90β. It is difficult to control for the relative levels of mutant versus native Hsp90α and Hsp90β, to achieve a selective siRNA knockdown of either Hsp90α or Hsp90β, or – as Hsp90 inhibitors strongly induce the heat shock response [42] – to overcome a strong induction of these native forms of Hsp90 as the inhibitor is added to the cells. Often superimposed upon these problems is the difficulty of obtaining comparable and physiological levels of expression of any Hsp90 gene introduced by transfection. All of these factors make it hard to generate good quantitative data that can satisfactorily distinguish the effects on the cells caused by drug inhibition of the mutant Hsp90 from those

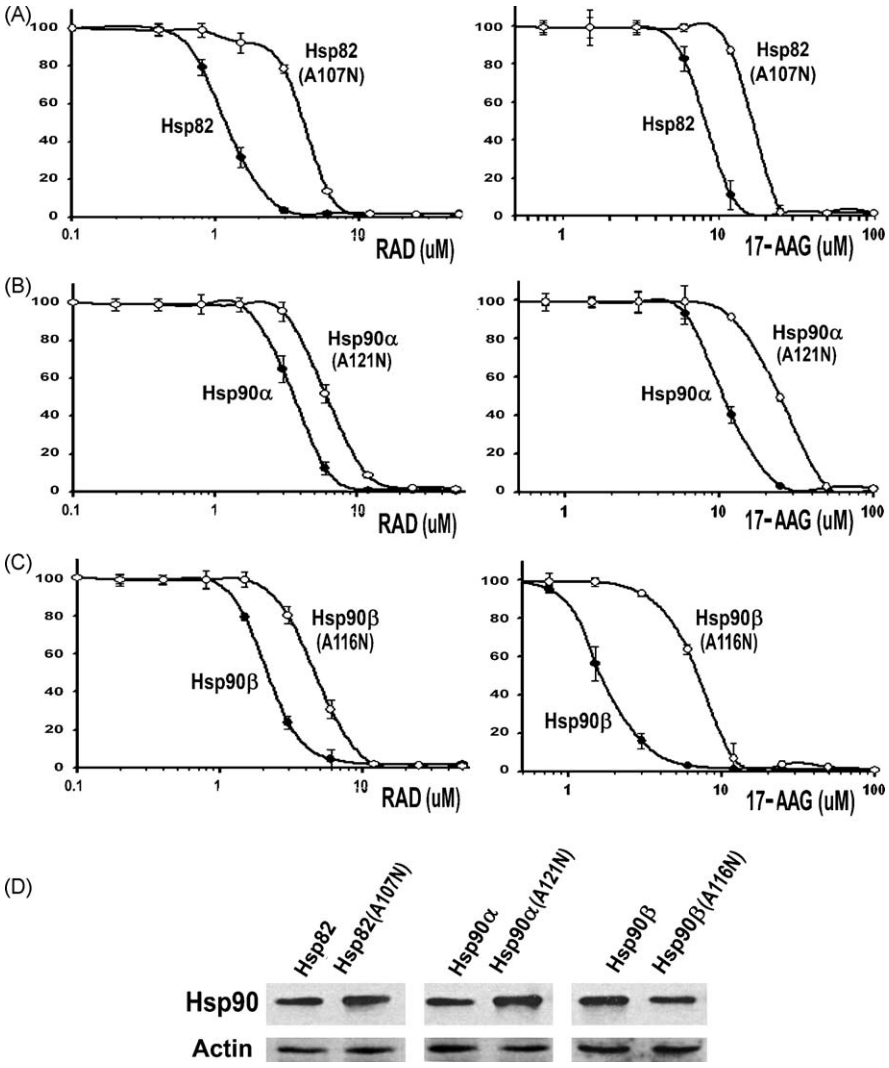

Fig. 4. Growth of PP1-9a-p/c cells expressing (a) Hsp82 (•), Hsp82(A107N) (○); (b) Hsp90α (•), Hsp90α(A121N) (○); and (c) Hsp90β (•), Hsp90β(A116N) (○) in the presence of increasing concentrations of RAD or 17-AAG. (d) Western blot analysis of the relative levels of wild type or mutant Hsp90 in these cells.

that result from the inhibition of these native, wild-type forms of Hsp90.

The yeast system can be designed so as to largely circumvent these problems. Yeast is readily engineered so that:

(i) any heterologous or mutant Hsp90 of interest, provided it is functional, now constitutes 100% of the Hsp90 of the cell (Fig. 2);
(ii) the levels of this Hsp90 drug target are similar in all of the different strains to be compared (Figs. 3 and 4);
(iii) this Hsp90 is now expressed from the promoter that normally drives most of the Hsp90 expression in yeast, a promoter that is induced no more than two- to three-fold with the induction of the heat shock response (Fig. 2).

Furthermore, we have now substantially overcome the two major drawbacks of the Hsp90α- or Hsp90β-expressing yeast strains generated in previous studies [23–26]. Firstly, the phenotypes of stress- and RAD-sensitivity often found to be associated with the expression of human Hsp90α or Hsp90β in these studies are largely absent in the strains used here (Fig. 3). Secondly, due mainly to the loss of multiple ABC transporter activities, the high intrinsic resistance of the yeast to Hsp90 inhibitor drugs is very greatly reduced. As a result it is possible to use these strains to determine relative IC50 values with high levels of confidence, using levels of Hsp90 inhibitor an order of magnitude lower than would be needed with normal yeast strains.

The robust, drug hypersensitive strains generated in this study are easily modified to express different forms of Hsp90, allowing their use in the analysis of how mutational changes to this chaperone might affect inhibitor resistance. We show how, by a single point mutation in the N-terminal domain that reinforces one conformational state of the chaperone, the cytosolic forms of human chaperone can acquire significant levels of resistance to 17-AAG, a drug now well-advanced in cancer clinic trials (Fig. 4). Furthermore, should there be any emergence of Hsp90 drug resistance in cancer studies and SNPs causing sequence changes to Hsp90α or Hsp90β be identified within these resistant cells, this system can provide a very rapid first test of whether these changes are a probable cause of the resistance. It is of course quite conceivable that the resistance might arise in ways other than through Hsp90 mutation, possibly in a manner that involves an alteration to the levels of those cochaperone proteins known to impact on cellular sensitivity to Hsp90 inhibitors [43–45].

All of the materials generated for this study are freely available for academic use. Commercial organizations should contact the corresponding author directly.

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