Medium-Throughput Detection of Hsp90/Cdc37 Protein-Protein Interaction Inhibitors Using a Split *Renilla* Luciferase-Based Assay

Farid Ahmad Siddiqui[1#], Hanna Parkkola[1#], Ganesh babu Manoharan[2] and Daniel Abankwa[*1,2]

[1] Turku Bioscience Centre, University of Turku and Åbo Akademi University, 20520 Turku, Finland; [2] Cancer Cell Biology and Drug Discovery Group, Life Sciences Research Unit, University of Luxembourg, 4362 Esch-sur-Alzette, Luxembourg

[*] Corresponding author: daniel.abankwa@uni.lu

[#] Authors contributed equally to this manuscript

Keywords: drug discovery, Hsp90/Cdc37 interaction inhibitors, protein-protein interface inhibitors, split *Renilla* luciferase, assay development

Abstract

The protein-folding chaperone Hsp90 enables the maturation and stability of various oncogenic signaling proteins and is thus pursued as a cancer drug target. Folding in particular of protein kinases is assisted by the co-chaperone Cdc37. Several inhibitors against the Hsp90 ATP-binding site have been developed. However, they displayed significant toxicity in clinical trials. By contrast, the natural product conglobatin A has an exceptionally low toxicity in mice. It targets the protein-protein interface (PPI) of Hsp90 and Cdc37, suggesting that interface inhibitors have an interesting drug development potential.

In order to identify inhibitors of the Hsp90/Cdc37 PPI, we have established a mammalian cell lysate-based, medium-throughput amenable split *Renilla* luciferase assay. This assay employs N-terminal and C-terminal fragments of *Renilla* luciferase fused to full-length human Hsp90 and Cdc37, respectively. We expect that our assay will allow for the identification of novel Hsp90/Cdc37 interaction inhibitors. Such tool compounds will help to evaluate, whether the toxicity profile of Hsp90/Cdc37 PPI inhibitors is in general more favorable than that of ATP-competitive Hsp90 inhibitors. Further development of such tool compounds may lead to new classes of Hsp90 inhibitors with applications in cancer and other diseases.

Introduction

Hsp90 is a molecular chaperone that plays an essential role in the folding and maturation of more than 200 client proteins. These clients include protein kinases, growth factor receptors and many enzymes.[1] Compared to normal cells, the expression of Hsp90 is 2- to 10-fold higher in a wide variety of human cancers, including breast, lung, prostate, colon and skin cancers, which indicates a crucial function of Hsp90 in transformed cells.[2,3] Higher expression of Hsp90 implies the increased stabilization of its client proteins, including mutant driver kinases, such as Raf, Her2 and Src in tumors.[4,5] Thus, cancer cells rely on the Hsp90

chaperone machinery to protect mutated and overexpressed oncoproteins from degradation. Cancer cells can therefore be considered to be addicted to the Hsp90 chaperone machinery.[6]

There are four different human Hsp90 paralogs: two cytoplasmic Hsp90 isoforms (Hsp90α and Hsp90β), Hsp90 of the endoplasmic reticulum (GRP94; 94 kDa glucose-regulated protein) and mitochondrial Hsp90 (TRAP1; tumor necrosis factor receptor-associated protein 1). They display similar ATPase activity and conformational changes, but differ in their interactions with co-chaperones, and due to their localization serve as chaperones to different sets of protein.[7] Cytosolic Hsp90 contains a conserved C-terminal motif that interacts with numerous co-chaperones, which is not present in the other paralogs.[8]

The Hsp90 chaperone machinery includes several co-chaperones and adaptor proteins such as Cdc37, Aha1, FKBP52, p23, STIP1, Hsp70, Hsp40.[9,10] Each of these proteins has its own function associated either with client selection or otherwise completing the Hsp90 chaperone machinery. Cdc37 plays a crucial role in loading kinase client proteins to the Hsp90 chaperone machinery.[11–14] While various kinases, transcription factors and E3 ligases interact with Hsp90, most of the interacting clients are kinases and their binding is mediated by Cdc37. Up to 60 % of the human kinome interacts with Hsp90/Cdc37.[15] Cdc37 selectively recruits clients by challenging their conformational stability, as it locally unfolds them.[16] Thus thermodynamically less stable kinases become clients for the Hsp90/Cdc37 complex.[17]

According to a model proposed for the Hsp90-Cdc37-kinase cycle by Verba et al. an open-state kinase binds the N-terminal domain of Cdc37. Through the Cdc37 middle domain, this complex interacts with the N-terminus of an open-state Hsp90, which closes upon N-terminal ATP-binding. Cdc37 then translocates to the middle domain of Hsp90. ATP hydrolysis results in Hsp90 opening and the kinase folding, while displacing Cdc37 from the complex.[18] Assistance from Hsp70/Hsp40 or other binding partners is probably required for the kinase

cycle, as in the case of steroid hormone receptors.[19] However, the precise process of how these proteins are involved still remains unclear.

In line with the significance of Hsp90 and kinases in tumorigenesis, Cdc37 itself is upregulated in many cancers. For example, in prostate cancer Cdc37 is overexpressed, whereas hepatocellular carcinoma cells overexpress both Cdc37 and Hsp90 as compared to normal cells.[20–23] Therefore, the collaboration of Hsp90 and Cdc37 likely plays an important role in the transformation and maintenance of cancer cells.

Several inhibitors of Hsp90 have been developed, but until now none has made it to the clinic. The first discovered Hsp90 inhibitor was geldanamycin. It is an N-terminal ATP-binding site inhibitor that blocks the ATPase cycle of Hsp90. Geldanamycin never entered clinical trials due to toxicity issues and poor pharmacological properties. Unfortunately its derivatives, including 17-AAG (17-N-allyloamino-17-demethoxygeldanamycin), failed in the clinic partly due to high toxicity.[24,25] Synthetic second generation Hsp90 inhibitors, such as luminespib, have shown more favorable results.[26,27]

Three other classes of inhibitors interfere with the complex formation of Hsp90 and its co-chaperones. Compounds of the first class bind the C-terminal nucleotide binding site.[28] (**Table 1**) While the C-terminal nucleotide-binding site does not display ATPase activity, it induces conformational rearrangements in Hsp90 upon ATP binding. These allosteric conformational changes are critical for the interactions of Hsp90 with its co-chaperones.[29]

Additional C-terminal allosteric sites exist and can be engaged by a second class of inhibitors, such as celastrol and withaferin A, which disrupt Hsp90/Cdc37 complex formation.[30–32] Finally, direct protein-protein interface inhibitors of the Hsp90/Cdc37 complex are known. For example, conglobatin A and the recently discovered platycodin D fall into this category.[33,34] Interestingly, conglobatin A selectively inhibits K-Ras membrane organization by an unknown mechanism and blocks stemness properties of cancer cells,

while it exhibits an unusually low toxicity.[35,36] This may suggest that Hsp90/Cdc37 inhibitors have an interesting potential against cancer stem cells. However, current Hsp90/Cdc37 inhibitors are complex natural products, which are typically difficult to synthesize. In order to discover chemo-synthetically more accessible compounds, we have developed a mammalian cell lysate-based assay that can be used for medium-throughput screening of Hsp90/Cdc37 interaction inhibitors.

Materials and Methods

Materials

Conglobatin A was purchased from BioAustralis (Cat. No. BIA-C1022, Smithfield, NSW, Australia). 17-AAG was purchased from Santa Cruz Biotechnology Inc. (Cat. No. sc-200641, CA, USA). 3-(2-Pyridyl)-5-(4-pyridyl)-1,2,4-triazole was purchased from Alfa Aesar (Cat. No. H50665, Karlsruhe, Germany). Celastrol (Cat. No. 70950) and (–)-epigallocatechin-3-gallate (EGCG; Cat. No. 70935) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Geldanamycin (Cat. No. T6343, TargetMol, Boston, MA, USA), luminespib (Cat. No. HY-10215, MedChemTronica, Sollentuna, Sweden), novobiocin (Cat. No. N825320, Toronto Research Chemicals, Toronto, Ontario, Canada), platycodin D (Cat. No. P578200, Toronto Research Chemicals, Toronto, Ontario, Canada) and withaferin A (Cat. No. NP-007425, AnalytiCon Discovery, Potsdam, Germany) were purchased through MolPort (Riga, Latvia). Stock solutions of these compounds (1 mM for measuring effects on *Renilla* luciferase activity at 20 μM and 10 mM for IC_{50} determination) were prepared in DMSO, which was purchased from Santa Cruz Biotechnology Inc. (Cat. No. sc-358801, CA, USA). JetPRIME transfection reagent was purchased from Polyplus transfection (Cat. No. 114-07, Illkirch, France) and the *Renilla* Luciferase Reporter Assay System from Promega (Cat. No. E2820, Nacka, Sweden).

Plasmid Constructs

The plasmids used in the study were described previously.[37] The full-length human Hsp90 and Cdc37 cDNAs were genetically fused to the N-terminal fragment (NRL, residues 1 to 229) and C terminal fragment (CRL, residues 230 to 311 of *Renilla* luciferase in a pcDNA3.1(+) vector backbone to produce pcDNA3.1(+)-NRL-Hsp90 and pcDNA3.1(+)-Cdc37-CRL, respectively. The NRL or the CRL fragments cloned into the pcDNA3.1(+) vector backbone were used as controls. pGL4.74 expressing full-length *Renilla* luciferase was used for counter assay experiments.

Cell Culture

Human Embryonic Kidney 293 EBNA (HEK 293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Cat. No. D6171, Sigma-Aldrich, Helsinki, Finland), supplemented with 10% fetal bovine serum (FBS, Cat. No. S1810, Biowest, Nuaille, France) and 2 mM L-glutamine (Cat. No. G7513, Sigma-Aldrich, Helsinki, Finland). Cells were incubated in a humidified incubator at 37 °C, with 5% CO_2. Cells were sub-cultured every 2-3 days and confirmed mycoplasma free.

DNA Transfections

One million HEK 293 cells were seeded in 10 cm culture dishes and incubated at 37 °C for 24 hours. The next day, cells were transfected with 10 µg of either pcDNA3.1(+)-NRL-HSP90 or pcDNA3.1(+)-Cdc37-CRL using 20 µl jetPRIME transfection reagent according to the manufacturer instructions. For some control experiments pcDNA3.1(+)-NRL or pcDNA3.1(+)-CRL were used. After 48 hours incubation, cells were harvested by

trypsinization. Cell pellets were washed thoroughly with PBS and used for cell lysate preparation or directly stored at -80°C for up to five months.

Cell Lysate Preparation

Cells were lysed for 10 minutes on ice in 1 ml 1X lysis buffer provided with the *Renilla* Luciferase Reporter Assay System kit. Lysates were cleared by centrifugation at 13,000 g for 1 minute at 4 °C. Lysates were kept on ice throughout the assay procedure. 1 ml of lysate prepared from one 10 cm culture dish was sufficient for 200 reactions or wells. Before performing the assay, the quality of lysates, reagents and positive control was checked by performing the assay in 3 to 5 wells of a 96-well plate as described in the standard split *Renilla* luciferase assay protocol below. If the relative luminescence unit (RLU) was between 40,000 to 50,000 after 40 seconds, lysates and other reagents were considered to meet the requirements to perform further experiments.

Split *Renilla* Luciferase Assay

20 µl assay buffer without *Renilla* luciferase substrate was added to each well of a white solid flat bottom opaque 96-well plate (Cat. No. 3917, Costar, Corning Incorporated, NY, USA). 20 µM test compound (typically 1 µl of a 1 mM stock solution) or 2% DMSO was added as negative control was added. The DMSO concentration was always adjusted to 2% at all compound concentrations. Next, 5 µl NRL-Hsp90-lysate was added in one row (12 wells) of the plate. The plate was agitated for a few seconds to mix reagents and then incubated for 5 minutes at RT. This readily allowed the compounds to bind to Hsp90. Then 5 µl Cdc37-CRL-lysate was added to the NRL-Hsp90-lysate containing wells and incubated at RT for 2 minutes. In the next step, 20 µl buffer containing 2X *Renilla* luciferase substrate was added to the same wells using an electronic pipette. The resulting 50 µl reaction mix was incubated for

30 seconds at RT and then shaken for 10 seconds. Finally, the row was read with a synergy H1 Hybrid Multi-Mode reader (BioTek, Winooski, VT, USA) in luminescence detection mode with a gain value of 250 and integration time of 1 second.

Data Analysis

The Z-factor (Z') was determined as, $Z' = 1 - \frac{3(\sigma_{max} + \sigma_{min})}{|\mu_{max} - \mu_{\min}|}$, where μ is the mean value, and σ is the standard deviation.[38]

Statistical significance of differences between samples was examined using one-way ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA).

Results

Assay Principle

The assay we describe here specifically detects Hsp90/Cdc37 interaction inhibitors, but not ATP-competitive Hsp90 inhibitors that bind the N-terminal ATP-pocket of the chaperone. Three classes of Hsp90/Cdc37 interaction inhibitors exist. The first two classes are allosteric inhibitors that bind the C-terminus of Hsp90, either at the nucleotide binding pocket or another allosteric site (**Table 1**). The third class comprises direct Hsp90/Cdc37 protein-protein interface (PPI) inhibitors, which bind between the N-terminal domain of Hsp90 and the C-terminus of Cdc37.

In order to measure the Hsp90/Cdc37 interaction, we employed a previously described split *Renilla* luciferase assay, where the N-terminal fragment of the luciferase was fused to the N-terminus of full length human Hsp90 and C-terminal fragment to the C-terminus of human Cdc37[39] (NRL-Hsp90 and Cdc37-CRL, respectively; **Figure 1A**). When Hsp90 and Cdc37

fusion proteins interact, the *Renilla* luciferase fragments recombine to form a functional enzyme that produces luminescence upon addition of the coelenterazine substrate (**Figure 1A**). When the interaction between Hsp90 and Cdc37 is inhibited, either by N-terminal PPI or C-terminal allosteric inhibitors, the recombination of the enzyme fragments is disrupted and luciferase activity is decreased (**Figure 1B**).

Assay Workflow

In order to obtain a high expression of the split luciferase fusion proteins NRL-Hsp90 and Cdc37-CRL in HEK 293 cell lysates, they were separately expressed for two days. After harvesting and cell lysis, lysates were cleared by centrifugation (**Figure 2A-C**). The standard assay protocol was carried out in a 96-well plate. A total reaction mixture volume of 50 µl per well was implemented for compatibility with the 384-well high-throughput screening (HTS) format. The reaction mixture included the assay buffer, test compound or vehicle, NRL-Hsp90-lysate, Cdc37-CRL-lysate and luminescent substrate, which were added sequentially to allow for optimal split luciferase maturation and luminescence signal generation (**Figure 2D-F**). With our current manual setup, this required the assay to be performed one row at a time. In the following, we describe the validation experiments for the critical assay steps.

Cell Pellets, but Not Lysates Can Be Stored Frozen

In order to determine whether cell lysates or cell pellets can be stored frozen for later use, we compared the signal of freshly prepared lysates to those of frozen and then thawed up lysates. As compared to the fresh lysate, storage of lysates at -20 °C for 1 or 3 days caused a 35% or 60% decrease in luminescence signal, respectively (**Figure 3A**). By contrast, normal luciferase activity was observed for lysates prepared from cell pellets that were stored at -80 °C for several months.

Background Recombination of Split Luciferase Fragments Is Negligible

The split luciferase fragments could spontaneously recombine and thus produce an unspecific luminescence signal that does not report on the Hsp90 and Cdc37 interaction. We therefore tested whether only the NRL- and CRL-fragments of *Renilla* luciferase without the Hsp90 and Cdc37 fusion could produce a signal in our assay. The specific luminescence signal resulting from the interaction of NRL-Hsp90 and Cdc37-CRL was 4.5-fold higher at our readout time in the standard protocol as compared to the signal produced by the NRL and CRL fragment mix (**Figure 3B**).

The Split Luciferase Assay Tolerates 2% DMSO

Next, we tested for the DMSO tolerance of the assay. For compound screening in biochemical assays, 1-5% DMSO concentration can be acceptable. When titrating increasing concentrations of DMSO in the split luciferase reaction mixture, we found that there was no significant effect of DMSO on the split luciferase Hsp90/Cdc37-fusion protein complex formation up to approximately 2% DMSO (IC_{50} = 5.6 ± 0.9%). (**Figure 3C)**. Therefore, we performed the assay with the tolerated DMSO concentration of 2%, which allowed for usage of typical compound stock concentrations of 1-10 mM in a HTS compatible reaction volume of 50 µl per well.

Compounds Can Be Preincubated with either NRL-Hsp90 or Cdc37-CRL

Before combining the N- and C-terminal fragments of the luciferase fusion proteins, we implemented a compound preincubation step (**Figure 2D,E**). We tested whether compound preincubation with either the NRL-Hsp90- or the Cdc37-CRL -lysate had any impact on the signal decrease. First NRL-Hsp90 was added to 20 µM conglobatin A (direct PPI inhibitor)

or withaferin A (allosteric inhibitor) and preincubated for up to 10 minutes. No difference was detected between the incubation times, suggesting that compound binding equilibrates fast (**Figure 4A**). Similar results were obtained when first preincubating Cdc37-CRL with conglobatin A or withaferin A, followed by NRL-Hsp90 addition (**Figure 4B**). From these results we conclude that the order of compound preincubation with the NRL-Hsp90- or the Cdc37-CRL -lysate does not matter. We decided to employ a 5-minute preincubation step of compounds with NRL-Hsp90 in our standard protocol.

Hsp90/Cdc37 Complex Matures in 2 Minutes

In order to determine the maturation time of the split luciferase when NRL-Hsp90 and Cdc37-CRL are combined, we recorded the complex maturation kinetics. To this end Cdc37-CRL-lysate and substrate were injected into the wells containing NRL-Hsp90-lysate in quick succession. After 10 seconds mixing time, it took approximately 150 seconds for the luminescence signal to reach its maximum (**Figure 4C**). Subsequently, the signal was stable up to 400 seconds.

We then validated this complex maturation time within our standard protocol setting (**Figure 2**). NRL-Hsp90 and Cdc37-CRL were combined with an electronic pipette and then matured for 0-10 minutes. Subsequent substrate addition following our standard protocol resulted in maximum signal within 2-5 minutes (**Figure 4D**), in agreement with the maturation kinetics (**Figure 4C**). Therefore, we employed 2 minutes maturation time for the split luciferase fragments before addition of the substrate.

Substrate Incubation Time and Temperature Dependency

Finally, we tested under standard protocol conditions how the luciferase signal evolves with time at different temperatures. After substrate addition, the NRL-Hsp90 and Cdc37-CRL

reaction was mixed for 10 seconds, before the signal was recorded. At 24 °C the maximum signal was reached after 30 seconds and remained stable up to approximately 180 seconds (**Figure 4E**). Low activities were detected at 30 °C or 35 °C, consistent with the optimal enzymatic activity of *Renilla* luciferase at approximately room temperature (20-25 °C). Therefore, we used in our standard protocol 24 °C as optimal temperature set within the plate reader. We measured the signal after addition of the substrate and a 10-second mixing period. Thus, our standard protocol with 2 minutes maturation and 40 seconds substrate incubation matched the observed maturation kinetics, which showed a maximum signal between 150-400 seconds (**Figure 4D**).

Validation of Assay Using Known Hsp90/Cdc37 Inhibitors

To validate the assay for screening of Hsp90/Cdc37 interaction inhibitors, we tested several compounds with previously reported mechanism of Hsp90 inhibition (**Table 1**), using our standard protocol (**Figure 2**).

All compounds were initially tested at the screening concentration of 20 μM to qualitatively determine their activity on Hsp90/Cdc37 interaction. At the tested concentration, N-terminal ATP competitors (geldanamycin, 17-AAG and luminespib) had no or little effect on Hsp90/Cdc37 complex formation, as expected. By contrast, the allosteric, C-terminal nucleotide pocket binder EGCG inhibited the complex. Novobiocin was very inefficient to inhibit complex formation at the tested concentration. Overall, allosteric, C-terminal Hsp90/Cdc37 interaction inhibitors (celastrol and withaferin A) seemed to have the highest inhibitory effect on complex formation; however, as the counter screen below revealed, the celastrol results were confounded. The direct PPI inhibitor conglobatin A potently inhibited complex formation, while platycodin D had no effect at the tested concentration (**Figure 5A**).[40] Finally, we tested whether an inhibitor of the Hsp90/Aha1 interaction, 3-(2-Pyridyl)-5-

(4-pyridyl)-1,2,4-triazole[41], would also affect the Hsp90/Cdc37 complex formation at 20 μM. This compound bears no chemical relatedness to the Hsp90 inhibitors, as a computational analysis using C-SPADE (https://cspade.fimm.fi) confirmed (**SI Figure 1A**).[42] In line with this no effect was observed, suggesting that compound targeted interfaces between Hsp90/Cdc37 and Hsp90/Aha1 do not overlap (**Figure 5A**).

In order to ensure that loss of luciferase signal is due to the disrupted complementation and not due to effects on the luciferase itself, we performed a counter screen using full-length *Renilla* luciferase. Celastrol strongly decreased the luminescence signal, while all other compounds had no effect; some significant loss was however observed with withaferin A (**Figure 5B**). We found that celastrol showed a strong absorbance at the luciferase emission peak of 480 nm, suggesting that it quenched the luminescent signal (**SI Figure 1B**). Thus, the split luciferase assay is not suitable for compounds with strong absorbance at the luciferase emission peak.

The screening-concentration results were corroborated by determining the IC_{50} of these Hsp90 inhibitors for the Hsp90/Cdc37 interaction in a 2-fold dilution series from 200 μM to 1.56 μM (**Table 1, Figure 5C-F)**. In agreement with the activity ranking at 20 μM, N-terminal ATP competitors had a high IC_{50} beyond the detection range of our assay (**Figure 5C)**. By contrast, C-terminal nucleotide pocket binders displayed an IC_{50} between 40-95 μM (**Figure 5D)**. The allosteric, C-terminal Hsp90/Cdc37 interaction inhibitor withaferin A was active at low micromolar concentration (**Figure 5E)**. Its activity was closely followed by the direct PPI inhibitor conglobatin A, while platycodin D was at least 10-fold less active than conglobatin A (**Figure 5F)**.

Finally, we evaluated the high/medium throughput compatibility of the assay by determining the Z-factor (Z'). The Z' typically ranges between 0 and 1, with Z' >0.5 being required for HTS-assays.[38] The allosteric, C-terminal Hsp90/Cdc37 interaction inhibitor, withaferin A and

the direct Hsp90/Cdc37 interface inhibitor conglobatin A, were used as the positive controls for the validation. The Z' was determined to be 0.71 and 0.52 for withaferin A (**Figure 5G)** and conglobatin A, respectively, using undiluted cell lysates (**Figure 5H)**. Diluting lysates 2-fold resulted in approximately 50% and 9% decreases of the DMSO control and withaferin A signals, respectively (**Figure 5G**). Consequently, the Z' value of the 50% dilution dropped, supporting that optimal results are obtained with undiluted cell lysates.

Discussion

The here reported cell lysate-based, split luciferase assay allows for the identification of two broad types of Hsp90/Cdc37 interaction inhibitors, allosteric C-terminal binders and direct PPI inhibitors. The assay has a very good Z' score and is therefore medium to high-throughput amenable. By using mammalian cell lysates, we can study proteins that are difficult to express and/ or purify from bacteria. When comparing our test set of inhibitors, it appears that C-terminal nucleotide binding pocket inhibitors possess the highest inhibitory potential. However, nucleotide binding to the N-terminus of Hsp90 negatively affects C-terminal domain binding[43], which may reduce the efficacy of inhibitors acting on this domain. Besides, cross-reactions between the nucleotide binding domains may occur, as C-terminal binders have been shown to disrupt both C- and N-terminal nucleotide binding.[44]

In line with previous reports, inhibition of Hsp90/Cdc37 by novobiocin was very inefficient.[45,46] However, the suggested direct PPI inhibitor platycodin D did not inhibit Hsp90/Cdc37 complex formation in our assay. This seems to contrast with previous findings, which demonstrated in immunoprecipitation experiments that platycodin D reduces levels of Hsp90-bound Cdc37, while it does not affect Hsp90 or Cdc37 protein levels.[40] Considering the absence of a response in our assay, the described activity of platycodin D may be due to indirect effects of the compound.

In general, cell-extract/lysate based immunoprecipitation assays can suffer from high false positive results when searching for direct protein-protein interaction inhibitors, as indirect mechanisms of action can remain undetected in the complex protein mixtures. Other biophysical methods can interrogate direct protein-protein interactions in the cellular environment. Förster Resonance Energy Transfer (FRET) methods employing steady-state or time-resolved fluorescence intensity (TR-FRET) measurements, as well as the Bioluminescence Energy Transfer (BRET) method are commonly used to study protein-protein interactions. Most of the high-throughput compatible assays involving these methods are carried out by ratiometric measurements of the donor and acceptor emission intensities. Thus these assays require a plate reader that is capable of measuring both donor and acceptor signals either simultaneously or sequentially.[47,48] Therefore, it is not possible to perform such assays with low-end plate readers that lack appropriate filters or a monochromator.

In recent years, other Hsp90 screening assays have been published. Thomas et al. introduced a yeast-based time-dependent turbidity-measuring liquid culture assay to detect Hsp90 antagonists. This high-throughput assay can identify compounds that modulate Hsp90 through different mechanisms.[49] However, the assay requires establishment of diverse, thoroughly characterized yeast strains and the differences between human and yeast protein interaction networks may affect the outcome. Here presented split luciferase assay could be employed as a secondary screening assay to this chemogenomic screening platform in defining the accurate mechanism of action. In another assay, HPLC-based affinity chromatography was combined with mass spectrometry, allowing detection of compounds that bind Hsp90 in a multicomponent mixture without preceding purification process. While the assay holds promise, it requires access to LC/MS equipment.[50] The amplified luminescence proximity homogenous (Alpha) technology and was used to identify Hsp90/Aha1 inhibitors.[51] Alpha screening technology is a widely adopted industry standard,

due to its versatility and sensitivity.[52] The assay principle is similar to ours, however, it required purified proteins, which makes its implementation more time consuming. This recent boost in development of screening assays to detect Hsp90 inhibitors supports its significance as a highly interesting drug target. Different assays can be used as complementary or alternative techniques depending on the objectives of the studies. We believe that given its advantageous characteristics in terms of sensitivity and usability, the presented split luciferase assay will be useful for the discovery of novel Hsp90/Cdc37 PPI inhibitors with applications in cancer and other diseases.

Acknowledgements

HEK 293 cells were a kind gift from Prof. Florian M. Wurm. Split-luciferase plasmid constructs were a kind gift from Prof. Duxin Sun.

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

Funding

DA acknowledges support from the Academy of Finland (#304638), the Sigrid Juselius Foundation and the Cancer Society Finland. FAS acknowledges support from the Finnish National Agency for Education and Åbo Akademi University.

References

1. Trepel, J.; Mollapour, M.; Giaccone, G.; et al. Targeting the Dynamic HSP90 Complex in Cancer. *Nat. Rev. Cancer* **2010**, *10*, 537–549.
2. Workman, P.; Burrows, F.; Neckers, L.; et al. Drugging the Cancer Chaperone HSP90: Combinatorial Therapeutic Exploitation of Oncogene Addiction and Tumor Stress. *Ann. N. Y. Acad. Sci.* **2007**, *1113*, 202–216.
3. Yamaki, H.; Nakajima, M.; Shimotohno, K. W.; et al. Molecular Basis for the Actions of Hsp90 Inhibitors and Cancer Therapy. *J Antibiot* **2011**, *64*, 635–644.
4. Ferrarini, M.; Heltai, S.; Zocchi, M. R.; et al. Unusual Expression and Localization of Heat-Shock Proteins in Human Tumor Cells. *Int. J. Cancer* **1992**, *51*, 613–619.
5. Solit, D. B.; Chiosis, G. Development and Application of Hsp90 Inhibitors. *Drug Discov. Today* **2008**, *13*, 38–43.
6. Mahalingam, D.; Swords, R.; Carew, J. S.; et al. Targeting HSP90 for Cancer Therapy. *Br. J. Cancer* **2009**, *100*, 1523–1529.
7. Johnson, J. L. Evolution and Function of Diverse Hsp90 Homologs and Cochaperone Proteins. *Biochim. Biophys. Acta - Mol. Cell Res.* **2012**, *1823*, 607–613.
8. Young, J. C.; Moarefi, I.; Ulrich Hartl, F. Hsp90: A Specialized but Essential Protein-Folding Tool. *J. Cell Biol.* **2001**, *154*, 267–273.
9. Young, J. C.; Agashe, V. R.; Siegers, K.; et al. Pathways of Chaperone-Mediated Protein Folding in the Cytosol. *we* **2004**, *5*, 781–791.
10. Weidemann, A.; Johnson, R. S. Biology of HIF-1α. *Cell Death Differ.* **2008**, *15*, 621–627.
11. Echeverria, P. C.; Mazaira, G.; Erlejman, A.; et al. Nuclear Import of the Glucocorticoid Receptor-Hsp90 Complex through the Nuclear Pore Complex Is Mediated by Its Interaction with Nup62 and Importin. *Mol. Cell. Biol.* **2009**, *29*, 4788–

4797.

12. Miyata, Y.; Nishida, E. Evaluating CK2 Activity with the Antibody Specific for the CK2-Phosphorylated Form of a Kinase-Targeting Cochaperone Cdc37. *Mol. Cell. Biochem.* **2008**, *316*, 127–134.

13. Smith, J. R.; Workman, P. Targeting CDC37: An Alternative, Kinase-Directed Strategy for Disruption of Oncogenic Chaperoning. *Cell Cycle*. **2009**, *8*, 362–372.

14. Pratt, W. B.; Morishima, Y.; Murphy, M.; et al. Chaperoning of Glucocorticoid Receptors. *Handb. Exp. Pharmacol.* **2006**, *172*, 111–138.

15. Taipale, M.; Krykbaeva, I.; Koeva, M.; et al. Quantitative Analysis of Hsp90-Client Interactions Reveals Principles of Substrate Recognition. *Cell* **2012**, *150*, 987–1001.

16. Keramisanou, D.; Aboalroub, A.; Zhang, Z.; et al. Molecular Mechanism of Protein Kinase Recognition and Sorting by the Hsp90 Kinome-Specific Cochaperone Cdc37. *Mol. Cell* **2016**, *62*, 260–271.

17. Verba, K. A.; Agard, D. A. How Hsp90 and Cdc37 Lubricate Kinase Molecular Switches. *Trends Biochem. Sci.* **2017**, *42*, 799–811.

18. Verba, K. A.; Wang, R. Y.-R.; Arakawa, A.; et al. Atomic Structure of Hsp90-Cdc37-Cdk4 Reveals That Hsp90 Traps and Stabilizes an Unfolded Kinase. *Science.* **2016**, *352*, 1542–1547.

19. Arlander, S. J. H.; Felts, S. J.; Wagner, J. M.; et al. Chaperoning Checkpoint Kinase 1 (Chk1), an Hsp90 Client, with Purified Chaperones. *J. Biol. Chem.* **2006**, *281*, 2989–2998.

20. Gray, P. J.; Prince, T.; Cheng, J.; et al. Targeting the Oncogene and Kinome Chaperone CDC37. *Nat. Rev. Cancer* **2008**, *8*, 491–495.

21. Stepanova, L.; Yang, G.; DeMayo, F.; et al. Induction of Human Cdc37 in Prostate Cancer Correlates with the Ability of Targeted Cdc37 Expression to Promote Prostatic

Hyperplasia. *Oncogene* **2000**, *19*, 2186–2193.

22. Wang, Z.; Wei, W.; Sun, C. K.; Chua, M.-S.; So, S. Suppressing the CDC37 Cochaperone in Hepatocellular Carcinoma Cells Inhibits Cell Cycle Progression and Cell Growth. *Liver Int.* **2015**, *35*, 1403–1415.
23. Zhu, J.; Yan, F.; Tao, J.; et al. Cdc37 Facilitates Cell Survival of Colorectal Carcinoma via Activating the CDK4 Signaling Pathway. *Cancer Sci.* **2018**, *109*, 656–665.
24. Butler, L. M.; Ferraldeschi, R.; Armstrong, H. K.; et al. Maximizing the Therapeutic Potential of HSP90 Inhibitors. *Mol. Cancer Res.* **2015**, *13*, 1445–1451.
25. Sidera, K.; Patsavoudi, E. HSP90 Inhibitors: Current Development and Potential in Cancer Therapy. *Recent Pat. Anticancer. Drug Discov.* **2014**, *9*, 1–20.
26. Felip, E.; Barlesi, F.; Besse, B.; et al. Phase 2 Study of the HSP-90 Inhibitor AUY922 in Previously Treated and Molecularly Defined Patients with Advanced Non–Small Cell Lung Cancer. *J. Thorac. Oncol.* **2018**, *13*, 576–584.
27. Piotrowska, Z.; Costa, D. B.; Oxnard, G. R.; et al. Activity of the Hsp90 Inhibitor Luminespib among Non-Small-Cell Lung Cancers Harboring EGFR Exon 20 Insertions. *Ann. Oncol.* **2018**, *29*, 2092–2097.
28. Li, Y.; Zhang, D.; Xu, J.; et al. Discovery and Development of Natural Heat Shock Protein 90 Inhibitors in Cancer Treatment. *Acta Pharm. Sin. B* **2012**, *2*, 238–245.
29. Donnelly, A.; Blagg, B. S. J. Novobiocin and Additional Inhibitors of the Hsp90 C-Terminal Nucleotide-Binding Pocket. *Curr. Med. Chem.* **2008**, *15*, 2702–2717.
30. Yu, Y.; Hamza, A.; Zhang, T.; et al. Withaferin A Targets Heat Shock Protein 90 in Pancreatic Cancer Cells. *Biochem. Pharmacol.* **2010**, *79*, 542–551.
31. Grover, A.; Shandilya, A.; Agrawal, V.; et al. Hsp90/Cdc37 Chaperone/Co-Chaperone Complex, a Novel Junction Anticancer Target Elucidated by the Mode of Action of Herbal Drug Withaferin A. *BMC Bioinformatics* **2011**, *12*, S30.

32. Zhang, T.; Li, Y.; Yu, Y.; et al. Characterization of Celastrol to Inhibit Hsp90 and Cdc37 Interaction. *J. Biol. Chem.* **2009**, *284*, 35381–35389.

33. Li, T.; Jiang, H. L.; Tong, Y. G.; et al. Targeting the Hsp90-Cdc37-Client Protein Interaction to Disrupt Hsp90 Chaperone Machinery. *J. Hematol. Oncol.* **2018**, *11*, 1–10.

34. Huang, W.; Ye, M.; Zhang, L. ru; et al. FW-04-806 Inhibits Proliferation and Induces Apoptosis in Human Breast Cancer Cells by Binding to N-Terminus of Hsp90 and Disrupting Hsp90-Cdc37 Complex Formation. *Mol. Cancer* **2014**, *13*, 1–13.

35. Westley, J. W.; Liu, C. M.; Evans, R. H.; et al Conglobatin, a Novel Macrolide Dilactone from Streptomyces Conglobatus ATCC 31005. *J. Antibiot. (Tokyo).* **1979**, *32*, 874–877.

36. Najumudeen, A. K.; Jaiswal, A.; Lectez, B.; et al. Cancer Stem Cell Drugs Target K-Ras Signaling in a Stemness Context. *Oncogene* **2016**, *35*, 5248–5262.

37. Jiang, Y.; Bernard, D.; Yu, Y.; et al. Split Renilla Luciferase Protein-Fragment-Assisted Complementation (SRL-PFAC) to Characterize Hsp90 / Cdc37 Complex and Identify Critical Residues in Protein-Protein Interactions. *J. Biol. Chem.* **2010**, *285*, 21023–21036.

38. Zhang, J. H.; Chung, T. D. Y.; Oldenburg, K. R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.* **1999**, *4*, 67–73.

39. Jiang, Y.; Bernard, D.; Yu, Y.; et al. Split Renilla Luciferase Protein Fragment-Assisted Complementation (SRL-PFAC) to Characterize Hsp90-Cdc37 Complex and Identify Critical Residues in Protein/Protein Interactions. *J. Biol. Chem.* **2010**, *285*, 21023–21036.

40. Li, T.; Chen, X.; Dai, X.-Y.; et al. Novel Hsp90 Inhibitor Platycodin D Disrupts

Hsp90/Cdc37 Complex and Enhances the Anticancer Effect of MTOR Inhibitor. *Toxicol. Appl. Pharmacol.* **2017**, *330*, 65–73.

41. Dutta Gupta, S.; Bommaka, M. K.; Banerjee, A. Inhibiting Protein-Protein Interactions of Hsp90 as a Novel Approach for Targeting Cancer. *Eur. J. Med. Chem.* **2019**, *178*, 48–63.

42. Ravikumar, B.; Alam, Z.; Peddinti, G.; et al. C-SPADE: A Web-Tool for Interactive Analysis and Visualization of Drug Screening Experiments through Compound-Specific Bioactivity Dendrograms. *Nucleic Acids Res.* **2017**, *45*, W495–W500.

43. Garnier, C.; Lafitte, D.; Tsvetkov, P. O.; et al. Binding of ATP to Heat Shock Protein 90. *J. Biol. Chem.* **2002**, *277*, 12208–12214.

44. Söti, C.; Rácz, A.; Csermely, P. A Nucleotide-Dependent Molecular Switch Controls ATP Binding at the C-Terminal Domain of Hsp90. N-Terminal Nucleotide Binding Unmasks a C-Terminal Binding Pocket. *J. Biol. Chem.* **2002**, *277*, 7066–7075.

45. Marcu, M. G.; Schulte, T. W.; Neckers, L. Novobiocin and Related Coumarins and Depletion of Heat Shock Protein 90- Dependent Signaling Proteins. *J. Natl. Cancer Inst.* **2000**, *92*, 242–248.

46. Allan, R. K.; Mok, D.; Ward, B. K.; et al. Modulation of Chaperone Function and Cochaperone Interaction by Novobiocin in the C-Terminal Domain of Hsp90. *J. Biol. Chem.* **2006**, *281*, 7161–7171.

47. Enkvist, E.; Vaasa, A.; Kasari, M.; et al. Protein-Induced Long Lifetime Luminescence of Nonmetal Probes. *ACS Chem. Biol.* **2011**, *6*, 1052–1062.

48. Ivan, T.; Enkvist, E.; Viira, B.; et al. Bifunctional Ligands for Inhibition of Tight-Binding Protein-Protein Interactions. *Bioconjug. Chem.* **2016**, *27*, 1900–1910.

49. Thomas, F. M.; Goode, K. M.; Rajwa, B.; et al. A Chemogenomic Screening Platform Used to Identify Chemotypes Perturbing HSP90 Pathways. *SLAS Discov. Adv. Life Sci.*

R&D **2017**, *22*, 706–719.

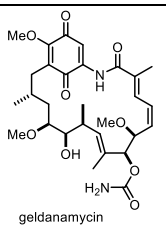
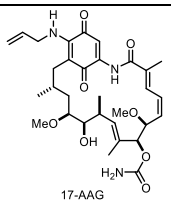
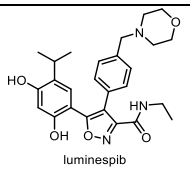
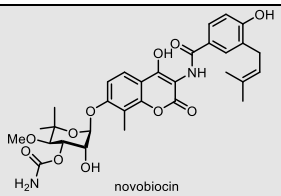
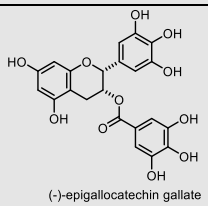
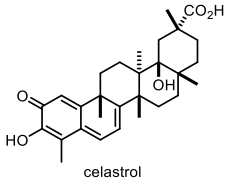
50. Singh, P.; Madhaiyan, K.; Duong-Thi, M. D.; et al. Analysis of Protein Target Interactions of Synthetic Mixtures by Affinity-LC/MS. *SLAS Discov.* **2017**, *22*, 440–446.

51. Ihrig, V.; Obermann, W. M. J. Identifying Inhibitors of the Hsp90-Aha1 Protein Complex, a Potential Target to Drug Cystic Fibrosis, by Alpha Technology. *SLAS Discov.* **2017**, *22*, 923–928.

52. Glickman, J. F.; Wu, X.; Mercuri, R.; et al. A Comparison of ALPHAscreen, TR-FRET, and TRF as Assay Methods for FXR Nuclear Receptors. *J. Biomol. Screen.* **2002**, *7*, 3–10.

Tables and Table Legends

Table 1. Hsp90 inhibitors used in this study and their activity in the split *Renilla* luciferase assay.

Compound	Structure	Hsp90 inhibition mechanism	IC_{50} in split luciferase assay (mean ± SEM), µM
geldanamycin	 geldanamycin	N-terminal ATP competitor	> 200
17-AAG	 17-AAG	N-terminal ATP competitor	
luminespib	 luminespib	N-terminal ATP competitor	
novobiocin	 novobiocin	allosteric, C-terminal nucleotide pocket binder	95.415 ± 0.005
EGCG	 (-)-epigallocatechin gallate	allosteric, C-terminal nucleotide pocket binder	40 ± 2
celastrol	 celastrol	allosteric, C-terminal Hsp90/Cdc37 interaction inhibitor	-

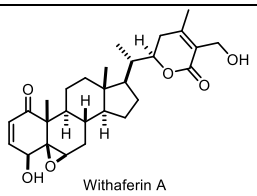
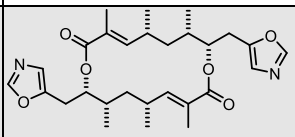
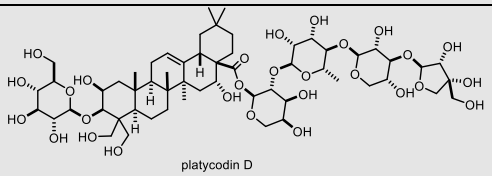
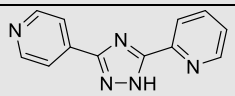
withaferin A	 Withaferin A	allosteric, C-terminal Hsp90/Cdc37 interaction inhibitor	4.07 ± 0.03
conglobatin A	 conglobatin A	direct Hsp90/Cdc37 interface inhibitor	7.2 ± 0.2
platycodin D	 platycodin D	direct Hsp90/Cdc37 interface inhibitor	> 100
3-(2-Pyridyl)-5-(4-pyridyl)-1,2,4-triazole		Hsp90/Aha1 interaction inhibitor	-

Figure Legends

Figure 1. Schematic representation of the split *Renilla* luciferase assay to detect inhibitors of the Hsp90/Cdc37 interaction. Hsp90 and Cdc37 are overexpressed in HEK 293 cells as fusion proteins with the N-terminal (NRL) and C-terminal (CRL) fragments of *Renilla* luciferase, respectively. (**A**) If combined, both NRL-Hsp90 and Cdc37-CRL mature into a functional luciferase enzyme that produces luminescence by converting the substrate coelenterazine. ATP competitive inhibitors have no or little impact on this. (**B**) In the presence of direct or allosteric Hsp90/Cdc37 interaction inhibitors, the formation of the NRL/CRL-complex is prevented, thus decreasing the luminescence signal.

Figure 2. Standard assay workflow. (**A**) HEK cells were plated, (**B**) transfected with NRL-Hsp90 and Cdc37-CRL constructs and (**C**) subsequently harvested and lysed as described in the methods section. (**D**) Here the split luciferase assay was performed in a 96-well plate format at RT. 1) 20 µl assay buffer was combined with 2) 20 µM test compound dissolved in DMSO (typically 1 µl of a 1 mM stock, resulting in 2% DMSO concentration) for screening purposes. For IC_{50} determination, the starting concentration was 200 µM (1 µl of a 10 mM stock, likewise resulting in 2% DMSO concentration). The compounds were mixed with the assay buffer by agitating the plate. Note, the remaining steps were performed one row at a time. 3) 5 µl NRL-Hsp90 lysate was added to the wells of a row. A preincubation step of test compound with NRL-Hsp90 was introduced. (**E**) Then 5 µl Cdc37-CRL lysate was added and the Hsp90/Cdc37 complex was allowed to mature. (**F**) Finally, 20 µl substrate-containing assay buffer was added and luminescence was measured after 40 seconds, including 10 seconds of final shaking.

Figure 3. Lysate storage, background recombination and DMSO tolerance. (**A**) Comparison of split luciferase activity of the NRL-Hsp90/Cdc37-CRL reaction using freshly prepared lysates or lysates stored at -20 °C for 1 and 3 days. Experiments were performed in assay buffer without DMSO following the standard protocol. Data show averages ± SEM of two independent experimental repeats of quintuplicate measurements. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison. Significance levels are annotated as; **p < 0.01; ****p < 0.0001. (**B**) Background fragment recombination was analyzed by comparing luciferase activity of non-fused *Renilla* luciferase fragments to that of Hsp90-and Cdc37-fused NRL and CRL fragments. This comparison was performed in the absence of DMSO following the standard protocol. Data show averages ± SEM of three independent experimental repeats of quintuplicate measurements. (**C**) The effect of increasing DMSO concentrations on *Renilla* luciferase activity in the standard protocol. Data show averages ± SEM of two independent experimental repeats of triplicate measurements.

Figure 4. Hsp90/Cdc37 complex maturation, signal stability and temperature dependence. (**A**) Split luciferase activity of the NRL-Hsp90/Cdc37-CRL reaction was measured in the presence of 2% DMSO, 20 μM conglobatin A or 20 μM withaferin A. NRL-Hsp90-lysate was first incubated with inhibitor/vehicle for up to 10 minutes, before Cdc37-CRL-lysate was added. Standard protocol was used. Data show averages ± SEM of two independent experimental repeats of triplicate measurements. (**B**) The reaction was carried out as in (**A**), however, now with an initial preincubation of inhibitor/ vehicle with Cdc37-CRL for the indicated time, followed by NRL-Hsp90 addition. Data show averages ± SEM of two independent experimental repeats of triplicate measurements. (**C**) NRL-Hsp90 and Cdc37-CRL maturation kinetics. Cdc37-CRL was added to the wells containing assay buffer and NRL-Hsp90 by injector, then immediately substrate was injected to the same wells.

Readings were taken after a 10-second mixing period after substrate addition. Data show averages ± SEM of two independent experimental repeats of triplicate measurements. (**D**) Split luciferase activity after different maturation times of the NRL-Hsp90/Cdc37-CRL complex within the standard protocol. Data show averages ± SEM of two independent experimental repeats of triplicate measurements. (**E**) Temporal evolution of the NRL-Hsp90 and Cdc37-CRL activity was measured at different temperatures. Readings were taken after a 10-second mixing period after substrate addition. Data show averages ± SEM of three independent experimental repeats of triplicate measurements.

Figure 5. Validation of Hsp90/Cdc37 split *Renilla* luciferase assay for compound characterization and screening. (**A**) Split luciferase activity of the NRL-Hsp90/Cdc37-CRL reaction in the presence of 20 μM Hsp90 inhibitors. 2% DMSO was used as negative control and the standard protocol was followed. Data show averages ± SEM of two independent experimental repeats of triplicate measurements. Statistical comparisons to DMSO control are annotated as ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. (**B**) Full-length *Renilla* luciferase activity in the presence of 20 μM Hsp90 inhibitors. 2% DMSO was used as negative control and the standard protocol was followed. Data show averages ± SEM of two independent experimental repeats of triplicate measurements. Statistical comparisons to DMSO control are annotated as ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. **(C-F)** Split *Renilla* luciferase activity of the NRL-Hsp90/Cdc37-CRL reaction incubated with increasing concentrations of (C**)** N-terminal ATP competitors (D) allosteric, C-terminal nucleotide pocket binders, (E) allosteric, C-terminal Hsp90/Cdc37 interaction inhibitor, and (F) direct Hsp90/Cdc37 PPI inhibitors. 2% DMSO was used as negative control and the concentration of DMSO was adjusted to 2% in all wells. The logarithmic concentration of compounds was plotted against RLU and the data were

fitted to log (inhibitor concentration) vs response using a four parameters equation using Prism (GraphPad) to obtain the IC_{50} of inhibitors. (**G,H**) Z' analysis using (G) withaferin A on 100% (empty circles, full circles) and 50% lysate (triangle, star) and (H) conglobatin A (100% lysate only) at 20 μM with 42 replicates. 2% DMSO was used as negative control.