Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 14 (2006) 1134–1142
Bioorganic & Medicinal Chemistry

Development and optimization of a useful assay for determining Hsp90's inherent ATPase activity

Christopher Avila,[a] Boris A. Kornilayev[b] and Brian S. J. Blagg[a,*]

[a]*The Department of Medicinal Chemistry and The Center for Protein Structure and Function, The University of Kansas, 1251 Wescoe Hall Drive, Malott 4070, Lawrence, KS 66045-7563, USA*
[b]*Biochemical Research Service Laboratory, The University of Kansas, 2121 Simons Drive, Lawrence, KS 66047, USA*

Received 6 August 2005; revised 9 September 2005; accepted 13 September 2005
Available online 4 October 2005

Abstract—The Hsp90 molecular chaperone is responsible for the conformational maturation of nascent polypeptides and the rematuration of denatured proteins. Inhibition of Hsp90 represents a promising approach towards the treatment of cancer because numerous signaling cascades can be simultaneously targeted by disruption of the Hsp90-mediated process. Hsp90's ATPase activity is essential to the Hsp90-mediated protein folding process, consequently, a coupled assay was developed and optimized for determination of Hsp90's inherent ATPase activity. Using maltose phosphorylase, glucose oxidase, and horseradish peroxidase as components of this assay, a highly reproducible assay with a *Z*-factor of 0.87 has been produced.

1. Introduction

The transformation of linear polypeptides into biologically active, three-dimensional structures is an essential process that has been regarded as the second half of the genetic code.[1] Generally, this protein maturation process is mediated by molecular chaperones which bind nascent polypeptides and transform them into native proteins that can function in subsequent biological processes. In addition to the maturation of newly formed polypeptides, several chaperones are overexpressed in response to cellular stresses to refold denatured proteins into their biologically active conformation. Heat shock proteins (Hsps) represent a family of chaperones that are overexpressed under cellular stress, including elevated temperature.[2–6]

Although there are several members of the Hsp family, one class has shown exceptional promise for the development of anti-tumor compounds. The 90 kDa heat shock proteins (Hsp90) are responsible for both refolding denatured proteins and folding newly formed polypeptides. They are overexpressed in cancer cells and are essential for cell viability.[7–9] The Hsp90-mediated protein folding mechanism is ATP dependent and energy derived from ATP hydrolysis is essential to this process.[10] In 1994, Whitesell and Neckers discovered that the anti-tumor compound, geldanamycin (GDA), bound Hsp90, and disrupted its ability to function as a molecular chaperone.[11] Later studies by Pearl and coworkers conclusively showed that GDA bound to Hsp90 in an ATP binding motif that was structurally different than most ATP-utilizing enzymes.[12] In fact, only bacterial DNA gyrase, and eukaryotic enzymes MutL and histidine kinase bind ATP in this unique bent conformation, suggesting that selective inhibitors can be made to bind this unusual motif.[13,14]

One of the consequences of Hsp90 inhibition by GDA is the rapid degradation of Hsp90-dependent client proteins. During the Hsp90-mediated protein folding process, a number of client and partner proteins bind the Hsp90/client protein complex, which results in an activated heteroprotein complex.[15,16] Awaiting ATP for continuation of the maturation process, this activated multiprotein complex can bind inhibitors of ATP and form an unstable structure. Ubiquitin ligase is recruited to the unproductive complex and ubiquitinates the protein substrate for degradation by the proteasome.[3,5] Consequently, inhibition of Hsp90 transforms the Hsp90 protein folding machinery into a catalyst for protein degradation.

Keywords: Hsp90; Inhibitors; Assay development; Cancer.
* Corresponding author. Tel.: +1 785 864 2288; fax: +1 785 864 5326; e-mail: bblagg@ku.edu

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.09.027

Forty eight Hsp90-dependent client proteins associated with oncogenesis have been identified.[16] These substrates include numerous kinases responsible for signaling cascades, transcription factors including the steroid hormone receptors, and other oncogenesis-related proteins, such as telomerase. Inhibition of Hsp90 simultaneously causes the degradation of these proteins and thus provides a combinatorial attack on multiple oncogenic pathways.[17–22]

The two most potent inhibitors of Hsp90 identified thus far are GDA[23,24] and radicicol.[25,26] GDA has been shown to produce toxicity unrelated to Hsp90 inhibition, which led to the development of two GDA derivatives, 17-AAG and 17-DMAG, both of which are currently in phases I and II clinical trials for the treatment of cancer (Fig. 1).[27,28]

Unfortunately, these molecules are plagued by poor solubility, which makes formulation difficult.[29] Furthermore, GDA has only recently been synthesized and improved analogues have not yet been prepared.[30,31] In contrast to GDA, radicicol is not active in vivo.[32] Consequently, a tremendous effort is currently underway to identify and develop new inhibitors of Hsp90 that can be easily modified for increased binding affinity and solubility, while at the same time decreasing toxicity.

As mentioned previously, inhibitors of Hsp90 act by competitive inhibition versus ATP. In fact, in the absence of partner/client proteins, Hsp90 maintains inherent ATPase activity that has been shown to be dramatically reduced by Hsp90 inhibitors.[33] Workman and co-workers described a malachite-green assay for identification of inhibitors of Hsp90's inherent ATPase activity[34] and the Chiosis and co-workers disclosed a fluorescence polarization assay for identification of Hsp90 inhibitors.[35] However, additional assays that can be used for elucidation of new Hsp90 inhibitory scaffolds by high-throughput screening have not been reported. Because of the tremendous interest in the development of new Hsp90 inhibitors and the lack of biochemical assays for identification of such inhibitors, we have sought to develop a coupled assay that can be readily used to screen Hsp90 inhibitors in a high-throughput manner.[36,37]

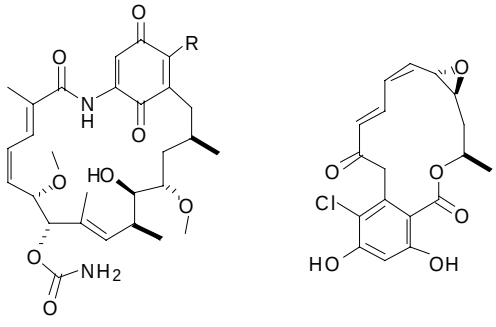

Figure 1. Inhibitors of Hsp90.

In this article, we report the development of a coupled enzymatic assay that was optimized for each enzyme and reagent, as well as time and temperature to provide a highly reproducible assay sufficient for determining Hsp90's ATPase activity.

2. Experimental

2.1. Materials

96-Well cell culture cluster clear, non-pyrogenic, polystyrene, flat- bottomed plates with low evaporation lid (catalog # 3595) were purchased from Corning Incorporated, Corning, NY. Maltose monohydrate (catalog # 4-7288) was purchased from Supelco, Bellefonte, PA. Horseradish peroxidase, Type VI, salt free, lyophilized powder (catalog # P-8375) was obtained from Sigma–Aldrich Chemical Co., St. Louis, MO. Glucose oxidase, from *Aspergillus niger*, a lyophilized powder (catalog # 49180) was purchased from Fluka, St. Louis, MO. Recombinant maltose phosphorylase from *Escherichia coli* (code # MAP2F) was purchased form Biozyme Laboratories Limited, San Diego, CA. 10-Acetyl-3,7-dihydroxyphenoxazine (amplex red, reference # BC043) was obtained from Synchem Laborgemeinschaft, Kassel, Germany. ATP, disodium salt of special quality (catalog # 10519979001), was purchased from Roche Applied Science, Indianapolis, IN. Dimethyl sulfoxide (DMSO molecular biology grade), potassium chloride (enzyme grade), tris hydroxymethyl aminomethane hydrochloride (electrophoresis grade), and magnesium chloride hexahydrate ($MgCl_2$) were purchased from Fisher Biotech. All aqueous solutions were prepared with Millipure water to minimize sources of inorganic phosphate. New plasticware was used for all solutions and mixtures. Absorbance was measured at 563 nm with a SpectraMAX 190 microplate reader (Molecular Devices). Geldanamycin was obtained from the NCI Development Therapeutics Program.

2.2. Purification of yeast Hsp82

Purification of yeast Hsp82 was based on a literature procedure previously described by Buchner and co-workers.[38] *E. coli* strain BL21-CodonPlus(DE3)-RIL-X was transformed by heat shock with pET28-HSP82 containing the full-length Hsp82 gene of *Saccharomyces cerevisiae* with an N-terminal His_6 tag for purification. Selection of transformed cells was performed on LB-Agar, containing kanamycin and chloramphenicol. Individual colonies were subjected to an analysis of their overexpressing ability and the clone with the highest Hsp82 expression was isolated for further studies. A 12 L culture was grown in the presence of kanamycin until OD_{595} 0.8 was reached (3–4 h), before induction of expression with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were then grown for 3 h and harvested by centrifugation at 7000 rpm for 30 min at +4 °C. Harvested cells were weighed and stored at −20 °C. Frozen cells (25–28 g) was thawed and re-suspended in ~250 mL of 40 mM potassium phosphate buffer (pH 8.0), containing 400 mM potassium chloride and

6 mM imidazole. Phenylmethanesulfonyl fluoride and benzamidine were added to the buffer at final concentrations of 1 mM. Cells were lysed with a constant cell disrupting system ('Basic Z' 0.75 KW benchtop model, Constant Systems Ltd, UK). To increase the efficiency of disruption, cells were passed through twice. The lysate was centrifuged at 8000 rpm for 1 h at +4 °C and the supernatant was centrifuged again at 18,000 rpm for 1 h at +4 °C. The supernatant was loaded onto a Ni–NTA column (ProBound Resin, Invitrogen) at a flow rate of 0.5 mL/min. The column was washed with 40 mM potassium phosphate buffer (pH 8.0), containing 400 mM potassium chloride and 20 mM imidazole. A linear gradient of imidazole (20–500 mM) in 40 mM potassium phosphate buffer (pH 8.0) containing 400 mM potassium chloride was applied to the column for Hsp82 elution, which was usually found in fractions corresponding to 170–200 mM of imidazole. The fractions were pooled and concentrated to a volume of 6–10 mL. The concentrate was subjected to gel filtration on Superdex 200 resin in 20 mM Tris–HCl, pH 7.5, containing 300 mM KCl and 1 mM EDTA. Fractions, corresponding to ~0.5 of the column volume typically contained Hsp82. These fractions were concentrated to a volume of 3.0–3.5 mL. Total protein yield was normally around 65–68 mg, which provides Hsp82 in greater than 90% homogeneity for use in subsequent studies.

2.3. ATPase activity assay

The ATPase activity assay procedure was based on that previously described.[36,37] Amplex red was diluted in DMSO to a 10 mM concentration. Maltose phosphorylase, maltose, glucose oxidase, and horseradish peroxidase were diluted in enzyme buffer (0.1 M Tris–HCl, pH 7.5) to a concentration of 200 U/mL, 40 mM, 200 U/mL, and 100 U/mL, respectively.

Test compounds were diluted to final concentrations of 40 μM and distributed into individual wells. Assay buffer (3 μL) (100 mM Tris, 20 mM KCl, and 6 mM $MgCl_2$, pH 7.4) was added to each well. The first and second rows in the 96-well plate were used for positive and negative controls and to determine the *Z*-factor for each plate. The last row contained only assay buffer to determine background absorbance. Hsp90 protein was thawed on ice and diluted in assay buffer to a 250 ng/μL stock solution. 25 μL of that solution was added to each well (total 6.25 μg of Hsp90), except for the background wells which received 25 μL of assay buffer. A working solution was prepared prior to addition. It was prepared by combining 2 U/mL glucose oxidase, 4 U/mL maltose phosphorylase, 0.4 mM maltose, 100 μM amplex red, 0.4 U/mL HRP, and enzyme buffer. Reaction solution (50 μL) was added to each well, except for the blank which contains buffer. Just before use, ATP was diluted in assay buffer to a concentration of 2.5 mM. ATP solution (20 μL) was added to each well for a final volume of 100 μL. Plates were mixed by pipette and then shaken for approximately 30 s to ensure homogeneity. Plates were covered with a plate cover and incubated for 3.0 h at 37 °C. The absorbance was measured at 563 nm. Each of the subsequent assays was performed in triplicate on at least two separate occasions.

2.4. ATP's effect on background activity

With varying sources of ATP commercially available, two sources were identified with the lowest inorganic phosphate concentration. These were from Sigma (catalog # A-6419) Chemical Co. and Roche Applied Science (catalog #10519979001). The working solution was mixed with concentrations of glucose oxidase, maltose phosphorylase, maltose, HRP, and amplex red as noted above. Each source of ATP was diluted in assay buffer to 2.5 mM before 20 μL of each solution was added to different wells containing 30 μL assay buffer and 50 μL working solution to provide a final volume of 100 μL. Plates were mixed by pipette and then shaken for approximately 30 s. The plates were incubated for 3.5 h and monitored every 10 min at 563 nm.

2.5. Effect of temperature

Hsp90 protein, GDA (20 μM), and assay components were added to two different 96-well plates at concentrations stated above. The plates were covered with a plastic plate cover and incubated at 37 °C and 42 °C, respectively, following the protocol outlined above.

2.6. Effect of time on ATPase assay

Absorbance was measured at 563 nm at 15 min intervals to determine the optimal length of the assay.

2.7. Effect of Hsp90 concentration

The concentrations of each of the components; glucose oxidase, maltose phosphorylase, maltose, HRP, and amplex red were kept at 8, 4 U/mL, 0.4 mM, 0.4 U/mL, and 100 μM, respectively. Hsp90 was added to individual wells so that the final amount of Hsp90 was 2.5, 3.75, 5.0, and 6.25 μg. Assay buffer was added to a final volume of 50 μL. Working solution (50 μL) was added to provide a final volume of 100 μL. Plates were mixed by pipette and then shaken for approximately 30 s to ensure homogeneity. The plates were incubated for 3.0 h and the absorbance was measured at 563 nm.

2.8. Horseradish peroxidase's activity

To determine the effect of horseradish peroxidase on the oxidation of amplex red, a reaction with H_2O_2 was used. 50 μL of 10 μM H_2O_2 was added to individual wells. The initial concentration of 0.4 U/mL of horseradish peroxidase was added to the reaction to determine response time. Amplex red was kept at a concentration of 100 μM. Other components of the coupled assay system were omitted. The reaction solution was added to the 50 μL test solutions for a final volume of 100 μL. Plates were mixed by pipette and shaken for approximately 30 s before incubation for 3.0 h at 42 °C and measured at 563 nm.

2.9. Effect of glucose oxidase concentration

Hsp90, GDA, and assay buffer were added as described above to a 96-well plate with positive controls

containing no GDA and two negative controls that included GDA/Hsp90/ATP and one with ATP. Glucose oxidase was added to an Eppendorf vial to a final concentration of 2, 4, and 8 U/mL. The concentrations of the other reactants; maltose phosphorylase, maltose, HRP, and amplex red were kept constant at 4 U/mL, 0.4 mM, 0.4 U/mL, and 100 μM, respectively. The reaction solutions were then added to 50 μL test solutions to a final volume of 100 μL. Plates were mixed by pipette and then shaken for approximately 30 s before incubation at 42 °C for 3.5 h. Absorbance was measured at 563 nm.

2.10. Glucose oxidase's effect on background activity

Various sources of glucose oxidase were purchased and tested to determine their effect on the background absorbance. Glucose oxidase from Sigma Chemical Company and Fluka Chemicals was purchased and used at a concentration of 8 U/mL. Concentrations of each of the other components, maltose phosphorylase, maltose, HRP, and amplex red were 4 U/mL, 0.4 mM, 0.4 U/mL, and 100 μM, respectively. The reaction solution was prepared by adding all enzymes to the enzyme buffer. The reaction solutions were then added to the 50 μL test solutions, mixed by pipette, and shaken before incubation at 42 °C for 3.5 h. Absorbance was measured at 563 nm.

2.11. Effect of maltose phosphorylase on ATPase activity

Hsp90, GDA, and assay buffer were added as described above, to a total volume of 50 μL. Positive controls (no GDA) and negative controls containing Hsp90/GDA/ATP/buffer and no Hsp90, ATP/buffer were also prepared. Increasing concentrations of maltose phosphorylase were added to determine relative absorbance as a function of ATPase activity. Maltose phosphorylase was added to Eppendorf tubes at concentrations of 4, 8, and 16 U/mL. Concentrations of the other reactants; glucose oxidase, maltose, HRP, and amplex red were maintained at 8 U/mL, 0.4 mM, 0.4 U/mL, and 100 μM, respectively. Reaction solutions were added to the 50 μL test solutions to produce a final volume of 100 μL. Plates were mixed by pipette and shaken for approximately 30 s before incubation for 3.5 h at 42 °C. Absorbance was measured at 563 nm.

2.12. Maltose phosphorylase's effect on background activity

Sources of maltose phosphorylase were purchased and tested to determine their effect on the background signal. Maltose phosphorylase from Sigma Chemical Company and Biozyme laboratories was purchased and used at a concentration of 4 U/mL. Concentrations of other reactants; glucose oxidase, maltose, HRP, and amplex red were 8 U/mL, 0.4 mM, 0.4 U/mL, and 100 μM, respectively. The reaction solutions were then added to the 50 μL test solutions, mixed by pipette, and shaken for approximately 30 s. The plate was then incubated for 3.0 h at 42 °C and absorbance was measured at 563 nm.

2.13. Suitability for high-throughput screening

Once optimal conditions were found, the *Z*-factor was calculated taking 10 positive controls with 6.25 μg Hsp90, 20 μL of 2.5 mM ATP, and 5 μL assay buffer and added to 50 μL working solution. Concentrations of glucose oxidase, maltose phosphorylase, maltose, HRP, and amplex red were 8 U/mL, 4 U/mL, 0.4 mM, 0.4 U/mL, and 100 μM, respectively. Ten negative controls were also prepared with 30 μL assay buffer, 20 μL of 2.5 mM ATP stock solution, and 50 μL reaction buffer for a final volume of 100 μL. Plates were mixed by pipette and then shaken for approximately 30 s to ensure homogeneity. Plates were covered with a plate cover and incubated for 3.5 h at 42 °C. The absorbance was monitored at 563 nm. The *Z*-factor was determined by solution of the following equation:[39]

$$Z\text{-factor} = 1 - \frac{3\text{SD of sample} + 3\text{SD of control}}{|\text{mean of sample} - \text{mean of control}|}$$

SD = standard deviation.

3. Results

The Hps90 N-terminal ATP binding site is highly conserved amongst all species.[1–6] ATP binding is believed to act as a conformational switch that allows binding of nascent client proteins when unoccupied. Once occupied, ATP binding promotes head to head dimerization of the N-terminal residues to afford a closed clamp conformation reminiscent of DNA gyrase.[40] In the absence of client proteins, Hsp90 maintains inherent ATPase activity that is substantially diminished in the presence of Hsp90 inhibitors.[31] To develop an assay suitable for measuring Hsp90's inherent ATPase activity, we turned to a coupled enzymatic assay to accurately determine the amount of ATP hydrolyzed by the Hsp90 chaperone.

Previous reports have shown that the coupled enzyme system containing maltose phosphorylase (MP), glucose oxidase (GO), and horseradish peroxidase (HP) is appropriate for determining the amount of inorganic phosphate produced by ATP hydrolyzing enzymes.[36,37] Inorganic phosphate released by Hsp90 is utilized by maltose phosphorylase, which phosphorylates maltose and then cleaves the disaccharide to produce glucose-1-phosphate and glucose (Fig. 2). The resulting product, glucose, is a substrate for glucose oxidase, which uses atmospheric oxygen to produce gluconolactone and hydrogen peroxide. Finally, horseradish peroxidase consumes hydrogen peroxide by the oxidation of amplex red (non-fluorescent) to provide the resorufin product. For greater sensitivity, the fluorescence emission of resorufin at 587 nm can be measured in lieu of absorbance at 563 nm.

Since the N-terminal ATP binding site is highly conserved amongst all Hsp90's, and previous studies have demonstrated that yeast Hsp90 (Hsp82) produces higher ATPase activity (~10-fold) than the mammalian homologues, we proceeded to develop an assay with yeast

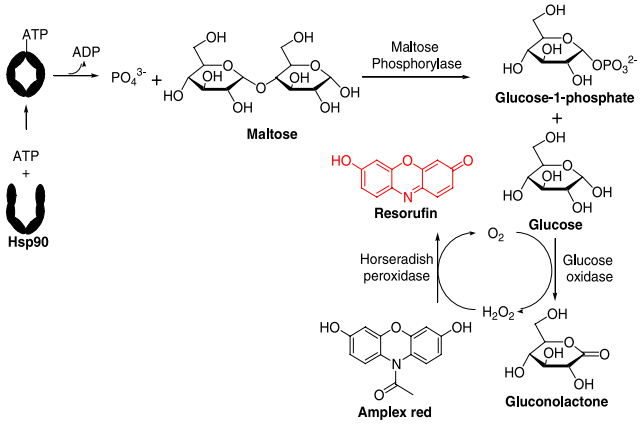

Figure 2. Mechanism of coupled assay.

Hsp90 (Hsp82).[38] For development of the assay, it was necessary to overexpress and purify large amounts of Hsp82 for optimization of the assay protocol. A plasmid encoding the gene for yeast Hsp90 was obtained from the laboratory of Buchner and co-workers.[38] [The plasmid encodes for a polyhistidine tag at the *N*-terminus of Hsp90 to aid purification of Hsp90 by separation on a nickel affinity column.] After inoculation of cultures, expression was induced by the addition of IPTG. The cells were lysed and the cell lysate was separated by SDS–PAGE to confirm the overexpression of yeast Hsp90. After purification by a Ni–NTA and gel filtration column, the protein was concentrated to 5 mg/mL. SDS–PAGE analysis showed Hsp90 to be greater than 90% homogeneous, as shown in Figure 3.

3.1. ATP as a source of inorganic phosphate

It is well known that commercially available ATP can contain large amounts of inorganic phosphate that can have detrimental effects on determination of enzyme-catalyzed ATP hydrolysis. Therefore, the first aim of this study was to identify a relatively pure source of ATP that did not cause formation of resorufin simply

Figure 3. Hsp90 purification. (A) molecular weight markers (200, 116, 97, 62, 45, and 31 kDa), (B) molecular weight markers (250, 150, 100, 75, 50, 37, 25, and 20 kDa), (C) cell lysate, (D) pool from Ni–NTA chromatography, and (E) pool of fractions after gel filtration on Superdex-200.

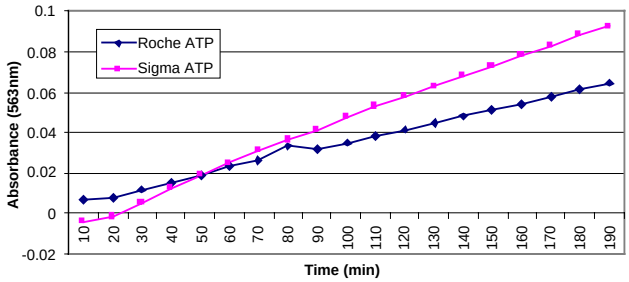

Figure 4. ATP's effect on the background absorbance.

by the addition of impure ATP containing phosphate. Six sources of ATP were purchased and analyzed at 37 °C in combination with the assay cocktail lacking Hsp90. The best sources of ATP that lacked significant phosphate impurities were obtained from Sigma (catalog # A-6419) and Roche Biosciences (catalog # 10519979001). Upon optimization of temperature (see below) we determined the background absorbance of each to be 0.09 and 0.06 absorbance units at 563 nM, respectively, at 42 °C (Fig. 4).

3.2. Effect of temperature on Hsp90 ATPase activity

With the purified recombinant protein in hand, we sought to explore the efficiency of the coupled assay system by monitoring the Hsp90-mediated hydrolysis of ATP. Previous studies have shown that Hsp90 is more active at 42 °C than at 37 °C. To determine whether the coupled assay was stable and the enzymes were active at an elevated temperature, we conducted studies at both 37 and 42 °C to evaluate the efficiency of this assay. As can be seen in Figure 5, Hsp90 ATPase activity was increased at 42 versus 37 °C, suggesting the feasibility of optimizing the assay by incubation at this temperature. In addition, it was determined that the *Z*-factor also increased at a higher temperature, indicating that elevated temperatures did not affect the reproducibility or the deviation of absorbance for this assay. Consequently, all assays described in this manuscript reflect the elevated temperature of 42 °C.

3.3. Length of assay

Upon identification of a temperature that afforded the greatest enzymatic activity, the stability of the enzymes involved in this multicomponent assay was surveyed. Hsp90-catalyzed hydrolysis of ATP was monitored by

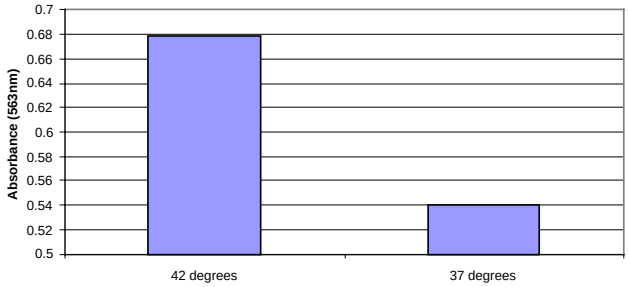

Figure 5. Hsp90 ATPase activity at 37 and 42 °C.

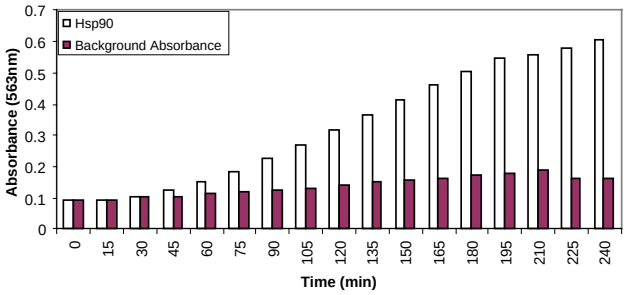

Figure 6. Hsp90 ATPase activity as a function of time.

measuring the amount of resorufin produced over the course of 4.0 h at 15 min intervals. As can be seen in Figure 6, the Hsp90-mediated reaction maintained linearity up to 3.5 h, after which the slope quickly decreased, indicative of the loss of enzymatic activity. Control experiments were run with inorganic phosphate to verify that the assay had no inherent problems associated with higher measurements of absorbance (data not shown).

3.4. Optimal Hsp90 concentration

With the background, temperature, and length of assay firmly established, we sought to optimize the amount of Hsp90 needed for a reproducible assay using the assay system described. Hsp90 was added to each well so that the final amount of Hsp90 was 2.5, 3.75, 5.0, and 6.25 μg. The working solution containing components of the enzyme coupled assay was added and incubated with ATP for 3.5 h at 42 °C. As can be seen in Figure 7, the Hsp90-catalyzed hydrolysis of ATP was linear under these conditions and correlated well with the amount of Hsp90 used in the individual assays. *Z*-factors were calculated for each concentration of protein and it was determined that 6.25 μg Hsp90 provided the highest *Z*-factor.

3.5. Horseradish peroxidase's effect on the absorbance of resorufin

To verify that HRP did not cause a lag time between the hydrolysis of ATP and the final measurement of resorufin absorbance, HRP was incubated with amplex red and hydrogen peroxide. As can be seen in Figure 8, amplex red was immediately oxidized to resorufin, which is consistent with the reported K_{cat} for this enzyme ($K_{cat} \sim 100$/min).[36,37]

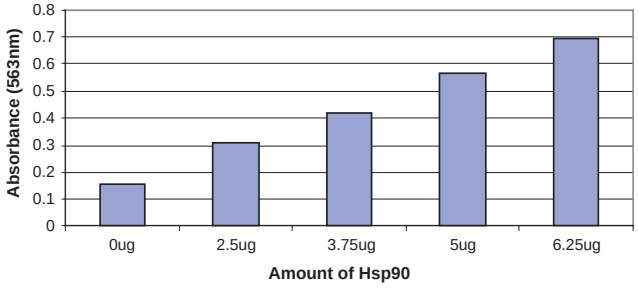

Figure 7. ATPase activity is dependent upon Hsp90.

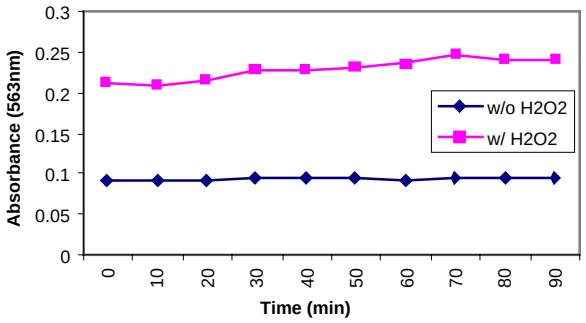

Figure 8. Effect of H_2O_2 on horseradish peroxidase.

3.6. Optimization of glucose oxidase on the Hsp90 ATPase reaction

To determine whether glucose oxidase produced a delayed response between Hsp90-catalyzed hydrolysis of ATP and resorufin production, three concentrations of glucose oxidase were surveyed in the multicomponent assay. Glucose oxidase, 2, 4, and 8 U/mL were added to individual wells followed by addition of the other components. As demonstrated in Figure 9, increasing the concentration of glucose oxidase to 8 U/mL resulted in an absorbance reading of 0.68 at 2.5 h, instead of the 3 h previously observed with 2 U/mL, suggesting that shorter time periods are appropriate or that increased time may provide greater sensitivity.

3.7. Effect of glucose oxidase on background absorbance

Although increased glucose oxidase concentrations provided higher absorbance readings, they also resulted in an elevated background as well. In the absence of Hsp90, the assays were reproduced with Roche's ATP and glucose oxidase purchased from Sigma and Fluka. As shown in Figure 10, background absorbance for the Sigma product was 0.12, while that obtained from Fluka produced a lower background of 0.06 absorbance units after 3 h of incubation at 42 °C. Attempts to remove phosphate impurities from the Sigma product via precipitation and redissolving in phosphate free buffer provided no decrease in background absorbance.

3.8. Maltose phosphorylase's effects on the Hsp90 ATPase reaction

Continuing to optimize each step of this coupled enzymatic assay, we looked at the previous enzyme in this

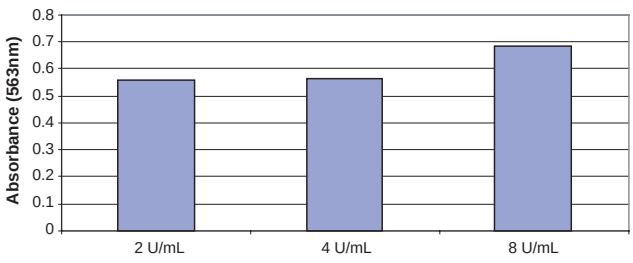

Figure 9. Effect of glucose oxidase concentration on Hsp90 ATPase activity.

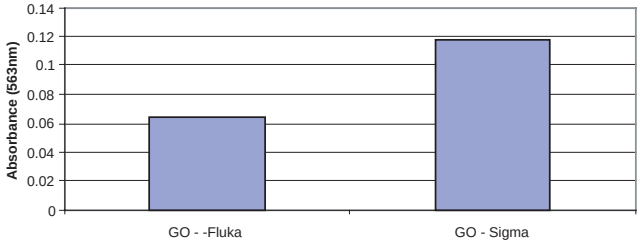

Figure 10. Background absorbance with different sources of glucose oxidase.

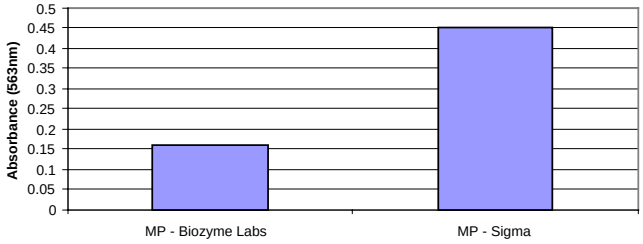

Figure 12. Background absorbance resulting from different sources of maltose phosphorylase.

multicomponent assay, maltose phosphorylase. Using the optimized conditions obtained thus far, three concentrations of maltose phosphorylase were added to individual wells before addition of other components. After incubation at 42 °C for 3 h with 4, 8, and 16 U/mL of maltose phosphorylase, it was determined that the previously maximized absorbance was reached within 1.8 h in the presence of 16 U/mL of maltose phosphorylase (Fig. 11). Once again, this suggested that shorter reaction times could be used if necessary, or that longer reaction times were likely to provide greater sensitivity.

3.9. Effect of maltose phosphorylase on background absorbance

Maltose phosphorylase was purchased from Sigma (catalog # M-8284) and Biozyme Laboratories (catalog # MAP2F) and added to the coupled assay system lacking Hsp90. In contrast to the other assay components, maltose phosphorylase had a significant effect on the production of resorufin. In fact, maltose phosphorylase from Sigma produced a background absorbance of 0.45, while the enzyme purchased from Biozyme provided a background of 0.16 in the same assay (Fig. 12). The results from this experiment suggest that the source and purification of maltose phosphorylase is critical to the development of this assay and that attention to phosphate contamination should be carefully analyzed prior to using in such an assay.

3.10. Optimized Hsp90 ATPase assay

The optimized assay for monitoring the amount of inorganic phosphate produced by Hsp90-catalyzed hydrolysis of ATP was found to be a working solution that contains glucose oxidase, maltose phosphorylase, maltose, amplex red, and horseradish peroxidase at concentrations of 8 U/mL, 4 U/mL, 0.4 mM, 100μM, and 0.4 U/mL, respectively. Hsp90 (6.25 μg) was added and incubated at 42 °C for 3.5 h. The amount of inorganic phosphate produced by this assay was compared to a standard curve prepared by incubation of various phosphate concentrations with the assay system in the absence of Hsp90. In accordance with previous results, yeast Hsp90 was found to produce a K_{cat} of ~0.6/min. A positive control for inhibition of Hsp90 ATPase activity was the introduction of 20 μM GDA, which completely abolished Hsp90's ATPase activity in this assay, but did not affect the coupled assay system in the presence of increasing concentrations of inorganic phosphate. The IC_{50} of GDA in this assay was determined to be 2.5 μM, which is well within the reported literature values for other measurements of Hsp90 inhibitory activity.[41,42] The background absorbance of this assay in the presence of Hsp90 and 20 μM GDA was typically between 0.15 and 0.17 absorbance units, while uninhibited Hsp90 produced an absorbance between 0.60 and 0.70 units after 3 h of incubation at 42 °C (Fig. 13).

To determine the efficiency of this assay, a *Z*-factor was calculated for the assay in the absence and presence of GDA. Using the equation previously described by Zhang et al., 20 assays were simultaneously run on a 96-well plate, which included 10 inhibited and 10 uninhibited reactions.[39] The average absorbance measurement was 0.64 for the Hsp90-catalyzed reaction and 0.15 for the inhibited assays. Using this equation, the *Z*-factor was determined to be 0.87, which represents a highly reproducible and optimized assay that should prove useful in subsequent high-throughput screening experiments.

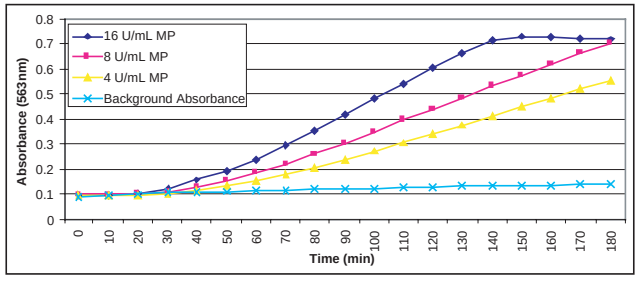

Figure 11. Maltose phosphorylase's effect on Hsp90-catalyzed hydrolysis of ATP.

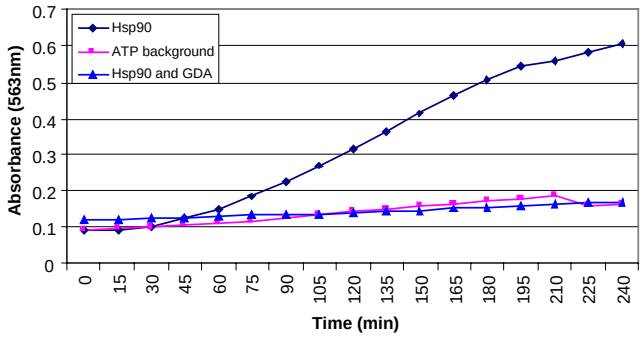

Figure 13. Hsp90 ATPase activity at optimized conditions with background activity.

4. Discussion

As Hsp90 continues to evolve as a promising target for the development of cancer chemotherapeutics, there will be an increasing need to identify new inhibitors that lack the undesirable properties associated with the natural product inhibitors. An increasingly powerful method for the identification of such lead compounds is high-throughput screening, which requires a highly robust and reproducible assay for evaluating structurally diverse compounds in an expedient manner. Because mammalian Hsp90 produces low ATPase activity, recombinant yeast Hsp90 was chosen for the development of such an assay. Yeast Hsp90 ATPase activity is significantly higher than that of the mammalian homologue. Furthermore, the N-terminal nucleotide binding pockets are highly conserved amongst species and the co-crystal structures of several inhibitors bound to yeast Hsp90 have been previously solved, providing adequate structural information for subsequent modification of molecules identified by high-throughput screening.[12]

Since the rate-determining step in this assay system is Hsp90-catalyzed hydrolysis of ATP (Hsp90 $K_{cat} \sim 0.4$/min, MP $K_{cat} \sim 20$/min, GO $K_{cat} \sim 24$/min, and HP $K_{cat} \sim 100$/min), the coupled assay described herein provides an effective measurement of Hsp90's ATPase activity.[36,37] There are numerous sources of ATP that are commercially available; however, many of these contain a significant amount of inorganic phosphate, which in this assay severely effects the measurement of background activity.

During our studies we determined that the most reliable source of ATP was catalog # 10519979001 from Roche. This source of ATP provided Hsp90 with a K_{cat} similar to that previously described by other researchers while simultaneously providing a low background absorbance.

Although several concentrations of Hsp90 were effective at providing signal-to-noise ratios between 3:1 and 5:1, 6.25 μg of Hsp90 per 100 μL well provided an outstanding *Z*-factor, which illustrates the highly reproducible nature of the assay. Previous reports have demonstrated that Hsp90's ATPase activity is increased at elevated temperature, which is not surprising when one considers that Hsp90 must refold rapidly accumulating heat denatured proteins at temperatures higher than 37 °C. Therefore, the results from our studies, which indicate that Hsp90's ATPase activity is greater at 42 °C are not surprising.[10] The ability to increase temperature enabled us to increase the Hsp90-catalyzed hydrolysis of ATP while increasing background absorbance only minimally. The stability of other enzymes involved in this multicomponent assay had not been previously determined at higher temperatures and it was unknown whether a suitable signal-to-noise ratio could be obtained. To address these issues we focused on the optimization of each reagent and commercially available enzyme in this assay. It was determined that a fourfold increase in glucose oxidase concentration provided shorter reaction times. Increased concentrations of MP also had a small impact on this assay; however, the cost of maltose phosphorylase from commercial sources is relatively high compared to other components of the assay. As a result, we settled upon a lower concentration, which proved to be appropriate for measuring Hsp90's ATPase activity in a reproducible and cost-effective manner.

Results from these experiments provide a number of mechanisms for optimizing the manner in which Hsp90 ATPase activity is determined. We have found that increasing the reaction time at 42 °C provides increasingly higher signal-to-noise ratios that are independent of the optimized reagents and enzymes described in this protocol. In the end the following conditions were found to be optimal for determining Hsp90's inherent ATPase activity: glucose oxidase (8 U/mL), maltose phosphorylase (4 U/mL), maltose (0.4 mM), amplex red (100 μM), horseradish peroxidase (0.4 U/mL), and Hsp90 (6.25 μg) were combined and incubated for 3.5 h at 42 °C and the absorbance of the resorufin product was measured at 563 nm.

5. Conclusion

Hsp90 has inherent ATPase activity, which is drastically reduced in the presence of inhibitors.[43] The assay described herein has been optimized for each component in an effort to provide a highly reproducible assay for determining Hsp90's inherent ATPase activity. Together, the optimized conditions obtained from these studies provide a valuable tool for identification of molecules that selectively inhibit Hsp90's N-terminal ATPase activity similar to that of GDA, which is one of the most potent inhibitors identified thus far. The cost- effective nature of this assay, the optimization of individual components described in this article, and the highly reproducible nature of this assay provide a reliable method for identification of Hsp90 inhibitors for future high-throughput screening efforts.

Acknowledgments

The authors gratefully acknowledge support of this project by the NIH (R01 CA114393), the NIH COBRE in Protein Structure and Function (RR017708), and the J.R. and INEZ Jay Fund. C.A. is the recipient of a K-INBRE Research Scholarship. The authors thank Mark Richter for helpful discussions.

References and notes

1. Frydman, J. *Annu. Rev. Biochem.* **2001**, *70*, 603–649.
2. Rutherford, S. L.; Lindquist, S. *Nature* **1998**, *396*, 336–342.
3. Toft, D. O. *Trends Endocrinol. Metab.* **1998**, *9*, 238–243.
4. Buchner, J. *Trends Biochem. Sci.* **1999**, *24*, 136–141.
5. Walter, S.; Buchner, J. *Angew. Chem., Int. Ed.* **2002**, *41*, 1098–1113.
6. Sreedhar, A. S.; Soti, C.; Csermely, P. *Biochim. Biophys. Acta* **2004**, *1697*, 233–242.
7. Neckers, L.; Schulte, T. W.; Mimnaugh, E. *Invest. New Drugs* **1999**, *17*, 361–373.

8. Neckers, L. *Curr. Med. Chem.* **2003**, *10*, 733–739.
9. Neckers, L.; Neckers, S. *Expert Opin. Emerg. Drugs* **2002**, *7*, 277–288.
10. Panaretou, B.; Prodromou, C.; Roe, S. M.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *EMBO J.* **1998**, *17*, 4829–4836.
11. Whitesell, L.; Mimnaugh, E. G.; De Costa, B.; Myers, C. E.; Neckers, L. M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8324–8328.
12. Roe, S. M.; Prodromou, C.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *J. Med. Chem.* **1999**, *42*, 260–266.
13. Ban, C.; Yang, W. *Cell* **1998**, *95*, 541–552.
14. Bilwes, A. M.; Quezada, C. M.; Croal, L. R.; Crane, B. R.; Simon, M. I. *Nat. Struct. Biol.* **2001**, *8*, 353–360.
15. Chen, S.; Sullivan, W. P.; Toft, D. O.; Smith, D. F. *Cell Stress Chaperones* **1998**, *3*, 118–129.
16. Ratajczak, T.; Carrello, A. *J. Biol. Chem.* **1996**, *271*, 2961–2965.
17. Adams, J.; Elliot, P. J. *Oncogene* **2000**, *19*, 6687–6692.
18. Csermely, P.; Schnaider, T.; Soti, C.; Prohaszka, Z.; Nardai, G. *Pharmacol. Ther.* **1998**, *79*, 129–168.
19. Neckers, L. *Trends Mol. Med.* **2002**, *8*, S55–S61.
20. Goetz, M. P.; Toft, D. O.; Ames, M. M.; Erlichman, C. *Ann. Oncol.* **2003**, *14*, 1169–1176.
21. Richter, K.; Buchner, J. *J. Cell. Physiol.* **2001**, *188*, 281–290.
22. Picard, D. *Cell. Mol. Life Sci.* **2002**, *59*, 1640–1648.
23. Grenert, J. P.; Sullivan, W. P.; Fadden, P.; Haystead, T. A. J.; Clark, J.; Mimnaugh, E.; Krutzsch, H.; Ochel, H.-J.; Schulte, T. W.; Sausville, E.; Neckers, L. M.; Toft, D. O. *J. Biol. Chem.* **1997**, *272*, 23843–23850.
24. DeBoer, C.; Meulman, R. J.; Wnuk, R. J.; Peterson, D. H. *J. Antibiot.* **1970**, *23*, 442–447.
25. Schulte, T. W.; Akinaga, S.; Soga, S.; Sullivan, W.; Stensgard, B.; Toft, D.; Neckers, L. M. *Cell Stress Chaperones* **1998**, *3*, 100–108.
26. Garbaccio, R. M.; Danishefsky, S. J. *Org. Lett.* **2000**, *2*, 3127–3129.
27. Banerji, U. *Proc. Am. Assoc. Cancer Res.* **2003**, *44*, 677.
28. Sausville, E. A. *Curr. Cancer Drug Targets* **2003**, *3*, 377–383.
29. Le Brazidec, J.-Y.; Kamal, A.; Busch, D.; Thao, L.; Zhang, L.; Timony, G.; Grecko, R.; Trent, K.; Lough, R.; Salazar, T.; Khan, S.; Burrows, F.; Boehm, M. F. *J. Med. Chem.* **2004**, *47*, 3865–3873.
30. Andrus, M. B.; Meredith, E. L.; Simmons, B. L.; Soma Sekhar, B. B. V.; Hicken, E. J. *Org. Lett.* **2002**, *4*, 3459–3462.
31. Andrus, M. B.; Hicken, E. J.; Meredith, E. L.; Simmons, B. L.; Cannon, J. F. *Org. Lett.* **2003**, *5*, 3859–3862.
32. Agatsuma, T.; Ogawa, H.; Akasaka, K.; Asai, A.; Yamashita, Y.; Mizukami, T.; Akinaga, S.; Saitoh, Y. *Bioorg. Med. Chem.* **2002**, *10*, 3445–3449.
33. Soti, C.; Racz, A.; Csermely, P. *J. Biol. Chem.* **2002**, *277*, 7066–7075.
34. Rowlands, M. G.; Newbatt, Y. M.; Prodromou, C.; Pearl, L. H.; Workman, P.; Aherne, W. *Anal. Biochem.* **2004**, *327*, 176–183.
35. Llauger-Bufi, L.; Felts, S. J.; Huezo, H.; Rosen, N.; Chiosis, G. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3975–3978.
36. Zhou, M.; Diwu, Z.; Panchuk-Voloshina, N.; Haugland, R. P. *Anal. Biochem.* **1997**, *253*, 162–168.
37. Mohanty, J. G.; Jaffe, J. S.; Schulman, E. S.; Raible, D. G. *J. Immunol. Methods* **1997**, *202*, 133–141.
38. Richter, K.; Muschler, P.; Hainzl, O.; Buchner, J. *J. Biol. Chem.* **2001**, *276*, 33689–33696.
39. Zhang, J. H.; Cheung, T. D.; Oldenburg, K. R. *J. Biomol. Screen.* **1999**, *4*, 67–73.
40. Prodromou, C.; Piper, P. W.; Pearl, L. H. *Proteins: Struct. Funct. Genomics* **1996**, *25*, 517–522.
41. Grenert, J. P.; Sullivan, W. P.; Fadden, P.; Haystead, T. A. J.; Clark, J.; Mimnaugh, E.; Krutszch, H.; Ochel, H.-J.; Schulte, T. W.; Sausville, E.; Neckers, L. M.; Toft, D. O. *J. Biol. Chem.* **1997**, *38*, 23843–23850.
42. Chiosis, G.; Huezo, H.; Rosen, N.; Mimnaugh, E.; Whitesell, L.; Neckers, L. *Mol. Cancer Ther.* **2003**, *2*, 123–129.
43. Panaretou, B.; Prodromou, C.; Roe, S. M.; O'Brien, R.; Ladbury Piper, P. W.; Pearl, L. H. *EMBO J.* **1998**, *17*, 4829–4836.