The complementation of yeast with human or *Plasmodium falciparum* Hsp90 confers differential inhibitor sensitivities

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Developing novel drugs against the unicellular parasite *Plasmodium* is complicated by the paucity of simple screening systems. Heat-shock proteins are an essential class of proteins for the parasite's cyclical life style between different cellular milieus and temperatures. The molecular chaperone Hsp90 assists a large variety of proteins, but its supporting functions for many proteins that are important for cancer have made it into a well-studied drug target. With a better understanding of the differences between Hsp90 of the malarial parasite and Hsp90 of its human host, new therapeutic options might become available. We have generated a set of isogenic strains of the budding yeast *Saccharomyces cerevisiae* where the essential yeast Hsp90 proteins have been replaced with either of the two human cytosolic isoforms Hsp90α or Hsp90β, or with Hsp90 from *Plasmodium falciparum* (Pf). All strains express large amounts of the Flag-tagged Hsp90 proteins and are viable. Even though the strain with Pf Hsp90 grows more poorly, it provides a tool to reconstitute additional aspects of the parasite Hsp90 complex and its interactions with substrates in yeast as a living test tube. Upon exposure of the set of Hsp90 test strains to the two Hsp90 inhibitors radicicol (Rd) and geldanamycin (GA), we found that the strain with Pf Hsp90 is relatively more sensitive to GA than to Rd compared to the strains with human Hsp90's. This indicates that this set of yeast strains could be used to screen for new Pf Hsp90 inhibitors with a wider therapeutic window.

1. Introduction

WHO estimates that over 3 billion people live under the threat of malaria. The malarial parasite *Plasmodium* infects over 500 million people annually, which results in 3 million deaths. Discovering and developing new inhibitors, for example for *Plasmodium falciparum* (Pf), is difficult as screening depends on the costly and laborious maintenance of parasite cultures and on determining parasitemia in rodent models. New molecular targets and experimental systems would be a welcome addition to the toolbox. Since unicellular parasites are exposed to extreme changes in their environment as they cycle between host and vector, their heat-shock proteins may play an important role. The molecular chaperone Hsp90 has been shown to be essential for viability in organisms ranging from yeast to mouse [1–3]. The life cycle of Pf has been shown to be blocked *in vitro* with two chemically distinct Hsp90 inhibitors, radicicol (Rd) and geldanamycin (GA), primarily at the transition from the Ring to the trophozoite stage in erythrocytes [4–7]. The stage-specificity of

the inhibition has been taken as an indication that the drugs might inhibit primarily through the parasite Hsp90.

Because Hsp90 has been recognized as an Achilles heel of cancer cells, there are already a considerable number of drugs available and more are being developed, and clinical trials are currently being conducted with GA derivatives [2,8,9]. These studies have provided a lot of information on the pharmacology of such inhibitors, and so far, they have revealed surprisingly few side effects. In addition, a recent study showed that Hsp90 inhibitors block infection of tissue culture cells by a wide panel of different viruses [10]. Not surprisingly, it has been suggested that the already available Hsp90 inhibitors could be further developed as anti-viral and anti-parasite agents. Hsp90 is highly conserved as illustrated by the fact that GA binds and inhibits both Pf and human Hsp90, and yet, within the constraints of 63% sequence identity at the amino acid level, there should be sufficient sequence and structural differences to identify inhibitors that are selective for the parasite Hsp90 [11].

The budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) was the first organism for which it was shown that Hsp90 is essential for viability [12]. This genetically easily manipulable organism has two genes, *HSC82* and *HSP82*, which encode two 95% identical Hsp90 proteins. Interestingly, heterologous Hsp90 proteins from many species are able to replace Hsp82 and Hsc82 of the budding yeast. These include the two human cytosolic isoforms Hsp90α [13] and

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Hsp90β [14], Hsp90 from *Trypanosoma cruzi* [15], Drosophila [16], *Caenorhabditis elegans* and *Candida albicans* [17]. *S. cerevisiae* lends itself perfectly to explore the functional conservation of proteins from other species, to study their homologous and heterologous biochemical interactions, and to screen for novel species-specific drugs. This provides the rationale for why we decided to generate and to study an isogenic set of yeast strains with human and Pf Hsp90.

2. Materials and methods

2.1. Plasmids

The backbone for all constructs is plasmid pRS313/GPD-PGK (a gift from A. Kralli). It is derived from the low copy number *CEN/ARS* yeast expression vector pRS313 [18] by insertion of the strong constitutive *GPD* promoter from the *TDH3* gene and the *PGK1* terminator. Plasmid pHCA/hHsp90β for expression of untagged human Hsp90β was derived from p2TG/hHsp90β [19] and has been described before [20]. Plasmid pRS313/PfHsp90 for expression of untagged *Pf* Hsp90 was made by transferring the Hsp90 open reading frame (ORF) from plasmid pGEX-PfHsp90 as a PCR fragment containing the sequence GGATC-CAAAAA and an EcoRI site preceding and following the ORF, respectively. For expression of Flag-tagged Hsp90 proteins, the expression vector pHGF was derived from plasmid pRS313/GPD-PGK by replacing the multiple cloning sequences encompassing the BamHI and HindIII sites with the sequence GGATCGCCACC-ATGGATTACAAAGATGATGATGATAAAGGGATCCAAGCTT (sequences encoding initiator methionine and Flag tag are underlined). Hsp90 ORFs were inserted into the BamHI or HindIII sites of pHGF to generate plasmids pHGF/Hsp82, pHGF/PfHsp90, pHGF/HsHsp90α and pHGF/HsHsp90β to express Flag-tagged yeast Hsp82 (one of the two yeast Hsp90 isoforms), *Pf* Hsp90, human Hsp90α and Hsp90β, respectively.

2.2. Yeast strains

All strains are derived from strain pp30#10 [21], which has the following genotype: *MATa ade2-101-o his3-Δ200 leu2-3,112 lys2-801-a trp1-289 ura3-52 Δhsc82::KanMx4 Δhsp82::KanMx4 / 2μ-HSC82-URA3*. It carries deletions of the two yeast *HSP90* genes *HSP82* and *HSC82* and is kept alive by expression of Hsc82 from an episome. To sensitize the strain for Hsp90 inhibitors [22], we deleted the gene encoding the export pump Pdr5 by using homologous recombination to replace the coding region with the loxP-LEU2-loxP cassette from plasmid pUG73 [23]. This generated yeast strain DP533. All others were derived from DP533 by plasmid shuffling. Specifically, strains DP549, DP551, DP552, DP553, DP554, and DP555, referred herein also as P, β, FY, FP, Fβ, and Fα, contain plasmids pRS313/PfHsp90, pHCA/hHsp90β, pHGF/Hsp82, pHGF/PfHsp90, pHGF/HsHsp90β, and pHGF/HsHsp90α, respectively. The strains with human Hsp90's being genetically highly similar, their correctness could also be double-checked with isoform-specific antibodies (data not shown, see also ref. [20]).

2.3. Preparation of yeast extracts and immunoblotting

Cells were grown to an OD600 of 0.7–0.8 in rich medium with 2% glucose and 200 μg/ml adenine (YAPD), collected and washed with ice-cold water. Cell pellets were resuspended in 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol, and a cocktail of protease inhibitors. Cells were broken mechanically with glass beads, and cleared lysates
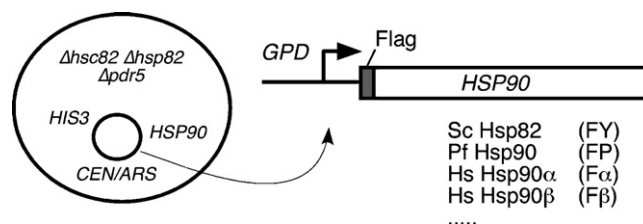

Fig. 1. Schematic representation of the isogenic set of Hsp90 tester strains. The species are abbreviated as follows: Sc, *Saccharomyces cerevisiae*; Pf, *Plasmodium falciparum*; Hs, *Homo sapiens*. The dotted line below the list of Hsp90 proteins indicates that this system can easily accommodate Hsp90 proteins from other species. Further details are given in the text.

quantitated to load equal amounts of proteins onto denaturing SDS-polyacrylamide gels. The immunoblots were processed as described before [20].

2.4. Growth assays

YAPD medium was used both for plates and for liquid cultures and cells were grown at 30 °C. Cells were freshly grown from an overnight inoculum to OD600 ~0.5 before starting the assays. For plate assays, 5 μl aliquots of a serial dilution series were spotted, starting with a dilution to OD600 = 0.05. Plates were scanned after 3 and 4 days. For assays with liquid cultures, triplicate samples of cells were diluted to OD600 = 0.05 except for FY, which was diluted further to 0.01. Cell densities were measured at appropriate dilutions with a spectrophotometer at OD600. Where indicated, Rd and GA were added from 10 mM stocks in dimethyl sulfoxide (DMSO). The untreated control plates and cultures received the same amount of DMSO (vehicle) needed as a solvent for the samples with the highest drug concentration.

3. Results

We used plasmid shuffling to replace the endogenous *HSP90* genes of *S. cerevisiae*, *HSC82* and *HSP82*, with *HSP90* from different species. The latter were expressed from a low copy plasmid under the control of the strong constitutive *GPD* promoter. To facilitate the comparison of Hsp90 protein levels, we also generated a set of strains expressing all Hsp90 proteins with an N-terminal Flag tag (Fig. 1). All complemented strains proved to be sufficiently viable (see below, and data not shown) to allow their in-depth analysis. Our strain sets included strains with *Pf* Hsp90, yeast Hsp82, human Hsp90α, and human Hsp90β. It is noteworthy that a continued auxotrophic selection for maintenance of the episomes was not necessary since the expression of an Hsp90 is essential for viability. Hence, the *HSP90* genes served themselves as a marker and all strains could be grown in rich medium.

We proceeded to determine the relative levels of the different Hsp90 proteins with an immunoblot experiment. Total cell extracts were prepared from all strains and probed with a monoclonal antibody against the Flag tag (Fig. 2A). An extract from a strain with untagged human Hsp90β (lane β) served as a negative control. By comparison with the total cellular proteins revealed on the filter by Ponceau S-staining, it is apparent that the levels of the four Flag-tagged Hsp90 proteins are overall quite comparable, human Hsp90α being the lowest with a 2-fold difference compared to yeast Hsp82. When a similar immunoblot was probed with antibodies that are specific for yeast Hsp90, no signal could be detected in any of the strains complemented with human or Pf Hsp90. The strain expressing Flag-tagged yeast Hsp82 served as a positive control. This result ascertained that the strains Fα, Fβ and FP expressing Flag-tagged human Hsp90α, Hsp90β and Pf Hsp90, respectively, indeed lack the endogenous yeast *HSP90* genes.
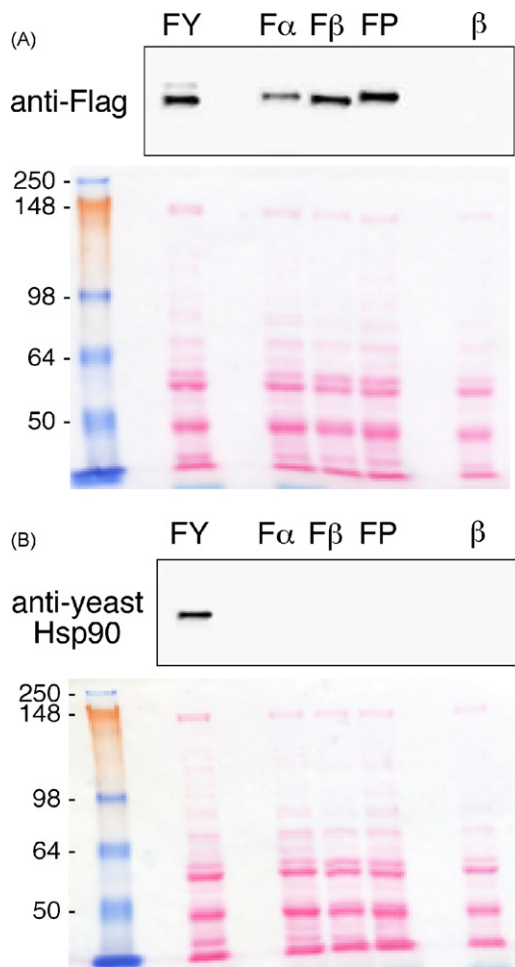
Fig. 2. Immunoblot analysis of Hsp90 proteins. Equal amounts of extracts from indicated strains were probed with a monoclonal against the Flag tag (panel A) or antibodies against yeast Hsp90 (panel B). The leftmost lanes on the Ponceau S-stained filters shown below the immunoblots in both panels are molecular weight markers (sizes indicated in kDa). The relative band intensities of the Flag-tagged Hsp90 proteins are 100%, 49%, 99% and 125% for FY, Fα, Fβ and FP, respectively.

We had previously compared the growth of cells with tagged or untagged yeast Hsp82 (data not shown) and had not noticed any particular difference. Since pilot experiments indicated that FP grew less well than FY (see below), we wanted to determine whether the Flag tag might adversely affect complementation by Pf Hsp90. Serial dilutions of equal numbers of cells of the two corresponding strains, P and Pf (with untagged and tagged Pf Hsp90, respectively), were spotted onto solid medium (Fig. 3A). It became clear that the Flag tag, if anything, slightly improved the function (or expression) of Pf Hsp90.

Thus, having established that Hsp90 protein levels were not too dissimilar and Flag-tagged versions of Hsp90 appropriate, we could start testing our set of strains consisting of the four Flag-tagged Hsp90 proteins. On plates, FP and Fα turned out to grow considerably more slowly than FY and Fβ although they eventually reached similar colony numbers and sizes. We realized that, if we were going to use plate growth assays to assess the effects of Hsp90 inhibitors, we would need more comparable cell densities. We therefore chose to spot FP and Fα 1 day earlier than FY and Fβ. With this 1 day advance, all four strains grow to comparable densities within an additional 4 days, FY being yet slightly more vigorous (Fig. 3B). To obtain more quantitative data, growth assays were repeated in liquid culture (Fig. 3C). In this case, all strains were subjected to identical conditions. These experiments confirmed the

slow and vigorous growth of FP and FY, respectively. Ultimately, all four strains reach similar saturating densities. Surprisingly, in liquid culture the yeast strain with human Hsp90α (Fα) does not display the prominent growth defect that is obvious on plates and grows even slightly faster than Fβ.

Next, we explored whether plate or liquid growth assays would allow us to test Hsp90 inhibitors and to identify differences between these tester strains. After pilot experiments with a range of inhibitor concentrations, we settled on concentrations that gave the largest differences without completely obliterating growth of all strains except the comparatively more resistant FY (data not shown). The result of a growth assay on plates is shown in Fig. 4. It must be pointed out again that Fα and FP were spotted 1 day earlier. However, this was the case for all treatments and, therefore, their relative sensitivities to the drugs can be compared. While FY is only slightly affected by either 40 μM GA or 2 μM Rd, FP is exquisitely sensitive to GA and to a lesser extent to 2 μM Rd. The strains with human Hsp90 isoforms (Fα and Fβ) are similarly affected and more resistant to GA than FP. FP in turn is more resistant to Rd than both Fα and Fβ. As could be expected from the results discussed above, the four strains behaved slightly differently in liquid medium (Fig. 5). Although the differences in Rd sensitivities between human (Fα and
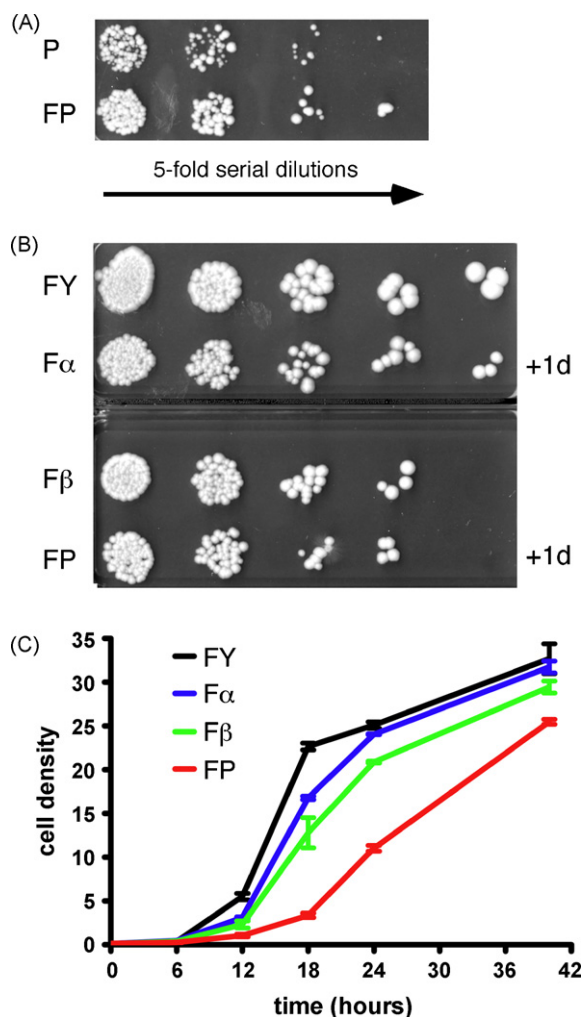

Fig. 3. Growth assays with Hsp90 tester strains in the absence of Hsp90 inhibitors. (A) Comparison of strains with untagged and Flag-tagged Pf Hsp90 (P and FP, respectively) with a plate growth assay. (B) Plate growth assay with all four Hsp90 tester strains. Fα and FP were spotted 1 day earlier (hence the indication +1d for one additional day) and the image was acquired on day 4 (relative to when FY and Fβ were spotted). (C) Growth curves determined with liquid cultures. Cell density refers to OD600 values. Error bars indicate the standard deviation.
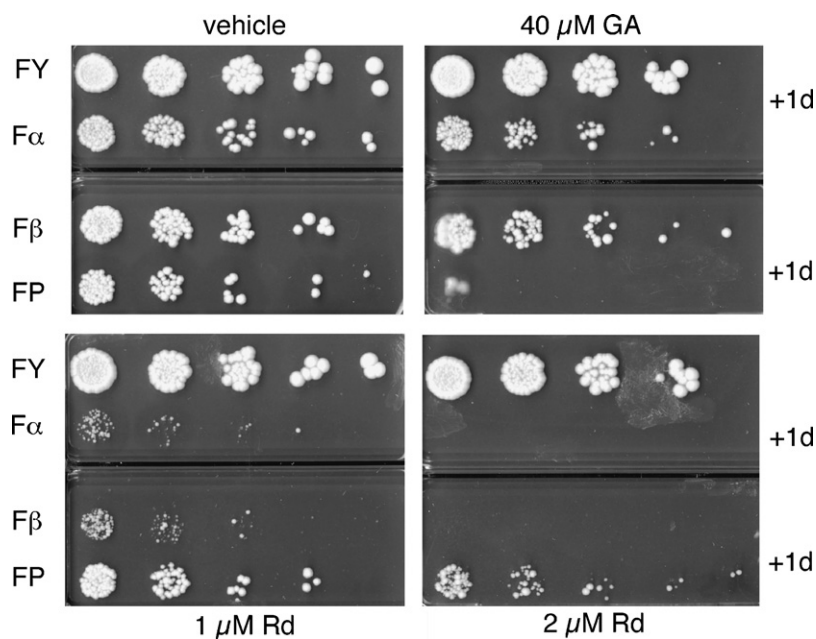
Fig. 4. Hsp90 tester strains show different sensitivities to Hsp90 inhibitors on plates. Fα and FP were spotted 1 day earlier (+1d) and the image was acquired on day 4. The solvent DMSO (vehicle) was added as indicated in Section 2.

Fβ) and Pf Hsp90 (FP) strains disappeared, GA was still more toxic to FP. To reveal the differential sensitivities of the four strains to the two drugs more clearly, cell densities in the presence of the drugs were standardized to those of untreated cultures and expressed as a ratio (Fig. 5D). FY, the strain that grows the most rapidly and is only slightly affected by either drug, is relatively more inhibited by GA for the first 18 h. In the presence of GA, it reaches saturation about 6 h later, but it does reach it, which is why the ratio drops back down to 1 at 24 h. Most importantly, this plot shows unambiguously that FP is hypersensitive to GA compared to Rd and that in that respect it differs substantially from the strains with human Hsp90 (Fα and Fβ). Moreover, this quantitative result agrees with the qualitative one from the plate assays, which already indicated that FP is more sensitive to GA than Rd and hypersensitive to GA by comparison with strains expressing human Hsp90.

4. Discussion

The number of known Hsp90 substrates is continuing to grow very rapidly (for an updated list, see http://www.picard.ch/downloads/Hsp90interactors.pdf) as could be expected for a molecular chaperone that may be the most abundant cytosolic protein even in unstressed cells [24]. It is still difficult to provide a comprehensive list of the substrates that account for the essential functions of Hsp90 for viability of both unicellular and metazoan organisms. We now add Pf Hsp90 to the list of heterologous Hsp90's that can apparently fulfill at least the essential functions of Hsp90 in yeast for vegetative growth in standard growth media. Whatever these functions are exactly, they must include many of the ones that have been highlighted by global biochemical and genetic analyses of Hsp90 interactions in this organism [25–28]. The results of these genetic complementation experiments underscore the high evolutionary conservation of Hsp90 [29–32]. For example, yeast Hsp82 compared to human Hsp90α and Pf Hsp90 is 59% and 58% identical, respectively. The sequence identity drops to only 36% with the *Escherichia coli* Hsp90 ortholog htpG, which may explain why htpG fails to complement yeast ([16], G. Palmer and DP, unpublished results). Despite the remarkable complementation by heterologous Hsp90's for viabil-

ity, this complementation is far from complete. In the absence of the Hsp90 co-chaperone Sti1, *C. elegans* Hsp90 and human Hsp90β cannot support viability of yeast mutants [17]. Moreover, human Hsp90β fails to support Ste11-dependent pheromone signaling in yeast [19], and yeast and human Hsp90's differ in their ability to support several other endogenous and exogenous substrates [33]. These shortcomings are mirrored by our findings that human and Pf Hsp90's do not support wild-type growth rates (Figs. 3–5). This is particularly evident for the strain with Pf Hsp90, which grows substantially more slowly than our control yeast strain with Hsp82.

It is noteworthy that the relative growth rates also differ between liquid and solid media. It is a well-known phenomenon in *S. cerevisiae* that the genetic background can differentially affect the ability of a strain to grow in a plate assay or in liquid culture (see for example refs. [34–36]). With respect to Hsp90, our findings may indicate that different environmental conditions impose subtly different Hsp90 substrate requirements, which are differentially fulfilled by different Hsp90 proteins. While our results demonstrate that heterologous Hsp90's do not behave differently because of vastly different expression levels, there are several other parameters that can be considered. They might differ in their ability to bind and to cooperate with the yeast Hsp90 co-chaperones or to process yeast substrates (see also afore-mentioned examples). Alternatively, they might not function optimally because of differences in posttranslational modifications or because of the particulars of the chemical milieu of a yeast cell.

We found that all strains of the Hsp90 tester set are sensitive to Hsp90 inhibitors, albeit to very different extents. Strain FY with the yeast Hsp82 was the least sensitive one and the only one that could eventually reach similar cell densities with drugs as without drugs both on plates and in liquid culture. Higher concentrations yet are necessary to inhibit growth of FY more strongly (data not shown), in agreement with previous reports about the toxicity of Hsp90 inhibitors to wild-type strains, notably in a Δpdr5 background [22,37]. At concentrations that were only minimally toxic to the strain FY, the other three strains were severely growth impaired. Surprisingly, Piper and co-workers [33] have reported that a strain with human Hsp90α displayed relative resistance to Rd compared to strains with human Hsp90β or yeast Hsc82 or Hsp82. This discrepancy could be due to any of a number of differences in the
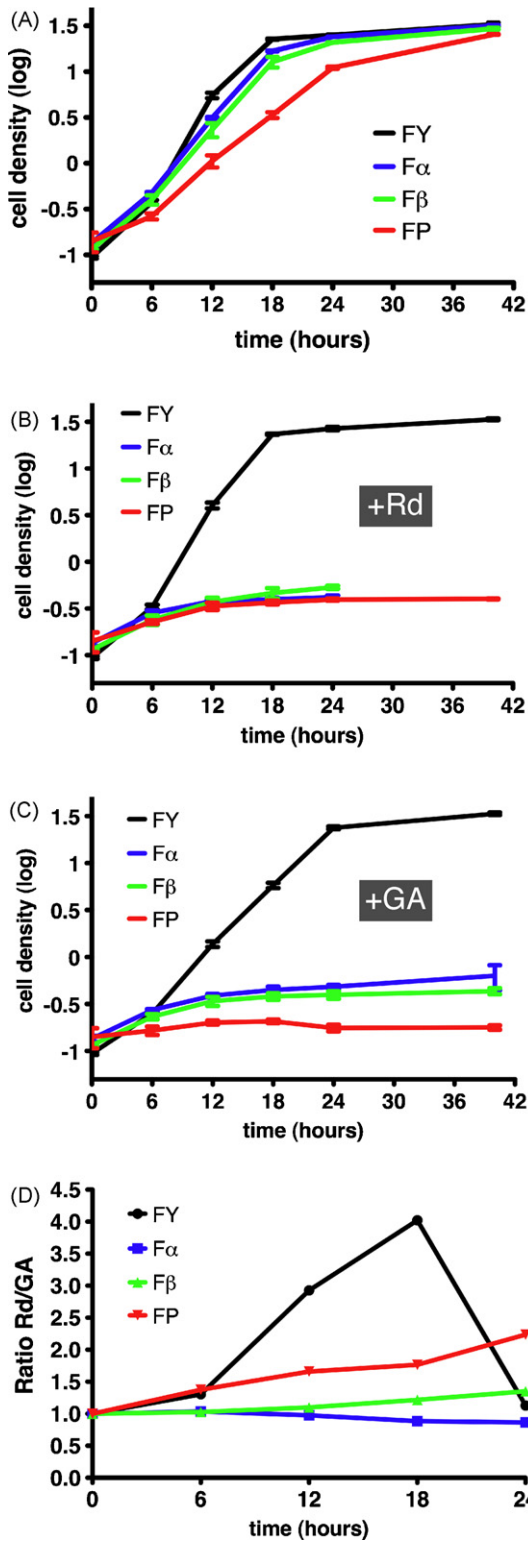
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Fig. 5. Hsp90 tester strains show different sensitivities to Hsp90 inhibitors in liquid culture. Panels A, B and C show growth curves of cultures that were left untreated (only solvent), treated with 2 μM Rd and 40 μM GA, respectively. The scale of the Y-axes is logarithmic. Error bars indicate the standard deviation. The data plotted in panel A is the same as that of panel C in Fig. 3, but here the scale of the Y-axis is different. (D) This graph shows the ratios of standardized cell densities observed with the two inhibitors. Note that the last time point of panels A–C is not computed here and that the scale of the Y-axis is linear.

experimental set-ups. Different expression levels of the drug target, Hsp90, could easily explain differences in drug sensitivity. While we do not know whether Hsp90α and Hsp90β levels were similar in the afore-mentioned study, we can assert for our strains that they differed by at most 2-fold (Fig. 2) because the two isoforms were marked by the same epitope tag and thus recognized by the same antibody with identical efficiency. Most notably, the levels of human Hsp90β and Pf Hsp90 were very close to those of the isogenic strain with yeast Hsp82.

Hsp90 inhibitor sensitivity is determined by a variety of parameters including cell permeability, export pumps, metabolism, and the binding affinity for Hsp90. Undoubtedly, the latter is the most relevant in the context of the differential sensitivities of different strains that only differ by the Hsp90 proteins they express. They may have intrinsically different binding affinities for one drug or another. Whether that is the case for yeast, human and Pf Hsp90 is not known since, to the best of our knowledge, they have not yet been measured side by side. Furthermore, the binding affinities of Hsp90 for inhibitors can be influenced by its interactions with co-chaperones [17,22,37–41], which in turn may be modulated by posttranslational modifications of Hsp90 (see for example refs. [42–44]). Since we had found that the Hsp90 co-chaperone p23 directly protects Hsp90 from binding inhibitors [22], we did introduce the Pf p23 ortholog into yeast. Instead of improving Pf Hsp90 function and resistance, expression of Pf p23 in FP further compromised growth (data not shown). To reconstitute Pf Hsp90 function fully in yeast, it may be necessary to introduce the entire or at least more of the Pf co-chaperone complement.

Whatever the reasons are for the observed differential sensitivities, we believe that our results constitute a proof of principle that it should be possible to use this set of yeast strains to screen for Hsp90 inhibitors with an even larger differential between the Hsp90 of the host and the Hsp90 of the deadly parasite. Moreover, the Hsp90 set could easily be expanded to include Hsp90 from other unicellular parasites such as Trypanosomes and Leishmania. In closing, we would like to emphasize that this is a powerful live cell, but nevertheless artificial, screening tool. The efficiency and parasite-specificity of candidate drugs will need to be ascertained and further characterized in appropriate *in vitro* and *in vivo* systems with the parasite itself.

Acknowledgments

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