Towards new antifungal drugs: A FRET-based HTS assay to identify selective *C. albicans* HSP90 and HSP90 - Sba1 interaction inhibitors

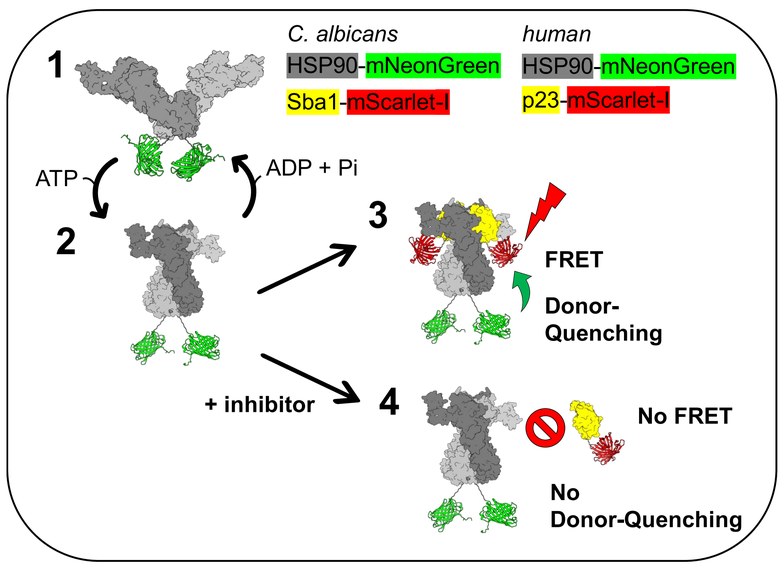
Philip Kohlmann* (1), Pascal Marchand (2), Marc-Antoine Bazin (2), Patrice Le Pape (2) and Joachim Jose (1)

1) University of Münster, Institute of Pharmaceutical and Medicinal Chemistry, PharmaCampus, Corrensstraße 48, 48149, Münster, Germany
2) Nantes Université, Cibles et médicaments des infections et de l'immunité, IICiMed, UR 1155, F-44000 Nantes, France
* E-mail: philip.kohlmann@uni-muenster.de

Abstract

Increase in drug resistance of pathogenic fungi as well as a growing immunocompromised patient population in risk of fungal infections create a need for novel anti-infective drugs [1].
HSP90 forms homodimers and is an essential chaperone involved in a plethora of protein-protein-interactions (PPI) [2]. The HSP90 conformational cycle is driven by the binding and hydrolysis of ATP by HSP90. Whereas HSP90 is highly conserved across species, co-chaperones of HSP90 like Sba1 are less conserved providing a potential target structure for the development of selective antifungal drugs [3]. A prerequisite of HSP90 – Sba1 interaction is the prior binding of ATP to HSP90. In this study, a high-throughput capable *in vitro* Förster Resonance Energy Transfer (FRET) based assay for the identification of HSP90 - Sba1 interaction inhibitors was developed. The assay is also suitable to identify ATP-competitive HSP90 inhibitors. HSP90-mNeonGreen (donor) and Sba1-mScarlet-I (acceptor) fusion protein constructs showed specific interaction in the assay. Known ATP-competitive HSP90 inhibitors such as geldanamycin can be reliably identified and characterized with the assay. This demonstrates the applicability of the assay to identify and characterize the activity of small molecule inhibitors. Competition experiments with Sba1 for Sba1-mScarlet-I binding to HSP90-mNeonGreen provide evidence for the suitability as a screening assay to also identify direct PPI inhibitors. Analogous setups for the homologous human HSP90 – p23 interaction enable the determination of selectivity for *C. albicans* PPI inhibition. A small nucleoside-mimetic library of 320 compounds was screened for inhibition of the HSP90 – Sba1 interaction. Overall, the developed assay showed low data variability and a robust separation, resulting in Z' and Z-factors of consistently > 0.5 [4].

Method



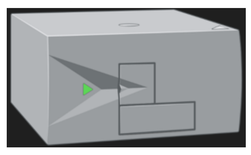
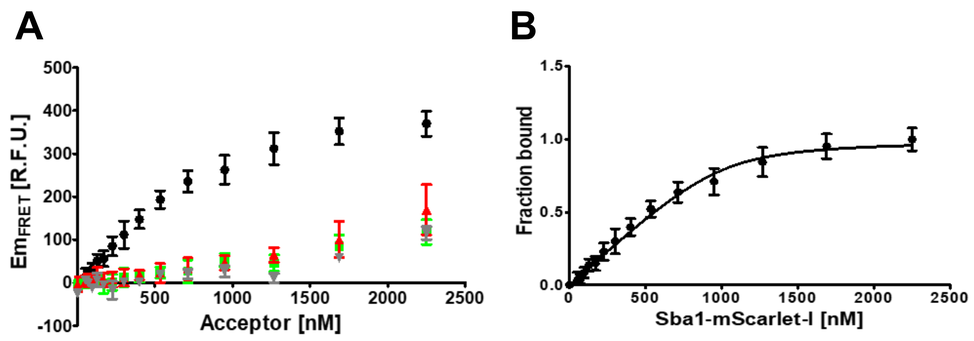


Figure 1. FRET Assay Setup. (**1**) HSP90 exists in the *apo*-state as a C-terminally dimerized homodimer. (**2**) Upon ATP binding, HSP90 adopts an N-terminally dimerized conformation. This conformation is a prerequisite for Sba1 binding. (**3**) HSP90-mNeonGreen - Sba1-mScarlet-I interaction leads to a high FRET emission as well as a Quenching of the Donor emission. (**4**) Inhibition of the interaction leads to a decrease in FRET emission and a higher Donor emission compared to the interacting sample.

Results

Characterization of the HSP90 – Sba1 interaction



Legend	Donor	Acceptor	5 mM ATP
●	HSP90-mNeonGreen	Sba1-mScarlet-I	+
■	mNeonGreen	Sba1-mScarlet-I	+
▲	HSP90-mNeonGreen	mScarlet-I	+
▼	HSP90-mNeonGreen	Sba1-mScarlet-I	-

K_D: 57 ± 8 nM

Figure 2. Assay readout shows high specificity for HSP90-Sba1 interaction.
A Interaction specificity of HSP90-mNeonGreen with Sba1-mScarlet-I (●) in comparison with Donor control (■) and Acceptor control (▲). When omitting ATP from the reaction buffer, HSP90-mNeonGreen - Sba1-mScarlet-I interaction is abrogated (▼). **B** Graph showing the fit of the HSP90-mNeonGreen – Sba1-mScarlet-I interaction and the determined binding affinity (K_D). Em_{FRET}: FRET emission. R.F.U.: relative fluorescence units. The limiting component (HSP90-mNeonGreen/mNeonGreen) was kept at a constant concentration of 1 µM.

Results

Validation of the HTS Assay

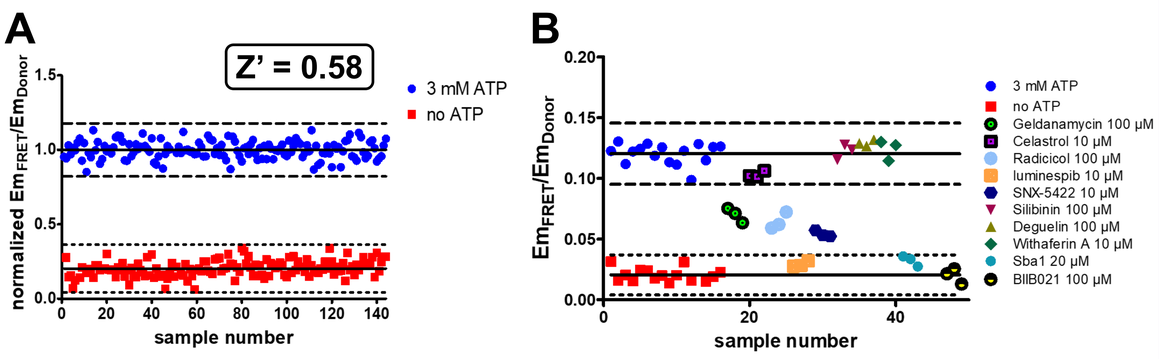


Figure 4. A Screening Assay shows low data variability and robust separation. HSP90-Sba1 interacting samples (blue circles) containing ATP show high FRET emission as well as lower Donor emission compared to control (red squares). Thus, calculation of the quotient of FRET Emission and Donor Emission (Em_{FRET}/Em_{Donor}) improves separation and leads to a Z'-factor of 0.58. **B** The screening assay can identify HSP90-Sba1 interaction inhibitors with a high degree of confidence. HSP90-mNeonGreen (1 µM) and Sba1-mScarlet-I (2 µM) with 3 mM ATP were incubated with various literature-described HSP90 inhibitors. Defining the hit threshold as 3 standard deviations (SD, dashed lines) of the 3 mM ATP samples mean, the assay reliably identifies known ATP-competitive inhibitors of HSP90 (geldanamycin, radicicol, NVP-AUY922, SNX-5422, BIIB021) as disrupting HSP90-Sba1 interaction, whereas non-ATP-competitive HSP90 inhibitors show no effect (celastrol, silibinin, deguelin, withaferin A). Furthermore, Sba1 without mScarlet-I can also be discerned as a HSP90-Sba1 interaction inhibitor by the assay. Solid lines represent means of each data set (3 mM ATP/no ATP). Dashed lines represent 3 SD from the respective mean.

Characterization of model HSP90 inhibitors

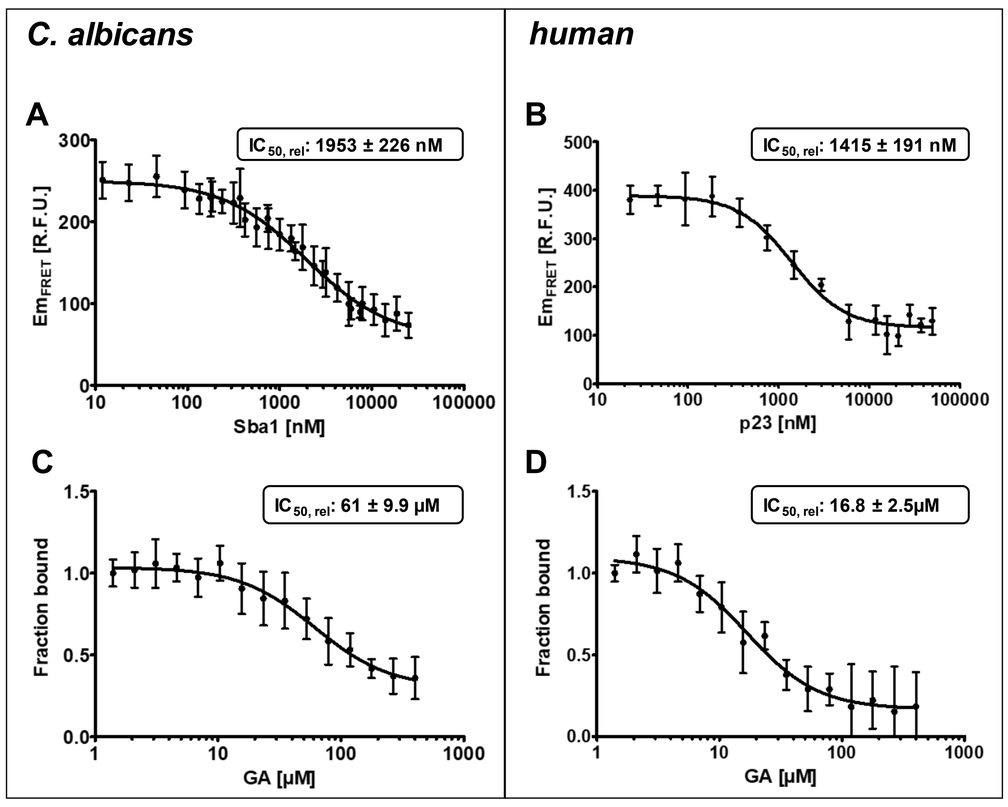


Figure 3. Characterization and selectivity profiling of model HSP90 and HSP90-Sba1/p23 interaction inhibitors. A Sba1 competes with Sba1-mScarlet-I (1 µM) for HSP90-mNeonGreen (1 µM) binding. **B** A similar influence can be observed for the human homologous HSP90α-p23 interaction. p23 was titrated to a fixed concentration of the FRET pair p23-mScarlet-I (1 µM) and HSP90α-mNeonGreen (1 µM). **C** Small molecule inhibitor geldanamycin (GA) disrupts interaction of Sba1-mScarlet-I (2 µM) and the ATP hydrolysis defective mutant HSP90E36A-mNeonGreen (1 µM) in a dose-dependent manner. **D** The experiment for GA influence on the human homologous interaction was analogous to **C**. All experiments were conducted in buffer containing 5 mM ATP.

Screening of nucleoside-mimetics library

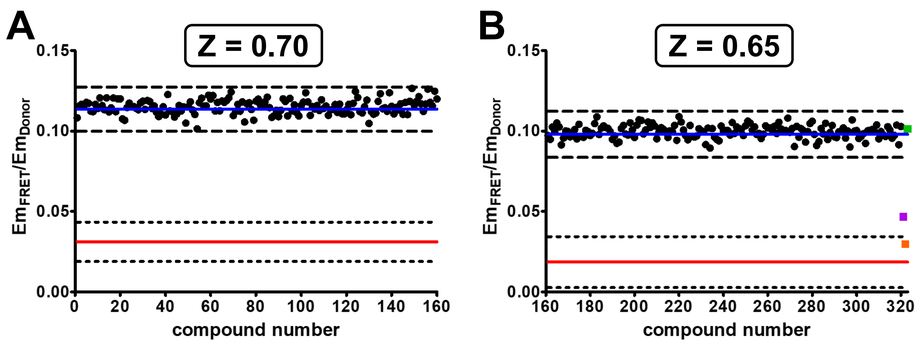


Figure 5. A nucleoside-mimetics library from Enamine (NML-320) was screened for HSP90-Sba1 interaction inhibitory activity. Compounds were screened in duplicates at a concentration of 100 µM. Shown in the graphs is the average Em_{FRET}/Em_{Donor} signal for each compound. The average of the interacting control (3 mM ATP) is shown as a solid blue line. The average of the non-interacting control (no ATP) is shown as a solid red line. Averages of inhibition controls geldanamycin (100 µM) and NVP-AUY922 (10 µM) are depicted as purple and orange squares, respectively. Non-inhibition control withaferin A (10 µM) is pictured as a green square. **A, B** Each graph shows the screening results of one 384-well plate with corresponding Z-factor (Z). Defining 3 SD (dashed lines) as the hit limit, none of the library compounds can be identified as a hit.

Conclusion

In this study, a high-throughput capable FRET-based assay for the characterization and identification of *C. albicans* HSP90-Sba1 interaction inhibitors was developed. Additionally, the selectivity against the homologous *human* HSP90-p23 interaction can be assessed. The screening assay shows consistent Z'- and Z-factors of >0.5. With the assay, large compound libraries should be screened to identify new ATP-competitive HSP90 and/or direct HSP90-Sba1 interaction inhibitors.

References
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