Chaperones

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Artificial Accelerators of the Molecular Chaperone Hsp90 Facilitate Rate-Limiting Conformational Transitions**

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Abstract: *The molecular chaperone Hsp90 undergoes an ATP-driven cycle of conformational changes in which large structural rearrangements precede ATP hydrolysis. Well-established small-molecule inhibitors of Hsp90 compete with ATP-binding. We wondered whether compounds exist that can accelerate the conformational cycle. In a FRET-based screen reporting on conformational rearrangements in Hsp90 we identified compounds. We elucidated their mode of action and showed that they can overcome the intrinsic inhibition in Hsp90 which prevents these rearrangements. The mode of action is similar to that of the co-chaperone Aha1 which accelerates the Hsp90 ATPase. However, while the two identified compounds influence conformational changes, they target different aspects of the structural transitions. Also, the binding site determined by NMR spectroscopy is distinct. This study demonstrates that small molecules are capable of triggering specific rate-limiting transitions in Hsp90 by mechanisms similar to those in protein cofactors.*

In eukaryotes, the molecular chaperone Hsp90 regulates the activity of hundreds of substrate proteins such as kinases, transcription factors, and steroid hormone receptors.[1] Many of them are important for cell fate and also involved in diseases ranging from cancer to neurodegeneration.[2–4]

Hsp90 consists of two identical subunits, each comprising an N-terminal ATP-binding domain (NTD), a middle domain (MD) involved in client binding, and a C-terminal dimerization domain (CTD). Hsp90 undergoes large conformational changes that result in an N-terminally closed form.[5] These are rate-limiting and modulated by co-chaperones which fine-tune Hsp90 towards specific needs.[6–8] These include inhibitors of the Hsp90 ATPase such as Sti1/Hop[9,10] and p23/Sba1[11] as well as activators such as Aha1.[11]

Because of its important function, Hsp90 has become a target for anticancer drugs.[2,12,13] Several specific low-molecular-weight Hsp90 inhibitors, which bind in a competitive manner to the ATP binding pocket of Hsp90, have been identified.[14–17] Some of them have been tested in clinical trials with promising results, among them the geldanamycin derivative 17-AAG and derivatives of purines like the compound PU-H71.[13,18] In addition, the antibiotic novobiocin was shown to inhibit the Hsp90 chaperone function.[19] In contrast to geldanamycin and radicicol, it interacts with a novel site located in the CTD of Hsp90.[20] Furthermore, the compounds 4-hydroxytamoxifen and tamoxifen have been reported to stimulate the Hsp90 ATPase activity, but their mode of action remains unclear.[21]

All clinically relevant inhibitors compete with ATP for binding to Hsp90. In contrast, the known co-chaperones of Hsp90 influence the reaction cycle in an allosteric manner; for example, Sti1/Hop inhibits conformational changes by binding to the MD and CTD[9,22] and Aha1 accelerates the cycle by interactions with the M- and N-domains.[23–25] For a better understanding of Hsp90 as well as for the design of novel strategies for therapeutic intervention, it is important to identify compounds that affect conformational changes by non-ATP-competitive mechanisms. Here, we monitored the closing reaction of Hsp90 by Förster resonance energy transfer (FRET)[5] and employed this assay for screening a compound library to identify novel Hsp90 modulators. The conformational changes of Hsp90 can be visualized by FRET using a dimer in which one subunit is labeled with a donor dye and the other subunit with an acceptor dye (Figure 1 A). In the presence of the non-hydrolyzable ATP analogue AMP-PNP, an increase in FRET signal is observed which reports on the N-terminal closing reaction.[5] We screened a library of roughly 10000 compounds in a 96-well format. The majority of the substances tested did not influence the FRET signal. We identifed 84 substances as potential modulators. Besides known derivatives of radicicol that inhibited the conformational rearrangements leading to the closed state of Hsp90 (Figure S1A), we also found several compounds that led to a marked acceleration of the closing reaction. As the conformational changes are the rate-limiting steps of the Hsp90 ATPase,[5,26] we determined their influences on the ATPase activity. While the majority of the substances tested

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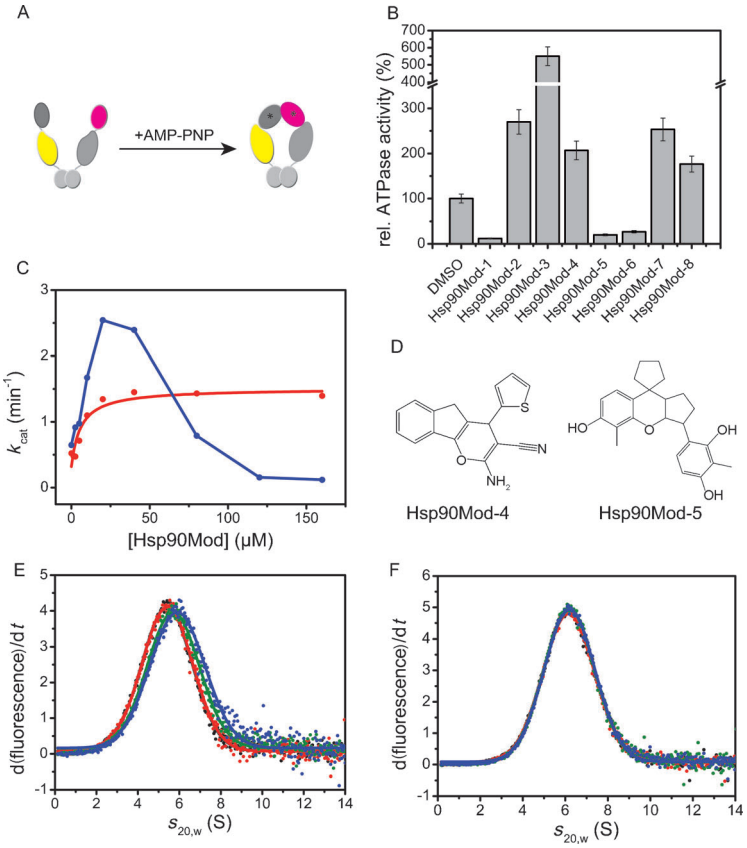

Figure 1. Identification of novel Hsp90 modulators. A) Principle of the FRET-based screen. The binding of AMP-PNP induces a closing reaction in the chaperone, which brings the yellow donor dye closer to the red acceptor dye. This leads to increased FRET efficiency and thus to a signal increase in the acceptor fluorescence channel. B) Influence of identified hits from the screening assay on the Hsp90 ATPase activity (k_{cat}). C) Concentration-dependent influence of identified modulators Hsp90Mod-4 (red) and Hsp90Mod-5 (blue) on the Hsp90 ATPase activity. D) Chemical structures of the further characterized Hsp90 modulators Hsp90Mod-4 and Hsp90Mod-5. E, F) Influence of the modulator Hsp90Mod-5 on the conformation of Hsp90 in the absence (E) or presence (F) of 2 mM AMP-PNP. The conformation of Hsp90 was analyzed by aUC with fluorescence detection using Q385C ATTO488 labeled yHsp90. Sedimentation was measured in the presence of 0 μM (black), 5 μM (red), 20 μM (green), and 40 μM (blue) Hsp90Mod-5.

did not affect the Hsp90 ATPase, eight compounds showed an influence, with three substances inhibiting and five of them accelerating the ATPase activity two- to fivefold (Figure 1B; Table 1). By varying the compound concentration in the ATPase assay we could determine their apparent affinity for Hsp90 based on half-maximal stimulation (Figure 1C and Table 1). For the compounds Hsp90Mod-1, Hsp90Mod-4, Hsp90Mod-5 (Figure 1D), Hsp90Mod-6, Msp90 Mod-7, and Hsp90Mod-8, the effects on the k_{cat} of the Hsp90 ATPase imply affinities between 4 and 58 μM. For Hsp90Mod-2 and Hsp90Mod-3 saturation could not be reached and thus the apparent affinity could only be estimated to be higher than 40 μM. For Hsp90Mod-5, an unusual behavior was observed: at concentrations up to 25 μM, the Hsp90 ATPase was stimulated and above it was inhibited (Figure 1C). Its affinity for Hsp90 seems to be below 10 μM. The most promising activator is Hsp90Mod-4. It stimulates the ATPase activity 2.8-fold and binds Hsp90 with an apparent dissociation constant of 6 μM (Figure 1C).

For further characterization, we chose the compounds Hsp90Mod-4 and Hsp90Mod-5. We found that the modulators do not affect the ATPase of the molecular chaperone Hsp70/Ssa1 (Figure S1B) but have a weak influence on human Hsp90 (Figure S1C), implying that the compounds specifically target the activity of Hsp90. To correlate the effects on the ATPase activity with those on the kinetics of conformational changes, we determined FRET kinetics in the presence of Hsp90Mod-4 and Hsp90Mod-5 (Figure S2A). For the activator Hsp90Mod-4, the rate constant for conformational change was 0.33 min^{-1}. This is about two times faster than in the absence of the modulator (0.18 min^{-1}). Thus the acceleration of the conformational rearrangements mirrors the influence on the ATPase activity, implying a direct activation mechanism. The modulator Hsp90Mod-5 tested at low concentrations led to a threefold acceleration. However, at higher concentrations no closing reaction was observed, consistent with its inhibitory effect (Figure S2A). Next, we determined the effect of the modulators on the affinity of Hsp90 for ATP. In the absence of modulators, a K_M value of 0.32 mM was observed. In the presence of activating concentrations of the modulators Hsp90Mod-4 and Hsp90Mod-5, K_M values of 0.18 mM and 0.07 mM were determined, respectively (Figure S2B). Importantly, both activators also affected the maximal ATP hydrolysis rate of Hsp90, leading to a more than twofold increase in ATP turnover under saturating ATP concentrations (see Figure 1C). Thus, these modulators represent a novel class of Hsp90-binding small molecules, which modulate Hsp90 activity by means of allosteric effects.

Analytical ultracentrifugation (aUC) allowed us to probe the effect of modulators on the overall conformation of the Hsp90 dimer. In the absence of

Table 1: Effects of modulators on the affinity for ATP and ATP turnover.[a]

Substance	$K_{D,app}$ ATP [μM]	rel. ATPase activity [%]
1% DMSO	–	100
Hsp90Mod-1	4±0.8	0
Hsp90Mod-2	>50	246
Hsp90Mod-3	>40	500
Hsp90Mod-4	6±1.5	280
Hsp90Mod-5	≈10	17
Hsp90Mod-6	66±4	8
Hsp90Mod-7	38±2	203

[a] ATPase measurements were performed as described in the Supporting Information. The $K_{D,app}$ for ATP was determined by assaying ATP turnover at different ATP concentrations and nonlinear curve fitting. The relative activity was determined by measuring the ATPase activity in the presence of saturating concentrations of the modulator relative to the turnover in the absence of modulators in the presence of 1% DMSO.

nucleotides, Hsp90 sedimented with an $s_{20,w}$ value of 5.4 S, which is representative of the open state. In the presence of Hsp90Mod-5, a conformational rearrangement to a more compact state was observed as the $s_{20,w}$ value increased with increasing concentrations of the compound to 5.7 S and further to 5.9 S (Figure 1 E). When the closed conformation of Hsp90 was induced in the presence of AMP-PNP, Hsp90Mod-5 had no detectable effect on the sedimentation behavior (Figure 1 F). This is in line with the notion that it affects conformational transitions preceding the closed state. At high concentrations of Hsp90Mod-5 a new peak with an $s_{20,w}$ of 10 S appears (Figure S2C), which likely corresponds to an Hsp90 tetramer. The peak is apparent both in the presence and absence of nucleotide. In contrast to Hsp90Mod-5, we observed only a slight increase of the sedimentation coefficient after addition of Hsp90Mod-4 (Figure S2D), implying that the two compounds employ potentially distinct mechanisms of activation. The acceleration of the conformational cycle of Hsp90 by compounds is reminiscent of the function of the co-chaperone Aha1, which also accelerates the conformational transitions leading to the closed state.[5,23] Therefore, we tested whether their modes of action are different. To this end, we performed ATPase assays in the presence of the accelerator Aha1 and different concentrations of the modulators (Figure S3A). For Hsp90Mod-4 or Hsp90Mod-5 and Aha1, we observed an increase in the maximum k_{cat} to 20 min^{-1} compared to the effect of Aha1 alone (16 min^{-1}). Interestingly, the affinity of Aha1 for Hsp90 is slightly increased in the presence of Hsp90Mod-4 (0.6 μM compared to 1.7 μM) and Hsp90Mod-5 (0.7 μM).

Based on the results obtained, Hsp90Mod-4 and Hsp90Mod-5 may exert their accelerating effects either on steps preceding formation of the N-terminally closed state or they may stabilize the closed state once it is formed. To distinguish between these scenarios, we utilized the co-chaperone p23, which binds exclusively to "closed" Hsp90 and inhibits its ATPase activity. To directly monitor their effect on the p23–Hsp90 interaction, the binding of p23 to Hsp90 was analyzed by aUC (Figure S3B, Figure 2 A,B). We found that Hsp90Mod-4 does not induce a detectable interaction between p23 and Hsp90 in the absence of nucleotides and ATP (Figure S3B, Figure 2 A), suggesting that it cannot promote the stable N-terminal dimerization required for efficient p23 binding. A similar pattern was observed for Hsp90Mod-5 (Figure S3B, Figure 2 A). However, in the presence of 80 μM Hsp90Mod-5 and ATP, a species sedimenting with 7 S was noted, suggesting that p23 can form a complex with Hsp90 under these conditions. These results imply that the two compounds act differently on Hsp90. It seems that Hsp90Mod-4 affects mostly the states preceding the completely closed state while Hsp90Mod-5 affects the closed state. To expand this analysis, we performed aUC experiments in the presence of ATPγS, which is an analogue slowly hydrolyzed by Hsp90 and thus populating the closed state.[5] Consistent with the results obtained above, we did not detect increased binding of p23 in the presence of Hsp90Mod-4, but Hsp90Mod-5 promoted the formation of the binding-competent closed conformation (Figure 2 B).

To gain further insight into the mode of action of the compounds, we tested their effects on an Hsp90 variant which by itself exhibits a strong tendency for N-terminal dimerization (Δ8Hsp90).[27] Surprisingly, for this Hsp90 variant, we did not detect an accelerating effect of the compounds on ATPase activity (Figure 2 C). This suggests that the modulators may only influence the conformational changes prior to hydrolysis, but are inefficient if this part of the reaction cycle is not rate-limiting.

To test for a possible involvement of the C-terminal domain in the interaction, we made use of an Hsp90 construct which lacks this domain (ΔCHsp90), but is dimeric as the N–M domains are covalently linked through a disulfide bridge.[28] Hsp90Mod-4 accelerated the ATPase of this mutant about twofold with an apparent K_D value of 9 μM (Figure S4A). This value is comparable to that of the full-length protein, strongly arguing for an Hsp90Mod-4 binding site in the N- or M-domain. Hsp90Mod-5 was also capable of stimulating the ATPase of ΔCHsp90 (Figure S4A). However, in this case, higher concentrations of the compound were needed. It is therefore possible that the C-domain is partially involved in binding of this compound.

Further, we tested two Hsp90 variants in which the long linker connecting the N- and M-domains was deleted. For ΔLHsp90-211-263, Hsp90Mod-4 had an affinity comparable

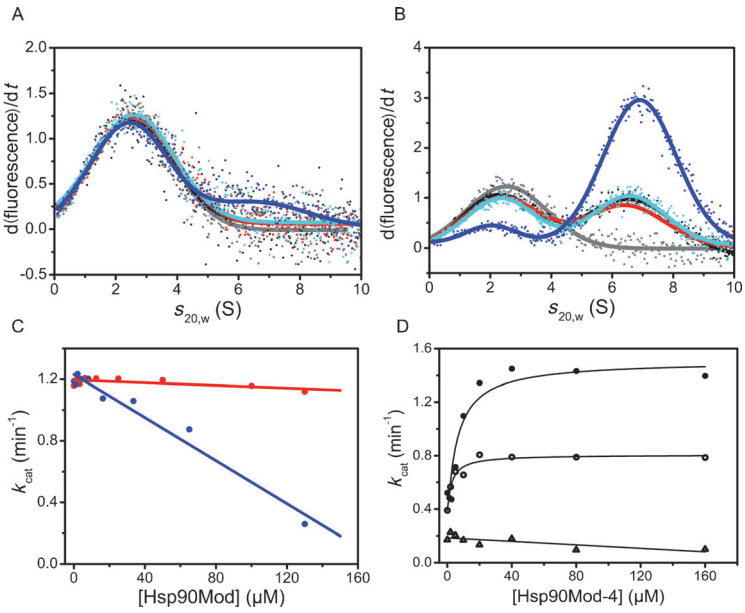

Figure 2. Influence of modulators on Hsp90 co-chaperone interactions. Interaction of Sba1 with yeast Hsp90 A) in the presence of 2 mM ATP or B) in the presence of ATPγS. (gray) Sba1/p23-Fluorescein alone, in the presence of Hsp90 with DMSO (black), 80 μM Hsp90Mod-4 (red), 25 μM Hsp90Mod-5 (cyan), and 80 μM Hsp90Mod-5 (blue). C) The concentration-dependent influence of the modulators (red) Hsp90Mod-4 and (blue) Hsp90Mod-5 on Δ8 Hsp90 was tested in ATPase assays. D) The influence of the modulator Hsp90Mod-4 was tested on constructs with different deletion lengths of the linker between the N- and M-domains (ΔL 211-263 yHsp90, open circles and ΔL 211-266, open triangles).

to that of the full-length protein (Figure 2D). The maximum k_{cat} was lower, consistent with previous studies with this Hsp90 variant.[29] A slightly more extensive deletion, ΔLHsp90-211-266, was not affected by this compound, possibly due to the additional conformational restriction.[29] Hsp90Mod-5 also stimulated ΔLHsp90-211-263 and did not affect ΔLHsp90-211-266 (Figure S4B), again pointing towards conformational restriction hindering the acceleration of the ATPase.

To map the binding site we performed titrations of the inhibitors on Hsp90 with NMR analysis. As titrations with Hsp90Mod-5 suffered from poor solubility and aggregation propensity, we focused on Hsp90Mod-4 and the interaction with the Hsp90 NTD and MD. Significant changes in signal intensities were seen in $^{1}H,^{15}N$ correlation NMR spectra of the NTD upon addition of Hsp90Mod-4 (Figure 3A). Residues affected cluster in three regions of the primary sequence (residues 32–41, 89–94, and 121–134) (Figure 3B). In contrast, titration of the MD with Hsp90Mod-4 showed no changes in chemical shift or peak intensity (Figure S5A and B). Mapping the spectral changes onto the structure of Hsp90 (Figure 3C and D) indicates that Hsp90Mod-4 affects the nucleotide binding site and the interface between NTD and MD. This suggests that Hsp90Mod-4 binds near helix α2 and then induces conformational changes in the ATP binding site. Interestingly, the residues affected by binding of Hsp90Mod-4 are distinct from the binding site of the co-chaperone Aha1[23] (Figure 3C and D), consistent with the synergistic acceleration by Hsp90Mod-4 and Aha1 (Figure S3A).

To test whether the modulators identified in this study also affect Hsp90 function in vivo, we made use of yeast (*S. cerevisiae*) as a model system to analyze the effects on a specific Hsp90 client protein. One of the most stringent clients known is the glucocorticoid receptor (GR). Its activity as a transcription factor can be assayed by a β-galactosidase-based reporter assay. Yeast cells transformed with the respective plasmids were grown in the presence of modulators and β-galactosidase activities were recorded (Figure 4A). Both substances, Hsp90Mod-4 and Hsp90Mod-5, reduced GR activity to similar levels. Radicicol exhibited stronger effects but it has to be considered in this context that its affinity for Hsp90 is much higher (19 nM).[16] The results obtained for the activators demonstrate that acceleration of the Hsp90 chaperone cycle in vivo can lead to decreased client processing thus offering a new strategy for intervention.

To understand the complex relationship between Hsp90, its clients, and our chemical modulators, we tested the formation of GR-LBD-Hsp90 complexes in vitro. Therefore we performed aUC with the fluorescently labeled ligand-binding domain of the GR (GR-LBDm*). Hsp90 binds to this protein weakly in the absence of ATP, but more strongly when ATP is added.[30] In the absence of ATP, Hsp90Mod-4 increased the interaction of Hsp90 with GR-LBDm* slightly and Hsp90Mod-5 to a much larger extent (Figure 4B). In the presence of ATP, binding was only slightly increased upon addition of the modulatory compounds (Figure 4C). Thus both modulatory compounds increase the affinity of yHsp90 towards the GR client protein and at the same time reduce client processing in vivo.

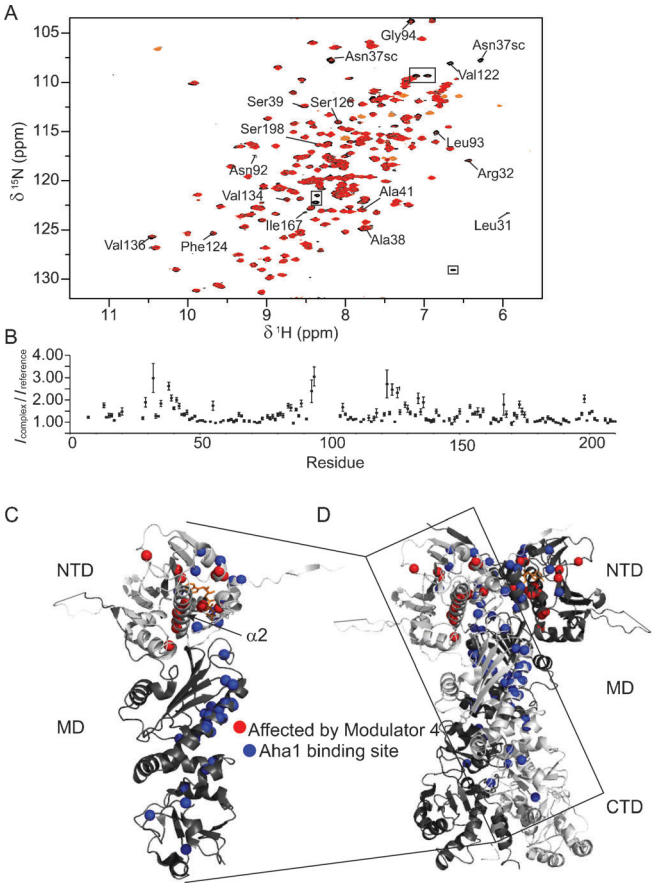

Figure 3. NMR analysis of the Hsp90Mod-4/Hsp90 interaction. $^{1}H,^{15}N$ HSQC NMR spectra of the NTD. A) free (black) and in the presence of HspMod-4 (red). Affected residues are annotated or marked by black boxes where chemical assignments were not available. Differences in peak intensities in free proteins versus the presence of HspMod-4 are shown for the NTD (B). Residues with significant intensity changes are shown as red spheres on the structure of (C) the Hsp90 NTD (light gray) and MD (dark gray) (PDB: 2CG9) and D) the full-length Hsp90 dimer. The known binding site of the co-chaperone Aha1[23–25] is indicated by blue spheres.

In our FRET-based screen we identified small molecules that affect both conformational changes and ATP turnover of Hsp90. Both Hsp90Mod-4 and Hsp90Mod-5 bind Hsp90 with reasonable affinity and activate its ATPase. The two modulators studied here seem to exert their effects by related mechanisms albeit differences exist. Hsp90Mod-5 is able to affect the conformation of Hsp90 already in the absence of nucleotides, leading to a more compact conformation. Thus, modulator binding seems to facilitate the steps preceding formation of closed states. This is similar to the effects of Aha1 on the conformational cycle even though the binding site may be distinct. The influences on p23 binding further suggest that the two modulators do not function identically. While Hsp90Mod-4 seems to affect only steps preceding the formation of the N-terminally closed state, Hsp90Mod-5 also seems to interact with closed states.

These findings are consistent with the binding site of Hsp90Mod-4 determined by NMR spectroscopy. Given that Hsp90Mod-4 binds to the Hsp90 NTD near the NTD/MD interface, it is conceivable that it modulates the ATPase

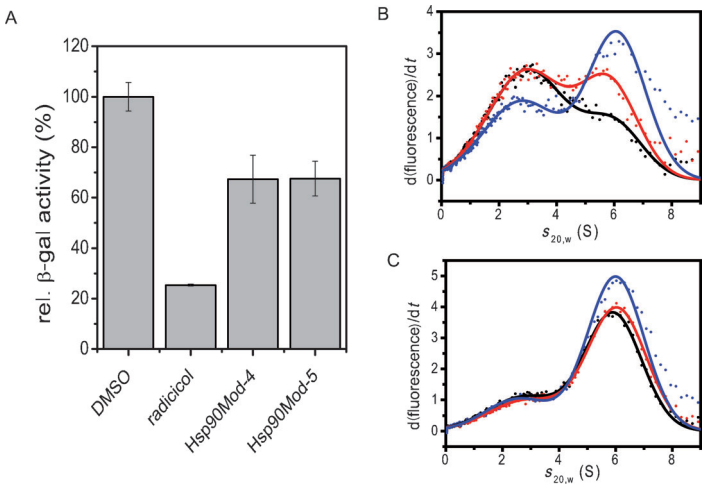

Figure 4. Influence of modulators on client processing. A) Influence of 10 μM of modulators on GR processing in *S. cerevisiae*. B, C) Interaction of Hsp90 with GR-LBD analyzed using aUC with fluorescence detection in the absence of modulator (black), in the presence of 120 μM Hsp90Mod-4 (red) or 25 μM Hsp90Mod-5 (blue) (B), in the absence of nucleotide and (C) in the presence of 2 mM ATP.

activity by changes in conformation and/or dynamics. It is important to note that Hsp90 is a very slow ATPase, hydrolyzing ATP on the timescale of minutes.[31,32] The initial rearrangement to release the N-terminal helix, which will subsequently interact with the other N-terminal domain in the Hsp90 dimer, is one of the critical first steps.[33] We propose that the modulators may facilitate these rearrangements which intrinsically inhibit the Hsp90 ATPase[33] (Figure 5). The effects of the modulators on the Hsp90 variant lacking the first eight residues (Δ8Hsp90) support this notion. Consistent with our hypothesis, it cannot be further stimulated by the modulators as the rate-limiting step is already facilitated by the mutation. Also, the diminished effect of the modulators on the conformationally restricted Hsp90 variants with a shortened linker between the N- und M-domains is in line with this idea.

Interestingly, the binding of the modulators does not interfere with the binding of the client GR. In vitro the client is bound more tightly in the presence of the modulators. This is consistent with previous results showing that GR binds most efficiently to a partially closed conformation of Hsp90.[30] Our results show that in the cell the compound has a negative effect on the maturation and activation of GR. Thus the coordination of cycling time and client affinity seems crucial for the efficiency of the Hsp90 system. Perturbing the system by artificial inhibition as well as by acceleration can therefore interfere with client processing and in consequence be potentially used for therapeutic intervention.

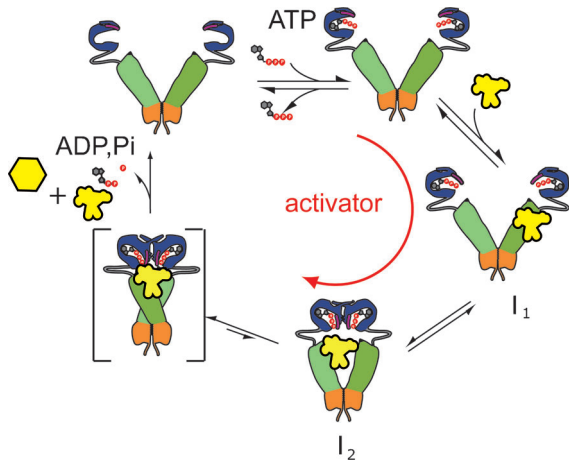

Figure 5. Schematic representation of the modulation of the conformational cycle. The conformational cycle of Hsp90 usually leads to the maturation of the steroid hormone receptor, which is depicted in yellow. The slow steps are the structural changes leading to the closed conformation. After ATP hydrolysis the processed client is released. In the presence of the activators, the conformational changes are accelerated leading to stronger client binding and a faster ATPase reaction. In vivo this results in impaired client maturation, which is visualized by the release of nonmatured receptor in addition to the matured receptor (yellow hexagon).

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