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Microarray-based screening of heat shock protein inhibitors

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ABSTRACT

Based on the importance of heat shock proteins (HSPs) in diseases such as cancer, Alzheimer's disease or malaria, inhibitors of these chaperons are needed. Today's state-of-the-art techniques to identify HSP inhibitors are performed in microplate format, requiring large amounts of proteins and potential inhibitors. In contrast, we have developed a miniaturized protein microarray-based assay to identify novel inhibitors, allowing analysis with 300 pmol of protein. The assay is based on competitive binding of fluorescence-labeled ATP and potential inhibitors to the ATP-binding site of HSP. Therefore, the developed microarray enables the parallel analysis of different ATP-binding proteins on a single microarray. We have demonstrated the possibility of multiplexing by immobilizing full-length human HSP90α and HtpG of *Helicobacter pylori* on microarrays. Fluorescence-labeled ATP was competed by novel geldanamycin/reblastatin derivatives with IC_{50} values in the range of 0.5 nM to 4 μM and Z^*-factors between 0.60 and 0.96. Our results demonstrate the potential of a target-oriented multiplexed protein microarray to identify novel inhibitors for different members of the HSP90 family.

1. Introduction

Protein folding by chaperones is one of the crucial ATP-dependent post-translational processes present in both lower and higher eukaryotes, as well as, in bacteria (Neckers et al., 2009). Stress or extreme environmental conditions, such as UV radiation, oxygen deficiency, the presence of ethanol or heavy metals, unfold proteins and destroy their function (Lindquist, 2009; Richter et al., 2010). Heat shock proteins (HSPs) can mediate both the initial folding and the rescue pathway of many damaged or unfolded client proteins without the need for *de novo* synthesis of the affected proteins (Trepel et al., 2010). In cancer cells, client proteins with incorrect genetic codes are not degraded since chaperones are also "rescuers" of these mutated clients, thereby preventing the induction of apoptotic pathways (Whitesell and Lindquist, 2005). In recent years, the chaperone machinery and in particular the HSP90 family have become main targets for developing drugs against cancer and other chaperone-dependent diseases (Holzbeierlein et al., 2010; Luo et al., 2008; Pesce et al., 2010). The N-terminus of HSP90α exhibits an ATP-binding pocket, which is responsible for the ATPase activity of the protein and represents an important target for small molecule inhibitors (Pearl and Prodromou, 2006). Several natural products such as geldanamycin, related ansamycin derivatives such as herbimycin, macbecin, as well as, the phenolic analogues, autolytimycin, reblastatin and radicicol have been found to bind to different positions of HSP90α (Matts and Manjarrez, 2009) (Fig. 1). Derivatives based on geldanamycin, such as 17-*N*-allylamino-17-demethoxygeldanamycin (tanespimycin and the corresponding hydrochloride salt (retaspimycin hydrochloride)) were implicated in phase III clinical trials for chemotherapy. Another derivative is the free 17-amino derivative (17-AAG) that has recently reached phase I in clinical trials. All these ansamycin derivatives bind to the N-terminal ATP-binding domain of HSP90α (Whitesell et al., 1994).

Abbreviations: 17-AAG, 17-*N*-allylamino-17-demethoxygeldanamycin; BSA, bovine serum albumin; Cy3, carbocyanin 3; DMSO, dimethylsulfoxid; FITC, fluoresceinisothiocyanat; GA, geldanamycin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSP, heat shock protein; HtpG, high temperature protein G; IC_{50}, half minimal inhibitory concentration; SUMO, small ubiquitin-like modifier; TEV, tobacco etch virus.

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These quinone-based geldanamycins and derived analogues induce severe toxic side effects, limiting their applicability as antitumor drugs (Franke et al., 2013). As a consequence, there is still a strong demand for improved HSP90α inhibitors (Amolins and Blagg, 2009; Matts et al., 2011; Tian et al., 2004). Several assays and screening methods have been recently developed to identify novel HSP90α inhibitors (Aherne et al., 2003; Du et al., 2007). Non cell-based assays rely on the following: (a) the detection of free phosphate; (b) the competition for binding of agents to the ATP-binding pocket; (c) the inhibition of HSP90-dependent protein folding; (d) conformational analysis by proteolytic stability; (e) techniques based on surface plasmon resonance (SPR) (Megraud, 1998).

Since small molecule libraries contain large numbers of different potential drugs, assays that can be operated in a high throughput manner are needed. To satisfy this need, we have developed a protein microarray based assay to screen for novel inhibitors of HSPs. The microarrays contain full-length target proteins that are immobilized. The arrays are cost effective because only 300 pg proteins per spot and much lower amounts of the inhibitors are required, compared to conventional assays.

Here, we report a new protein microarray assay that is based on the competitive displacement of ATP from the ATP-binding pocket of HSP by potential inhibitors using Cy3-ATP as the fluorescent probe (Scheme 1). The assay design allows the parallel spotting of different ATP-dependent proteins on a single microarray for multiplexed testing of potential inhibitors. In this study we selected purified full-length human HSP90α and bacterial HtpG from *Helicobacter pylori* as target proteins. We chose HtpG, because it represents an emerging target in infectious diseases. Besides known inhibitors of HSPs we also screened novel compounds derived by mutagenesis in order to demonstrate the applicability of the developed microarray to the identification of new inhibitors of the ATP-binding pocket.

2. Materials and methods

2.1. Purification of the human HSP90α and bacterial HtpG of H. pylori

The *Escherichia coli* strain BL21pLysS/BL21(DE3) (Invitrogen, Carlsbad, CA, USA) was either used to host the plasmid pET 15.1, containing the human HSP90α gene, or the plasmid pET SUMO (Invitrogen, Carlsbad, CA, USA), with the HtpG gene of *H. pylori*. The genes were cloned into the frame of the N-terminal hexahistidine and the TEV and SUMO protease cleavage site, respectively. All protein purification steps were carried out at 4 °C and 0.005% (w/v) protease inhibitor (P-8465 from Sigma-Aldrich, St. Louis, MO, USA) added to the lysis buffer (20 mM Tris–HCl, pH 8.0, 500 mM KCl, 5 mM β-mercaptoethanol, 2 mM imidazol, 10% (v/v) glycerol) in order to minimize the degradation of recombinant proteins by endogenous proteases. Cell pellets were lysed by two French Press cycles (French® Press Amicon 20000, Amicon, USA) at 12,000 psi and adjusted to 100 ml with lysis buffer. Soluble proteins were separated from cellular debris by centrifugation at 25,000 × *g* for 50 min at 4 °C. The recombinant protein was purified from the supernatant by metal chelating Talon resin purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA) after incubation for 2 h on ice. Unbound proteins were removed by washing buffer containing 500 mM KCl, 6 mM β-mercaptoethanol, 2 mM imidazol, 20 mM Tris pH 8.0. For stability reasons, the resin was washed with one column volume of buffer containing 1 M KCl, 6 mM β-mercaptoethanol, 2 mM imidazol, 1 mM Mg–ATP, 0.1% Tween and 20 mM Tris pH 8.0. Subsequently, bound proteins were eluted with two column volumes elution buffer (500 mM KCl, 6 mM β-mercaptoethanol, 250 mM imidazol and 20 mM Tris pH 8.0). The N-terminal tag of the HSP90α was removed by TEV protease from Promega (Mannheim, Germany) and the N-terminal tag of the HtpG was cleaved by SUMO protease from Invitrogen (Carlsbad, CA, USA). Dialysis was performed against 500-fold volume of buffer containing 20 mM Tris pH 8.0, 20 mM KCl, 6 mM β-mercaptoethanol and 10% (v/v) glycerol at 4 °C for 24 h. The human HSP90α protein was further purified by anion exchange chromatography on a Mono Q column in 20 mM Tris–HCl pH 8, and 6 mM β-mercaptoethanol using a linear gradient to 0.5 M KCl. The protein was concentrated with an YM50 centrifugal filter device, Amicon (EMD Millipore Corporation, MA, USA). Homodimeric HSP90α was isolated by SEC 16/60 chromatography in 20 mM Tris–HCl pH 8.0, 500 mM KCl and 6 mM β-mercaptoethanol. Finally, the human HSP90α and the bacterial HtpG was dialyzed into 20 mM Tris pH 8, 20 mM KCl, 6 mM β-mercaptoethanol, 10% (v/v) glycerol (storage buffer), and samples were frozen at a concentration of 3 mg ml^{-1} in liquid nitrogen and stored at −80 °C.

2.2. Direct competitive displacement assay

HSP90α (3 mg ml^{-1}) and HtpG (1.4 mg ml^{-1}) were spotted in the storage buffer in 4 × 5 matrices on UniSart® 3D nitro slide (Sartorius Stedim Biotech, Göttingen, Germany) using a contactless GeSim Nano-Plotter™ (GeSiM mbH, Großerkmannsdorf, Germany) with a nanotip pipette with a volume of 800–1600 pl (8 drops/dot) onto the nitrocellulose surface of a microarray slide. Nitrocellulose membrane-coated microarrays were chosen because they are known to enable a high binding capacity along with a stabilization of the immobilized protein (Lubbecke et al., 2012; Walter et al., 2010). The slide was air dried for 30 min at room temperature. Before printed protein arrays were subjected to the direct competitive displacement assay, nonspecific sites were blocked with 1% (w/v) BSA in storage buffer for 4 h at room temperature. 16 well hybridizations chambers of Nexterion (Schott Nexterion, Mainz, Germany) were used to separate the subarrays.

For screening of inhibitors, the subarrays were incubated with a mixture of 100 nM Cy3-ATP (NU-833-CY3 purchased from Jena Bioscience GmbH, Jena, Germany) and GA derivatives (see Section 2.3) or radicicol in binding buffer (20 mM HEPES–KOH, pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, 0.01% (v/v) Tween 20, 2% (v/v) DMSO, 0.1 mg ml^{-1} BSA) for 16 h at 4 °C. Per subarray 50 μl of the mixture were applied and to determine IC_{50} values of the potential inhibitors, the concentration of GA derivatives was varied from 50 μM to 50 pM. As a positive control a mix of binding buffer, 100 nM Cy3-ATP and geldanamycin derivative **12** (50 μM) were and the negative control was binding buffer with 100 nM Cy3-ATP with lack of the potential inhibitor. The slides were washed after the incubation three times for 5 min with binding buffer and afterwards dried with compressed air. Fluorescence originating from Cy3-labeled ATP was determined by GenePix 4000B Laser Scanner (Molecular Devices, Inc.) with 532 nm emission wavelength, laser power 33%, PMT gain 350 and calculated using the program GenePix 6.1 (Molecular Devices, Inc., Sunnyvale, CA, USA) and ImaGene 5 of BioDiscovery, Inc. (Hawthorne, CA, USA). The evaluation of the displacement was done with the IC_{50} value. The dose–response curves were calculated with Origin 7G (OriginLab Corporation, Northampton, MA, USA) and fitted with the non-linear function logistic, $A1 = 0$, $A2 = 1$.

2.3. Preparation of potential inhibitors

All geldanamycin derivatives, except for 17-AAG, used in this study were obtained as previously reported (Eichner et al., 2012; Scheibel et al., 1997). The synthetic strategy relied on mutasynthesis (Rinehart, 1979; Shier et al., 1969) using a mutant strain of *Streptomyces hygroscopicus* var. geldanus that was blocked in