



Mutations that increase both Hsp90 ATPase activity in vitro and Hsp90 drug resistance in vivo

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ABSTRACT

Hsp90 inhibitors are currently tested in clinical trials as anticancer agents. We investigated whether inhibitor resistance may arise as a result of a point mutation in Hsp90. We used yeast cells that expressed human Hsp90 β to select inhibitor-resistant mutants from the randomly mutagenized library. Single amino acid substitution, I123T, in a selected mutant was sufficient to confer inhibitor resistance. Transfection of human cells with the HSP90 β I123T and the corresponding HSP90 α I128T yielded cell lines resistant to inhibitors of the Hsp90 ATPase. Unexpectedly, mutations did not result in diminished inhibitor binding in vitro. Similarly resistant cells were obtained after transfection with previously described A116N and T31I mutants of HSP90 β that cause increase in ATPase activity in vitro. Inhibitor-resistant phenotypes of the I123T and A116N mutants depended on their increased affinity for Aha1, whereas T31I mutation did not result in increased Aha1 binding. These results show possible scenario by which resistance may arise in patients treated with Hsp90 inhibitors. Additionally, our results show that each isoform of Hsp90 can alone sustain cellular functions.

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1. Introduction

Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone essential for the viability of eukaryotic cells (for review see [1,2]). Hsp90 machinery is required for proper conformation, activation and function of hundreds of client proteins involved in virtually all cellular processes.

Human genome contains two genes that encode highly homologous cytoplasmic isoforms of Hsp90, α and β (hHsp90 α , hHsp90 β). These isoforms exist in a cytoplasm as homodimers. To date, only few examples of substrates specifically regulated by one of the Hsp90 isoforms exist in literature, such as hHsp90 β -dependent regulation of cell differentiation by c-IAP1 and progesterone receptor maturation

Abbreviations: 17-AAG, 17-(Allylamino)-17-demethoxygeldanamycin; Hsp90, heat shock protein 90; yHsp90, *S. cerevisiae* Hsp90; hHsp90, human Hsp90; RNAi, interfering RNA; ITC, isothermal titration calorimetry

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by GCUNC-45 [3,4]. hHsp90 α , but not hHsp90 β , chaperones proteolytic intermediates in the MHC Class I antigen processing pathway [5]. An enhanced interaction of the hHsp90 α with various substrates during heat shock was also reported [6]. Little is known about effects of deletion or inactivation of one of the cytoplasmic hHsp90 isoforms. Disruption of both alleles of HSP90 β does not seem to be detrimental for viability of chicken DT40 cells [7]. However, HSP90 β knockout in mice resulted in lethality in embryonic development indicating necessity of this protein for the proper formation of placental labyrinth [8]. There are no data on the effects of Hsp90 α inactivation other than results of short-term cultures made with isoform-specific small interfering RNA (siRNA). In particular, mouse HSP90 α knockout was never reported, probably due to the fact that oocytes express almost exclusively this isoform [9].

Hsp90 ATPase activity is essential for its chaperoning action (reviewed in [1]). The most notable structural rearrangement in the N-terminal domain that occurs upon ATP binding is movement and remodeling of the structure known as the “lid” consisting of amino acids 94–125 in yeast yHsp90 (103–135 in hHsp90 β) [10]. This change leads to N-domains dimerization, structure compaction and ATPase activation. These changes are believed to be directly responsible for the

maintenance of competent state of substrates. Several cochaperones were shown to interact with Hsp90 depending on its ATP-binding status and modulate its ATPase activity. Sba1/p23 interacts with the ATP-bound N-domain of Hsp90 and stabilizes compacted conformation, thus inhibiting ATPase activity [11,12]. In contrast to p23, Aha1 is the only co-chaperone that upon binding to Hsp90 stimulates ATPase activity by rearrangement of the active site [13–15].

Two natural compounds, radicicol and geldanamycin, and their chemically modified analogues such as 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) were identified as inhibitors of the Hsp90 that compete with ATP for the binding site in the N-terminal domain [16–19]. Hsp90 has become an attractive target for anticancer therapy, due to its general function in maintaining various pathways perturbed in cancer progression, such as growth signaling, apoptosis, angiogenesis and metastasis (for review see [20]). ATP competing inhibitors are currently tested in clinical trials [21].

Here we present novel hHsp90 mutants that render human and yeast cells resistant to Hsp90 inhibitors. We show that the resistance depends on the increased ATPase turnover due to enhanced interaction with Aha1.

2. Materials and methods

2.1. Yeast strains

HSP82 and *HSC82* genes were replaced with a G418 resistance marker in the haploid strains SEY6210 and SEY6211 [22] as described previously [23]. These strains were mated and the resulting diploid was transformed with plasmid expressing *hHSP90β* and *URA3* genes. Transformants were subjected to sporulation and random spore dissection [24]. The haploid strain BY516 that carried deletions of both yeast *HSP90* genes was selected based on its inability to lose plasmid assayed by grow on 5-FOA containing media [24]. Deletions in this strain were confirmed by PCR with *HSC82* and *HSP82* specific primers. Strain BY516 was used to construct strain BY517 by transformation with plasmid pB549 (p423TDH3-hHSP90β) followed by selection for HIS+ ura- phenotype. Yeast strains that expressed different alleles of *Caenorhabditis elegans* or human Hsp90 as a sole source of this protein were obtained by transformation of strain BY517 with plasmids obtained by cloning different forms of Hsp90 into plasmid p416TDH3. Transformants were selected for the histidine prototrophy and loss of the *HIS3* marker.

The library of the mutagenized *hHSP90β* alleles was used to transform strain BY516. Transformants were selected on media lacking histidine. Transformed colonies were collected in pools, each containing about 1000 clones, ten pools in total. Pools were tested for the radicicol resistance in liquid YPD medium supplemented with 5 μM radicicol. Only one pool grew up to saturation whereas growth of the remaining nine pools was completely inhibited. Radicicol-resistant clones were colony-purified and used to isolate plasmids containing mutated alleles of *hHSP90β*. Growth of the yeast strains carrying wild-type and mutant *hHSP90* was monitored by the optical density at 600 nm.

2.2. Plasmids

Yeast TDH3 promoter was amplified by PCR from the yeast genomic DNA and used to replace GAL1 promoter in plasmids p416GAL1 and p423GAL1 [25]. The resulting plasmids, p416TDH3 and p423TDH3, were used for expression of the human and *C. elegans* *HSP90* genes in yeasts. For expression in human cells, wild-type and mutant alleles of *hHSP90α* and *hHSP90β* (I128T and I123T respectively) with N-terminal FLAG and HA tags were cloned into the pcDNA3.1(+) vector. Sequences of the PCR primers used to generate inserts and detailed information on plasmids generated for this study are provided in [Supplementary Tables 1 and 2](#). Random mutations

were introduced into *hHSP90β* sequence during PCR amplification with Taq polymerase. To increase mutation rate, product of the first amplification was used as a template in the next PCR reaction. Re-amplification was repeated four times. Product of the fourth round of the amplification was cloned in p423TDH3 vector, yielding a library of about 10000 independent clones. Site-directed mutagenesis was performed by PCR with mutagenic primers.

2.3. Cell line experiments

Plasmids carrying wild-type and various mutants of the *hHSP90α* and *hHSP90β* were transfected into human cell lines using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfectants were selected in the IMDM supplemented with 10% FCS and 1 mg/ml G418. This initial selection was followed by the selection where G418 was replaced with 1 μM 17-AAG. Measurements for the growth curves and IC50 of human cells were performed using CellTiter-Glo reagent (Promega) according to the manufacturer's protocol. For Aha1 knockdown cells were transfected with Silencer Select siRNA (Applied Biosystems/Ambion, cat. no. s20802, s20903) according to the manufacturer's instructions. Radicicol, 17-AAG and 17-DMAG were purchased from Sigma.

2.4. Protein expression and purification for geldanamycin-binding and ATPase assays

Human wild-type and I128T mutant of *hHSP90α* and *C. elegans daf-21* were cloned in vector pTYB4 (New England Biolabs). Expression of the proteins was carried out in *Escherichia coli* BL21 strain. Proteins were purified by an affinity chromatography according to the New England Biolabs recommendations, followed by the chromatography on heparin and mono-Q sepharose. Human *AHA1* was cloned in pET28 vector (Novagen) to obtain C-terminal His-tagged fusion and was purified by chromatography on Talon resin (Invitrogen).

2.5. In vitro assays

Geldanamycin immobilized on agarose beads was prepared according to [26]. Geldanamycin-beads were incubated with purified Hsp90 proteins in buffer containing 100 mM NaCl, 20 mM Tris pH 7.5, 1% BSA and 0.1% Triton X-100 and washed four times with the same buffer. Bound proteins were eluted with SDS-loading buffer and visualized by western blotting with an anti-Hsp90 antibody. ATPase activity was measured by direct hydrolysis of the ATP γ -P³³. Reaction was performed in a buffer containing 25 mM Hepes-KOH pH 7.5, 25 mM KCl, 5 mM MgCl₂, 1 mM ATP and 1 μM ATP γ -P³³. Samples containing Hsp90 protein at the 10 μM concentration and Aha1 (when indicated) were incubated at 37° for 2 h. Produced radioactive phosphate was separated from the substrate by the TLC chromatography, radioactivity was assessed using Storm Phosphoimager (Amersham Biosc.) and quantified with ImageQuant 5 software. Activity of the non-specific ATPases contaminating Hsp90 preparations was measured under the same conditions with addition of the 200 μM radicicol. Obtained values as well as the value of non-specific ATPases contamination of Aha1 preparation were used to correct measured Hsp90 activities.

2.6. Western blot and co-immunoprecipitation

FLAG-specific antibody covalently linked to agarose beads (Sigma) was used to precipitate FLAG-HA-tagged exogenous hHsp90 protein. Western blot with anti-HA antibody was used to confirm that equal amounts of the precipitated hHsp90 were loaded on the gel in each case. Aha1 was detected using Aha1-specific ab56721 antibody (Abcam), GAPDH with sc-32233 antibody (Santa Cruz). hHsp90 was detected with SPA 846 antibody (Stressgen). To detect specific isoforms SPS 771

antibody for hHsp90 α (Stressgen) and PA 012 for hHsp90 β (Affinity Bioreagents) were used.

2.7. Modeling

Computational studies were carried out on the structure of single N-domain of the yHSP90 (Protein Data Bank code 1AM1, 2.0 Å resolution) and on the structure containing all three domains (N, M and C) of the yHSP90 (PDB code 2CG9, 3.1 Å resolution). In both structures the loop containing V114 (residues 97–121) is clearly visible and temperature factors for atoms in this loop are within range 15–50 for 1AM1 structure in within range 70–75 for 2CG9 structure. YASARA software v. 9.1.25 (YASARA Biosciences) using Yamber3 forcefield (modification of Amber 99 forcefield) was used for protein visualization and modification (mutation V114T). Subsequent optimization was performed only for the part of protein structure containing amino acids within 1.0 nm around modified residue.

2.8. Isothermal titration calorimetry

ITC experiments were carried out on VP-ITC isothermal titration calorimeter (Microcal Inc., MA, USA) with the active cell volume of 1.4315 ml. The concentration of wild-type or mutant Hsp90 α protein in the cell was 6–10 μ M, while the syringe contained 60–100 μ M of 17-AAG, radicicol or Aha1. For the titrations of Hsp90 with Aha1 in the presence of 17-AAG, 20 μ M of 17-AAG was added to both, the cell and the syringe, to saturate the Hsp90 binding sites. Stock solutions of 17-AAG or radicicol were prepared in DMSO at 10–50 mM concentration and stored at –20 °C. The ITC buffer contained 20 mM HEPES, pH 7.5, 25 mM NaCl, and up to 1% DMSO. Titrations were carried out at 25 °C, using 25 injections of 10 μ l each, injected at 200 second intervals. All experiments were repeated at least twice. Radicol was purchased from A. G. Scientific, Inc, 17-AAG and geldanamycin were purchased from Sigma.

2.9. Protein preparation for ITC

Human wild-type and I128T mutant of *hHSP90 α* were inserted into pET-15 vector (Novagen). Proteins with N-terminal His₆-tag were expressed in *E. coli* strain BL21 (DE3) and purified as previously described [27]. Purified proteins were dialyzed against the storage buffer: 20 mM Tris, pH 7.5, 50 mM Na₂SO₄ and 1 mM DTT and stored at –80 °C. Human *AHA1* was cloned in pET-28 vector (Novagen) to obtain the C-terminal His₆-tag fusion. Protein was expressed in *E. coli* strain BL21 (DE3) and purified using a Ni-IDA affinity column (Amersham Biosciences), followed by an anion exchange chromatography on Q-sepharose (Amersham Biosciences). Protein was stored at –80 °C in a buffer containing 20 mM HEPES, pH 7.5, 0.5 M NaCl and 0.5 mM DTT. For ITC experiments proteins were dialyzed against ITC buffer and/or concentrated using Microcon filter devices (Milipore) and resuspended in the ITC buffer.

3. Results

3.1. Selection of the radicicol-resistant Hsp90 mutants

We used an error-prone Taq polymerase to introduce random mutations into *hHSP90 β* coding sequence. Amplification yielded a population of *hHSP90 β* alleles containing a single nucleotide substitution every 50–60 bp. We created a library of these PCR products in a yeast vector under the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene. To select for the radicicol-resistant mutants, we transformed the library into a yeast strain which carried the deletion of *yHSC82* and *yHSP82* genes and a plasmid with *hHSP90 β* gene. One of these transformants was able to grow in liquid YPD medium in the presence of 5 μ M radicicol. Plasmid isolated from radicicol-resistant

yeast carried *hHSP90 β* gene that contained multiple single amino acid substitutions (Suppl. Fig. 1). To find the mutations responsible for the radicicol-resistant phenotype, we introduced these mutations separately in the human *hHSP90 β* ORF cloned in a yeast expression vector. Tests of the resulting plasmids demonstrated that substitution of the single amino acid, I123T, is sufficient to confer the radicicol-resistant phenotype on yeast, whereas growth of the control strain transformed with plasmid that carried wild-type *hHSP90 β* was completely inhibited, despite similar expression level of both proteins (Suppl. Figure 2a). It was shown previously that yeast cells expressing *daf-21*, the *C. elegans* homolog of Hsp90, were able to grow in the presence of the Hsp90 inhibitors [28]. Yeast that expressed I123T mutant of hHsp90 β or hHSP90 α I128T mutant that corresponds to I123T in hHsp90 β demonstrated equal or greater resistance to radicicol when compared to *daf-21* (Fig. 1a). Compared to wild-type hHsp90,

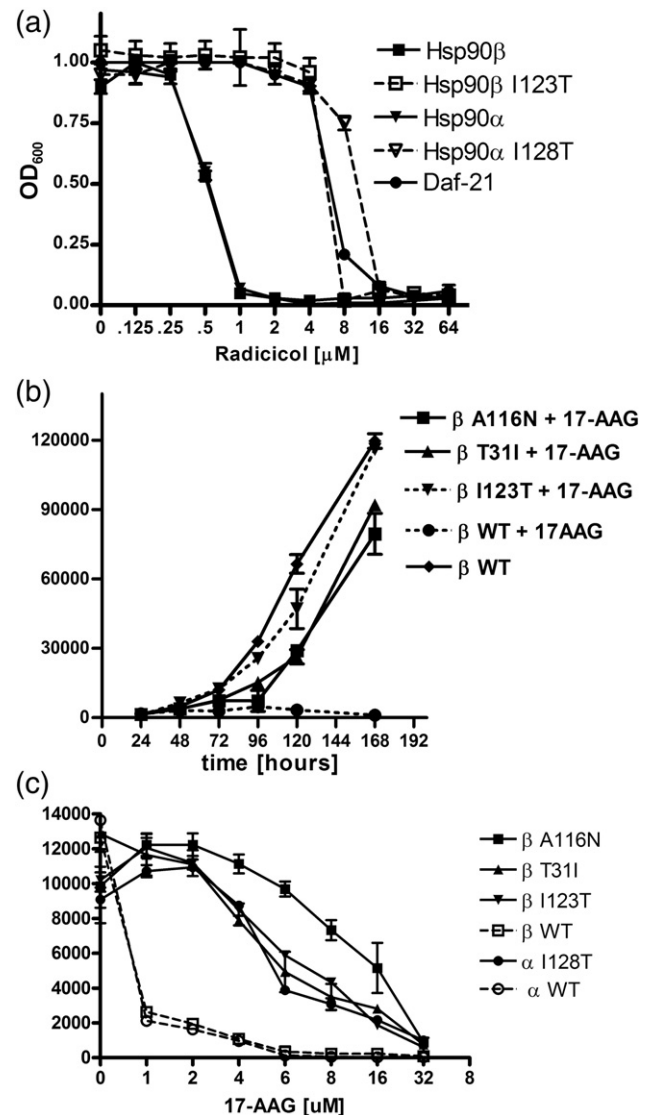


Fig. 1. Growth of yeast and human cells that express different mutants of hHsp90 on media supplemented with Hsp90 inhibitors. (a) yeast strains that express indicated forms of hHsp90 and *C. elegans daf-21* as a sole source of this protein were grown on YPD with different concentrations of radicicol for 4 days. Growth was measured on 96-well plates as OD₆₀₀. Plot presents representative results of one of the three independent experiments. (b) Growth of HEK293 cells transfected with indicated mutants of *hHSP90* cultured on media with 1 μ M 17-AAG was measured using luciferase-based assay. Results were plotted in arbitrary luminometric units. (c) Growth of HEK293 cells transfected with indicated mutants of *hHSP90* in 17-AAG was measured as in (b). Results in (b) and (c) represent mean and SD values from three independent experiments

resistance of the *hHsp90β* and *hHsp90α* mutants increased 10 and 20-fold respectively. The corresponding IC₅₀ values were 0.5 μM for both wild-type proteins, 6 μM for *hHsp90β* I123T and 10 μM for *hHsp90α* I128T. Similar results were obtained for geldanamycin and 17-AAG, however, due to the low sensitivity of yeast to these drugs the effect was less pronounced (Suppl. Figure 3).

3.2. Selection and growth of the inhibitor-resistant human cells

Transfection of the HEK293 cells with plasmids containing *hHSP90α* I128T and *hHSP90β* I123T mutants, followed by selection with 17-AAG allowed us to establish cell lines that were able to grow continuously in media supplemented with 1 μM 17-AAG. Similarly treated cells transfected with plasmids carrying wild-type *hHSP90* genes or plasmid pcDNA3.1 did not acquire inhibitor resistance and died after 8–10 days of culture (Fig. 1b and Suppl. Fig. 4a). This transformation and selection process was repeated 9 times at different occasions, always resulting in resistance after transformation with mutants, whereas transformation with wild-type Hsp90's never yielded 17-AAG resistant cells. Growth rate of the cells resistant to 17-AAG cultured in a presence of this inhibitor and cells transfected with control plasmid grown without inhibitor were nearly identical, indicating that either isoform of hHsp90 is able to perform all chaperone functions essential for growth of HEK293 cells (Suppl. Fig. 4b). The IC₅₀ of the *hHSP90β* I123T and *hHSP90α* I128T transfectants was in 5 μM, compared to 0.5 μM for the cells transfected with wild-type hHsp90 α and β (Fig. 1c). We observed similar difference in resistance of the mutated and wild-type transfectants to other inhibitors of Hsp90 – 17-DMAG and radicicol (Suppl. Fig. 5). Expression of the exogenous wild-type and mutant hHsp90 after transfection was similar (Suppl. Fig. 2b). We observed moderate and sometimes dramatic increase in the expression of exogenous Hsp90 after exposure to 17-AAG depending on the initial transfection efficiency and whether transfectants were pre-selected for the G418 resistance (Fig. 2a, Suppl. Fig. 2b). This, together with substantial cell death 3–8 days after beginning of selection, suggests that during selection on 1 μM 17-AAG only cells with highest expression of the mutated Hsp90 proteins survived. The exogenous Hsp90 in cells after selection reached levels equal to or higher than that of the endogenous protein (Fig. 2b, Suppl. Fig. 2b). Further increase of the 17-AAG concentration did not result in additional increase in the Hsp90 mutant production (Fig. 2b). This observation shows that cells do not benefit from increasing of the Hsp90 synthesis beyond level sufficient to acquire initial resistance to inhibitor.

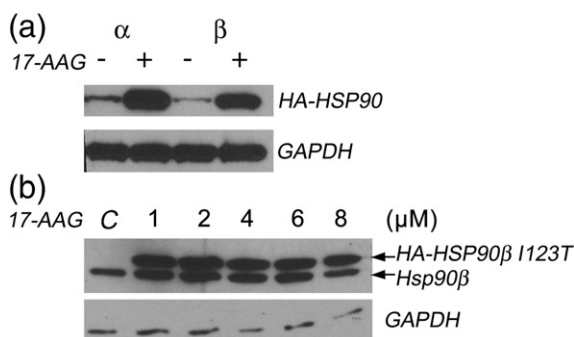


Fig. 2. Expression of the hHsp90α I128T and hHsp90β I123T mutants in HEK293 cells. (a) Exogenous Flag-HA-tagged hHsp90 was detected with anti-HA antibody in protein extracts from the cells before and after selection with 1 μM 17-AAG. (b) Cells transfected with hHsp90β I123T and selected on 1 μM 17-AAG were grown on media with increased concentrations of 1 μM 17-AAG. Anti-Hsp90 antibody (SPA 846) was used to detect exogenous, tagged hHsp90β I123T (upper band) and endogenous hHsp90β (lower band). Lane C represents transfected cells before 17-AAG selection.

3.3. Inhibitor-resistant mutants bind 17-AAG and radicicol

We hypothesized that decreased affinity for radicicol and geldanamycin might be responsible for resistance to inhibition of the Hsp90 mutants. Therefore we tested binding of the wild-type hHsp90α and hHsp90α I128T to geldanamycin immobilized on agarose beads. We compared these proteins with daf-21, *C. elegans* homolog of Hsp90, that does not bind geldanamycin [29]. We did not detect any daf-21 binding, whereas both hHSP90α proteins demonstrated similar interaction with geldanamycin (Fig. 3).

We have used hHsp90α I128T, but not hHsp90β I123T, mutant in all in vitro experiments since the expression of the latter in *E. coli* was very poor.

We also measured the binding of 17-AAG and radicicol to wild-type and I128T mutant of hHsp90α by isothermal titration calorimetry (ITC). Titration curves are shown in Fig. 4 a–b and the values of dissociation constant (K_d) are listed in Table 1. Wild-type protein showed a slightly stronger binding of 17-AAG ($K_d \sim 0.3$ μM) compared to I128T mutant ($K_d \sim 1.0$ μM). However, there was no detectable difference in radicicol binding to wild-type and mutant protein ($K_d \sim 0.5$ nM for both proteins). It is unlikely that small difference in 17-AAG binding between two proteins contributes substantially to the inhibitor-resistant phenotype of the mutants.

3.4. 17-AAG resistant alleles of Hsp90 have increased affinity for Aha1

A pull-down assay of the Hsp90 mutants with immobilized geldanamycin and calorimetric measurements excluded decreased inhibitor binding as a likely explanation of their resistance to inhibition. In a search for an alternative explanation, we used co-immunoprecipitation to compare binding of cochaperones to the wild-type and mutant hHsp90. Results presented on Fig. 5a demonstrate that hHsp90α I128T and hHsp90β I123T mutants bind Aha1 protein much stronger than wild-type hHsp90α or hHsp90β. This suggests that stimulation of ATPase activity of the mutants by Aha1 binding most likely resulted in a decreased sensitivity to competitive inhibitors of the Hsp90 ATPase such as radicicol and 17-AAG in vivo. We noticed also that both wild-type and mutant hHsp90α had higher affinity for Aha1 than hHsp90β.

We also studied the interaction of Aha1 with the wild-type and mutant form of hHsp90α by ITC in the absence or presence of 17-AAG. The ITC curves showed a striking difference in Aha1 binding to the wild-type hHsp90 (Fig. 4c) and I128T mutant (Fig. 4d). Wild-type protein bound Aha1 with the K_d of ~ 3 μM, while the K_d obtained for mutant protein was ~ 0.02 μM (Table 1), showing more than 100 times higher affinity of Aha1 for Hsp90 mutant compared to wild-type. The presence or absence of 17-AAG had essentially no effect on Aha1 binding to hHsp90 proteins (Fig. 4c–d).

3.5. Stimulation of the ATPase activity results in the inhibitor resistance of hHsp90

Two mutations that increased ATPase activity of the yHsp82, T22I and A107N (T31I and A116N in the hHsp90β sequence, respectively),

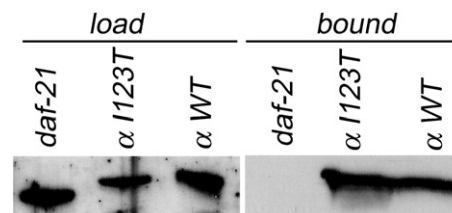


Fig. 3. Mutant and wild-type Hsp90 bind geldanamycin with similar affinity. Geldanamycin immobilized on agarose beads was used to pull-down purified wild-type hHsp90α, hHsp90α I128T or *C. elegans* daf-21.

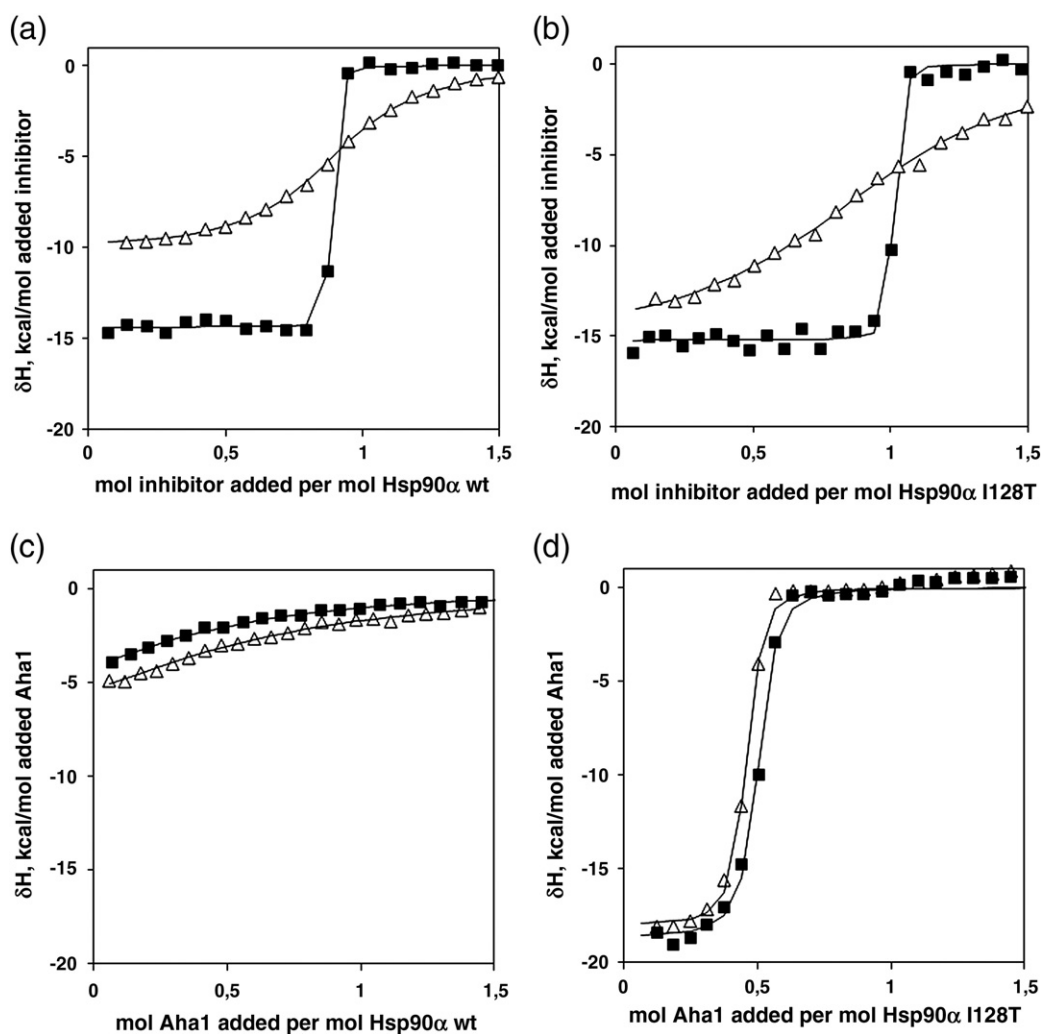


Fig. 4. Isothermal titration calorimetry of wild-type and I128T mutant of Hsp90 α with 17-AAG, radicicol, and Aha1. All experiments were performed at 25 °C. Errors shown in the table are standard deviations or uncertainties of the fit of the data, whichever is greater. (a) ITC curve of 17-AAG (Δ) or radicicol (\blacksquare) binding to wild-type Hsp90 α . (b) ITC curve of 17-AAG (Δ) or radicicol (\blacksquare) binding to mutant Hsp90 α I128T. (c) ITC curves of Aha1 binding to wild-type Hsp90 α in the absence (\blacksquare) or presence (Δ) of 20 μ M 17-AAG. (d) ITC curves of Aha1 binding to I128T mutant in the absence (\blacksquare) or presence (Δ) of 20 μ M 17-AAG.

were reported before [10]. Transfection of HEK293 cells with plasmids carrying T311 or A116N mutants yielded cells that grew in a presence of 1 μ M 17-AAG with IC50 values similar or greater to IC50 for hHsp90 β I123T and hHsp90 α I128T transfectants (Fig. 1c). Results of the co-immunoprecipitation presented on Fig. 5b demonstrate that A116N mutation caused the increased affinity for Aha1. In contrast,

T311 mutation located on the opposite surface of the Hsp90 N-terminal domain did not cause the increase in Aha1 binding. These results point at the importance of the “lid” structure, containing amino acids 116 and 123, for the interaction with Aha1. When we transfected cells with anti-Aha1 siRNA, we observed marked decrease in Aha1 expression up to 48 h after transfection. Decreased level of

Table 1
Dissociation constant of 17-AAG, radicicol and Aha1 binding to wild-type Hsp90 α and I128T mutant, obtained by isothermal titration calorimetry.

Protein (in the cell)	Ligand/protein (in the syringe)	K_d , μ M ^a	n , mol/mol ^b	ΔH , kcal/mol ^c
Hsp90 α wt	17-AAG	0.3 ± 0.06	1.03 ± 0.01	-10.2 ± 0.3
Hsp90 α I128T	17-AAG	1.0 ± 0.3	1.07 ± 0.02	-15.2 ± 0.4
Hsp90 α wt	Radicicol	0.0005 ± 0.0002	0.99 ± 0.03	-14.4 ± 0.2
Hsp90 α I128T	Radicicol	0.0005 ± 0.0002	0.98 ± 0.02	-15.3 ± 0.3
Hsp90 α wt	Aha1	3.0 ± 1.5	0.40 ± 0.15	-10 ± 4
Hsp90 α wt + 17-AAG	Aha1 + 17-AAG	3.0 ± 1.5	0.55 ± 0.12	-11 ± 4
Hsp90 α I128T	Aha1	0.02 ± 0.004	0.47 ± 0.02	-17 ± 1.6
Hsp90 α I128T + 17-AAG	Aha1 + 17-AAG	0.015 ± 0.005	0.43 ± 0.01	-18 ± 1.8

^a K_d – dissociation constant.

^b n – stoichiometry of binding.

^c ΔH – enthalpy of binding.

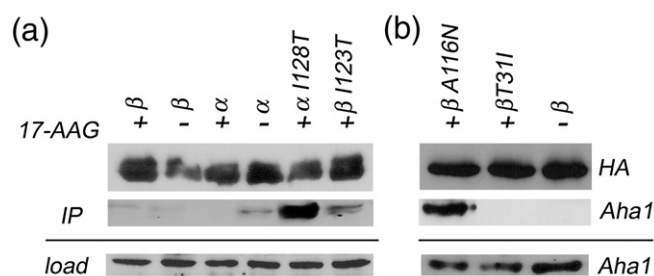


Fig. 5. Hsp90 mutants in the “lid” structure interact strongly with Aha1. (a–b) HEK293 cells transfected with Flag-HA-tagged mutant and wild-type forms of hHsp90 were cultured for 24 h before immunoprecipitation with or without 1 μ M 17-AAG. hHsp90. Flag-HA-hHsp90 was precipitated using anti-FLAG antibodies immobilized on agarose beads. Aha1 and hHsp90 eluted from beads were detected with anti-Aha1 and anti-HA antibodies respectively.

Aha1 expression in the A116N and I123T transfectants resulted in slower growth that was not worsened further by the addition of 17-AAG (Fig. 6). We noticed similar effect of Aha1 silencing in the cells transfected with the wild-type hHsp90 β . However, cells carrying mutation T31I were less sensitive to the decreased expression of Aha1. These results underscore the differences between “Aha1-dependent” A116N and I123T mutants and “Aha1-independent” T31I mutant. The A116N mutation was previously predicted to stabilize closed “lid” conformation by interaction of the aspartate

with residues within the N-terminal domain [10]. We have used molecular modeling to explain augmented Aha1 binding by I123T mutant. Based on our model, we predicted that a closed conformation of the I123T mutant (corresponding to V114T in yHsp82) is stabilized by interaction of threonine114 with arginine 346 located in the middle domain (R366 and R358 in hHsp90 α and β respectively). On the other hand, such mutation destabilizes hydrophobic interactions of V114 with the loop 22–26 in the open conformation of the lid (Fig. 7a–b).

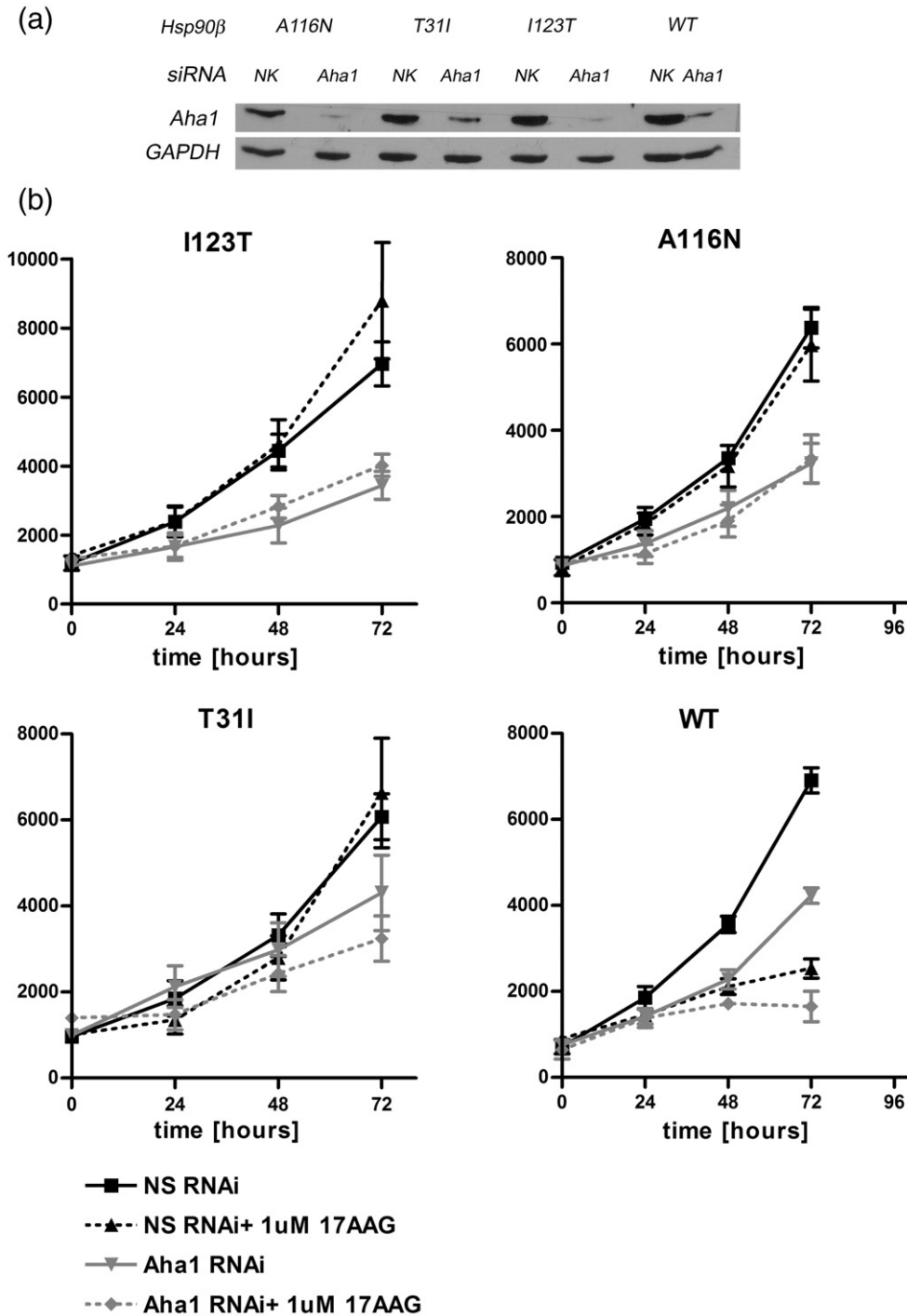


Fig. 6. Inhibitor resistance of I123T and A116N mutants depends on Aha1. Cells that expressed indicated mutants of Hsp90 β were transfected with Aha1-specific siRNA (Aha1 RNAi) and non-specific siRNA (NS RNAi). (a) expression of Aha1 in cells transfected with control and Aha1-specific siRNA 48 h after transfection. (b) growth curves of transfectants cultured with and without 1 μ M 17-AAG. Growth curves were plotted in arbitrary luminometric units. Error bars represent standard deviation calculated from 3 independent experiments.

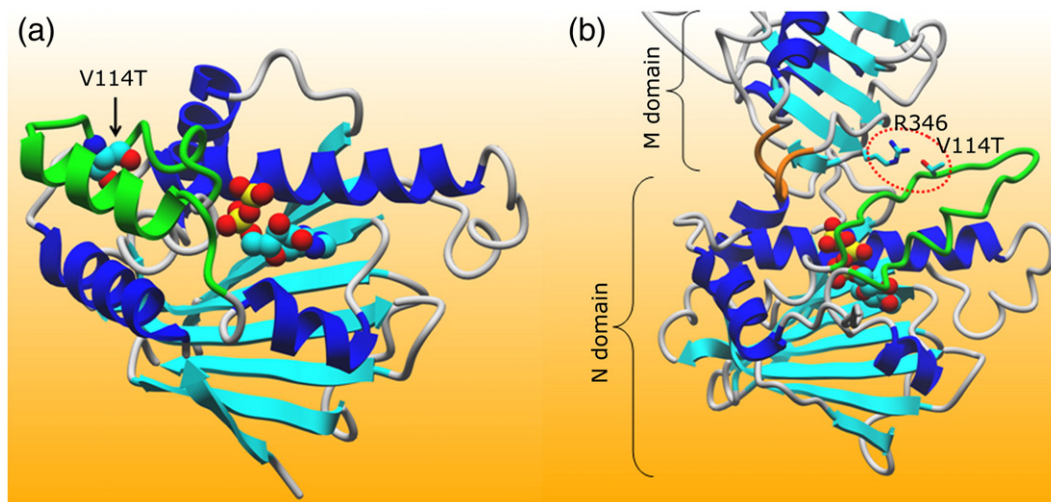


Fig. 7. Computational modeling of the V114T mutation in the yHsp82 structure. (a) in a model based on the complex of HSP90 N-domain with ADP loop containing V114T (residues 97–121 in green) is ordered (contains α -helix). (b) binding of ATP causes large movement of 97–121 loop (in green) and this loop stays disordered. The catalytic loop 377–381 (orange) protrudes to the space occupied previously by loop 97–121.

3.6. ATPase activity of the HSP90 α inhibitor-resistant mutant is strongly stimulated by Aha1 in vitro

We measured ATPase activity of the purified wild-type and I128T mutant of hHsp90 α . Both proteins demonstrated similar ATPase activity in the absence of Aha1 (Fig. 8). However, upon addition of Aha1, the rate of ATP hydrolysis by the hHsp90 α I128T increased at lower Aha1 concentration. At equimolar concentration of Aha1 ATPase activity of hHsp90 α I128T mutant was 3-fold higher compared to wild-type hHsp90 α .

4. Discussion

Yeast was previously used as a model system to assay properties of different Hsp90 proteins [30,31]. We used this system to select for the hHsp90 inhibitor-resistant mutants from a library of plasmids with randomly mutagenized *hHSP90 β* genes. We identified mutation I123T that is sufficient for the inhibitor resistance in yeasts. This mutation is localized in a highly conserved region of the N-terminal domain, known as the “lid” [10]. Mutants hHsp90 α I128T and hHsp90 β I123T

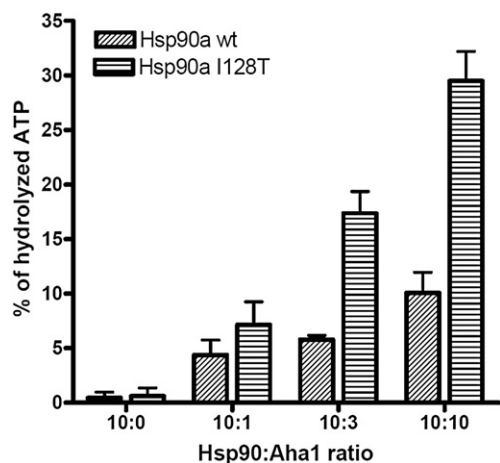


Fig. 8. Stimulation of the ATPase activity of hHsp90 α and hHsp90 α I128T with Aha1. Constant amount of the purified hHsp90 was mixed with Aha1 at different molar ratios and incubated for 30 min at 20 °C. After that time, the reaction was initiated by addition of ATP- γ P³³. Error bars represent standard deviation calculated from 3 independent experiments.

introduced into HEK293 human cells allowed us to select cell lines that were 17-AAG resistant, whereas introduction of the wild-type alleles into the cells, therefore increasing the wild-type protein level, does not protect the HEK293 cells against the inhibitors. Our experiments provide for the first time evidence that point mutations in Hsp90 may induce resistance to 17-AAG in human cells. Hsp90 is a potential attractive target for an anti-cancer therapy and the ATPase inhibitors attract a lot of attention [20,32,33]. Selection of the cells that acquired resistance to inhibitors of oncogenic kinases is a well known problem in therapy [34–36]. Our results indicate that similar process may lead to mutational alterations of Hsp90 resulting in acquired resistance to ATPase inhibitors. So far no mutations in genes encoding Hsp90 in human cells were identified that would alter Hsp90 activity. However, predicted wide use of Hsp90 inhibitors as anti-cancer drugs may favor selection of such mutations. Such scenario is further supported by our results indicating that inhibitor tolerance could be in principle induced by whole class of mutations increasing ATPase activity, irrespectively of the exact localization of these mutations in HSP90 protein.

The increased expression of Hsp90 in cancer cells, sometime correlated with decreased survival of the patients, was reported in several studies [37–39]. This increased expression is most likely a physiological adaptation that compensates an increased requirement for the Hsp90 chaperoning activity in actively proliferating cells and by itself does not give cells extra protection from Hsp90 inhibitors. Our data that expression of transfected Hsp90 does not increase resistance of the cells to inhibition supports this explanation.

Decreased expression or activity of NQO1 oxidoreductase was reported previously as a mechanism of the acquired resistance for 17-AAG and 17-DMAG [40]. The involvement of other oxidoreductases was also postulated [41]. To exclude that inhibitor resistance of our cells depends on the oxidoreductase activity we tested them for resistance for radicicol. Unlike 17-AAG and 17-DMAG, toxicity of radicicol does not depend on the reduction to hydroquinone. Resistance of our cell lines to radicicol proved that this phenotype was not NQO1 dependent. We observed inhibitor resistance only in cultures transfected with mutated hHsp90s and never in cultures transfected with the wild-type hHsp90s. Therefore we concluded that these mutations are sufficient to cause resistance and no mutations or changes in expression of other genes are involved.

One naturally occurring geldanamycin-resistant Hsp90 homologue, *C. elegans* daf-21, was previously described and it was shown that this protein was unable to bind geldanamycin [29]. Amino acids

changed in the inhibitor-resistant human Hsp90s are conserved in daf-21, indicating that the mechanism of their resistance may not involve decreased affinity to geldanamycin. In pull-down in vitro experiments I128T mutant and wild-type hHsp90 α bound geldanamycin equally well. Moreover, ITC measurements of 17-AAG and radicicol binding also did not show dramatic changes in the affinity of hHsp90 α I128T for the inhibitor. Relatively small, 3-fold increase in K_d of the I128T mutant may contribute to the resistance phenotype, but it is unlikely to explain more than 10-fold increase in the resistance observed in vivo.

Binding of Aha1 to both hHsp90 β I123T and hHsp90 α I128T was dramatically increased. We found that hHsp90 β A116N mutation, previously reported to increase ATPase activity in vitro, also resulted in an increased affinity for Aha1 in vivo. Unambiguous results were obtained from ITC assays, which showed that I128T mutation increased the affinity of hHsp90 α for Aha1 over 100 times.

Results of the ATPase activity assays reflected increased Aha1 binding to hHsp90 α I128T mutant compared to wild-type. Both proteins hydrolyzed ATP at a similar rate when Aha1 was not present. However, upon addition of Aha1 ATPase activity of the I128T mutant increased at lower Aha1 concentrations and reached higher maximal level. Our findings that a strong Aha1 binding by hHsp90 may protect it from ATPase inhibitors complement earlier report that decreased Aha1 expression causes hyper sensitivity to 17-AAG [42]. Our results suggest that changes in Aha1 expression level may affect effectiveness of the Hsp90 ATPase inhibitors in treatment of particular tumors.

Aha1 was reported as a cochaperone that binds to the middle domain of Hsp90 and stimulates ATPase activity by moving arginine 380 (yHsp82) closer to the catalytic site [13,15]. However, affinity of Aha1 for the full-length Hsp90 is almost 6 times higher than for the middle domain, suggesting that interaction involves also N-terminal or C-terminal domain of Hsp90 [15]. Our results provide for the first time evidence that sequences located in the N-terminal part of hHsp90 β , in particular amino acids 116 and 123, are important for the Aha1–Hsp90 interaction. These residues lie within the “lid” structure (amino acids 108–139 in hHsp90 α and 103–134 in hHsp90 β), which is predicted to undergo structural remodeling upon ATP binding and hydrolysis [10]. According to our model T123 (T128) may stabilize “lid” in a closed conformation by interaction with R358 (R366) in the middle domain. This interaction may increase stability of the Hsp90–Aha1 complex by promoting conformational change that naturally occurs upon Aha1 binding. Isoleucine 123 is involved in Hsp90–p23 complex formation and mutation of this residue could affect function of p23 [43]. However, we did not see obvious negative influence of the I123T mutation on the growth of the cells that could be attributed to the decreased p23–Hsp90 affinity. Moreover, I123T mutation did not affect p23 binding to Hsp90 in the co-immunoprecipitation experiment (result not shown). Therefore we concluded that the effect of I123T substitution is most likely restricted to Hsp90–Aha1 interaction.

To test whether an increased ATPase activity is sufficient for the inhibitor resistance we studied properties of two mutations that in yHsp90 constitutively increased rate of ATP hydrolysis in vitro [10]. These mutants carried T22I and A107N substitutions. Transfections of the human cells with corresponding hHsp90 β mutants (T31I and A116N) yielded cells that grew continuously on 17-AAG but only A116N mutation increased Hsp90–Aha1 binding, whereas T31I mutant demonstrated wild-type affinity for Aha1. Similar 17-AAG growth phenotypes of A116N and T31I mutants despite differences in their ability to bind Aha1 support conclusion that high ATPase activity and not co-chaperone binding is crucial for the inhibitor resistance. These observations, together with results of the geldanamycin-binding assay, strongly suggest that increased rate of ATP hydrolysis is sufficient for the cells to acquire resistance to the whole class of the ATPase competitive inhibitors, even if affinity of these inhibitors to ATP-binding site is not diminished. Our results complement also previous report that located in the lid segment yHsp90 T101I

mutation, that causes severe decrease of the ATPase activity and decreased expression of Aha1, results in the radicicol and geldanamycin sensitive phenotype [30,42].

It was shown before that the other Hsp90 co-chaperone, HOP/Sti1, contributes to the inhibitor resistance in yeast [30] most likely due to the decreased binding affinity of inhibitor to Hsp90–Sti1 complex, compared to Hsp90 alone [44]. Recently published results proved that Sba1/p23 has also protective effect against inhibitors of Hsp90 [45]. Sba1 binds to the N-terminal domain of Hsp90 and decreases rate of ATP hydrolysis, resulting in the stabilization of the ATP-bound form of Hsp90 [12]. An interaction of Sba1 with Hsp90 also decreases Hsp90 affinity for ATPase inhibitors [45]. Therefore, Aha1, Sti1 and Sba1 binding to Hsp90 can reverse effects of Hsp90 inhibitors, but the mechanism of action of each co-chaperone is different.

In the cells transfected with hHsp90 α I128T or hHsp90 β I123T only these proteins remain active when activity of the endogenous Hsp90 α and β is blocked by 17-AAG present in the medium. In all respects, most of the Hsp90 chaperoning activity in these cells is provided by sole isoform of Hsp90. Therefore, our results proved that neither hHsp90 isoform is essential for the viability of HEK293 cells, even in cultures lasting for several weeks.

A radicicol-resistant mutant of the *S. cerevisiae* Hsp90, designed basing on the structural information on Hsp90 from the radicicol-resistant yeast *H. fuscoatra* was recently reported [46]. This work demonstrated that replacing amino acids crucial for radicicol binding results in resistance to this inhibitor but not to geldanamycin. In contrast, we demonstrated that mutations of amino acids not involved directly in binding of ATP or inhibitor may confer resistance to two different types of inhibitors: radicicol and 17-AAG.

In conclusion, we demonstrated for the first time that various single amino acid substitutions can result in Hsp90 resistance to known inhibitors. Resistance for inhibitors does not correlate with decreased binding affinity for inhibitor of the mutated Hsp90. We provided genetic and biochemical evidence that the “lid” region located in N-terminal domain of Hsp90 is crucial for interaction with Aha1 in addition to the described Aha1 binding site in the middle domain of Hsp90. Our results suggest also that the expression level of Aha1 in a cancer cells may be a valuable prediction factor for the efficiency of the Hsp90 inhibitors in therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2010.03.002.

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