Design of a Flexible Cell-Based Assay for the Evaluation of Heat Shock Protein 70 Expression Modulators

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ABSTRACT

Heat shock protein 70 (Hsp70) is a chaperone protein that helps protect against cellular stress, a function that may be co-opted to fight human diseases. In particular, the upregulation of Hsp70 can suppress the neurotoxicity of misfolded proteins, suggesting possible therapeutic strategies in neurodegenerative diseases. Alternatively, in cancer cells where high levels of Hsp70 inhibit both intrinsic and extrinsic apoptotic pathways, a reduction in Hsp70 levels may induce apoptosis. To evaluate and identify, in a single assay format, small molecules that induce or inhibit endogenous Hsp70, we have designed and optimized a microtiter assay that relies on whole-cell immunodetection of Hsp70. The assay utilizes a minimal number of neuronal or cancer cells, yet is sufficiently sensitive and reproducible to permit quantitative determinations. We further validated the assay using a panel of Hsp70 modulators. In conclusion, we have developed an assay that is fast, robust, and cost efficient. As such, it can be implemented in most research laboratories. The assay should greatly improve the speed at which novel Hsp70 inducers and inhibitors of expression can be identified and evaluated.

INTRODUCTION

In the cytoplasm of eukaryotic cells, the presence of an insult, whether chemical, thermal, or in the form of misfolded or aggregated protein, triggers a complex biological response referred to as the heat shock response.^{1–3} This phenomenon is associated

with expression of heat shock proteins (HSPs), which also function as molecular chaperones, and of proteins involved in the ubiquitin-proteasome pathway.

Impaired induction of the heat shock response may lead to a defective stress-induced synthesis of HSPs and potentially the accumulation of aggregated proteins.^{1–3} As a result, protein folding-related diseases may occur. Due to the very limited proliferation potential of neurons, the nervous system is most prone to such diseases, and the ultimate result is neurodegeneration. In neurons, toxicity caused by misfolded proteins may result from an imbalance between normal chaperone capacity and production of dangerous protein species.^{1–3} Therefore, increased chaperone expression can potentially suppress protein neurotoxicity, suggesting possible therapeutic strategies.^{4,5} Indeed, several studies have reported a reduction in cellular toxicity upon expression of Hsp70 and Hsp40 in neurodegenerative aggregation disease models of polyglutamine diseases, such as Huntington's disease, spinal and bulbar muscular atrophy, and several ataxias (SCA1–3).^{6–9} In various cellular models of Alzheimer's disease, increased levels of Hsp70 promoted tau solubility and tau binding to microtubules¹⁰ and inhibited the propensity of A β to aggregate.¹¹ In Parkinson's disease models, directed expression of Hsp70 or pharmacologic HSP modulation prevented the neuronal loss caused by α -synuclein.^{12,13} An effect of Hsp70 in conferring protection to the presynaptic and postsynaptic termini in response to stress has also been reported.¹⁴ The neuroprotective effect of Hsp70 extends to astrocytes, where Hsp70 induction reduces apoptosis and necrosis by glucose and oxygen deprivation.^{15,16} Altogether, in the diseased brain, Hsp70 induction may play a multifaceted, protective role on the damaged neuronal protoplasm, on specialized synapses and on supporting astrocytes.

On the other hand, elevated Hsp70 expression, such as detected in cancer cells, facilitates the malignant phenotype.^{17–19} This effect derives from the ability of Hsp70 to inhibit key effectors of the apoptotic machinery, including the apoptosome, the caspase activation complex, and apoptosis-inducing factor. Hsp70 also plays a role in the proteasome-mediated degradation of apoptosis-regulating proteins.^{17–19} Elevated expression of Hsp70 appears to be high enough to control apoptosis, because downregulation of Hsp70 using antisense approaches increases the sensitivity of tumor cells to serum withdrawal and apoptosis inducing factor (AIF).²⁰ Further, a reduction in

ABBREVIATIONS: DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; Hsp, heat shock protein; SD, standard deviation; S:N, signal-to-noise ratio; TBST, Tris buffer saline +0.1% Tween 20.

endogenous Hsp70 levels in and of itself promotes *in vitro* the apoptotic death of tumor cells derived from a wide variety of cancers, including breast, colon, prostate, hepatocellular carcinoma, and glioblastoma, while displaying no toxicity toward normal epithelia derived from breast or prostate, or toward fetal lung fibroblast.^{21–24} In cancer cells, Hsp70 also contributes to the Hsp90 chaperone machine, a protein complex with important roles in regulating the function of several onco-proteins.^{25,26} Overall, the Hsp70 protein is overexpressed in most cancer cells and is induced by other stresses, including anticancer drugs, yet these events occur as a result of a general stress response. In contrast, the protective functions of Hsp70 manifest in a manner that depends upon the specific wiring and function of apoptotic elements within a cell.²⁷

The data presented above indicate that modulation of Hsp70 expression offers several therapeutic avenues to ameliorate a range of human diseases. In neurodegenerative diseases, in which Hsp70 induction may confer a protective advantage, induction of Hsp70 by pharmacological means is a potentially viable therapy. On the other hand, in cancer, downregulation of Hsp70 is a valuable strategy to induce apoptosis and a potential effective means to overcome tumor cell resistance.

To speed the rate at which modulators of Hsp70 expression can be evaluated and identified, a strategy that probes an increase or decrease in Hsp70 levels in neuronal or cancer models is needed. Here we describe a simple, cell-based assay to quantify Hsp70 protein levels in un-engineered cells. The method, which relies on whole-cell immunodetection of Hsp70 in human cells, utilizes a minimal number of cells, yet is sufficiently sensitive and reproducible to permit quantitative determinations. The assay has been developed for the microtiter plate format, which requires expenditure of minimal amounts of compound, thus making this an ideal platform for small molecule testing in low- to medium-throughput format. The assay also uses commercially available reagents and requires an ordinary plate reader with chemiluminescence capability; thus, our assay can be implemented in most research laboratories. This fast and reliable assay should greatly improve the speed with which novel Hsp70 protein expression inducers or inhibitors can be identified and evaluated.

MATERIALS AND METHODS

Reagents

We synthesized and characterized the purine-scaffold Hsp90 as previously described.^{28,29} We purchased 17-AAG, Quercetin and Bouin's solution from Sigma-Aldrich.

Cell Lines

We purchased the human neuroblastoma cells SK-N-SH and the human breast cancer cells SKBr3 from the American Type Culture Collection. Cells were cultured routinely in ATCC-formulated Eagle's minimum essential medium (ATCC, Cat. no. 30-2003) for SK-N-SH and Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 for SKBr3, supplemented with 10% fetal bovine serum, and 1% penicillin and streptomycin.

Primary Neuronal Culture

Cortical neuron cultures were prepared from Sprague-Dawley rats at 18 days of gestation (E18) as previously described.^{30,31} Briefly, cortical regions were dissected in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution under a stereomicroscope and incubated in 0.1% trypsin (Invitrogen) at 37°C for 30 min. The tissue was triturated by aspirating 7 to 10 times with a narrowed plastic pipette. Cells were plated onto poly-D-lysine-coated 96-well microplates at $\sim 5 \times 10^6$ cells per cm^2 in Neurobasal medium supplemented with 10% fetal bovine serum plus B27 and N2 supplement for optimum survival of central nervous system (CNS) neurons. Half of the medium was exchanged with fresh serum-free medium a day after the initial plating. Fluorodeoxyuridine (10 μM ; Sigma) was added to inhibit proliferation of non-neuronal cells. The medium was changed every other day. All cultures were grown in a humidified atmosphere containing 5% CO_2 at 37°C. To determine the effects of Hsp90 inhibitors on Hsp70 protein steady states, the inhibitor was added at day 6 of culture, and cells were incubated at 37°C as indicated.

Hsp70 Assay

The assay consists of plating SK-N-SH or SKBr3 cells (at the indicated number or at 8,000 cells per well in 96-well plates) and incubating them at 37°C and 5% CO_2 for 24 h. After the attachment period, the growth medium (100 μL) containing drug or vehicle (dimethyl sulfoxide [DMSO]) was added and the plate was incubated for an additional 12–24 h. After the incubation period, ice-cold Tris buffer saline +0.1% Tween 20 (TBST) (1 $\times$ 200 μL) was added to wash the plate. TBST should be placed on ice at least 30 min before use. To avoid accidental removal of adherent cells from the microtiter well plates, the pipet tips should be positioned to the side of each well when removing liquid from the plates by using an 8-channel tip aspirator. Cells were fixed and permeabilized by the addition of 100 μL MeOH (-20°C) for 10 min at 4°C. Methanol was removed by vacuum suction and the cells were washed once with ice-cold TBST. Plates were then blocked with 200 μL of SuperBlock® (Pierce #37535) for 90 min, and for the optimization steps, the anti-Hsp70 antibodies presented in Figure 1 (100 μL at a 1:1,000 dilution in SuperBlock) were placed in each well. For the optimized assay, the anti-Hsp70 antibody (Stressgen #SPA-810) was used in similar conditions. Control wells contained 100 μL of mouse IgG (NeoMarker) at the same dilution with SuperBlock. The plate was left in the cold room overnight, and then washed with TBST (2 $\times$ 200 μL). The secondary antibody (100 μL) (Amersham; Sheep Anti-Mouse IgG HRP #NXA931 or Sigma, #A-9044 for the optimized assay, or as indicated in Figure 1 for all other primary antibodies) at a 1:2,000 dilution in SuperBlock was added to all the wells for 2 h. Unreacted antibody was removed by washing with ice-cold TBST for 5 min on a shaker. ECL reagent (RPN2134; Amersham) (100 μL) was added and plates were read in an Analyst GT plate reader (Molecular Devices). Luminescence readings were then imported into SOFTmax PRO® 4.01. Readings from IgG Control wells were used as background to be subtracted from all measured values. Readings were then graphed in relative luminescence units. For identification of compounds with

Fig. 1. Hsp70 assay development and optimization. The effect of antibodies and fixatives on assay performance. SK-N-SH neuroblastoma cells were plated at 8,000 cells/well and treated in triplicate with an Hsp70 inducer (PU24FCI at 30 μ M, gray bars, or 17-AAG at 200 nM, dark gray bars) or DMSO (Vehicle, black bars) for 24 h. After fixation and permeabilization with Bouin's reagent (**A**) or MeOH (**B**), the indicated combinations of primary and secondary antibodies were added. Antibodies were used at 1:1,000 and 1:2,000 dilutions for primary and secondary, respectively. Corresponding IgG or IgM were added as appropriate to indicate potential unspecific binding (Control IgG or IgM, white bars). Error bars represent the SD of the mean ($n = 3$). (**C**, **D**) Signal/background ratios were calculated as ratios of readings obtained in Hsp70-inducer- and vehicle-only-treated wells. DMSO, dimethyl sulfoxide; Hsp70, heat shock protein 70; SD, standard deviation.

potential activity on cell number (*i.e.*, cytotoxics or proliferation inducers), the plate was washed with TBST (2 $\times$ 200 μ L) and stripped with Pierce Stripping buffer (Pierce #21059) for 15 min at room temperature. After stripping, the plate was re-blotted by the addition of a primary antibody against β -actin (A1978; Sigma) (1:5,000 in SuperBlock) followed by a HRP-labeled secondary antibody (NXA931, Amersham) (1:3,000 in SuperBlock), and processed in a manner identical to the Hsp70 assay. For the rat cortical primary neurons Hsp70 assay, the 96-well plates precoated with poly-D-lysine for cell attachment (Corning #3603) were prepared with 600,000 cells/well. The assay was conducted otherwise similarly to SK-N-SH cells, with the difference that

the anti-Hsp70 antibody (Stressgen #SPA-810) was used at a 1:100–1:500 dilution in SuperBlock.

Assay Parameter Calculations

The signal-to-noise ratio (S:N) was calculated by using the equation $S:N = (\text{StdSample} - \text{StdBkg})/(\text{StdSample}^2 + \text{StdBkg}^2)^{0.5}$. Z' values were determined as $1 - 3 \times (\text{StdSample} + \text{StdBkg})/(\text{AvgSample} - \text{AvgBkg})$, where StdSample is the standard deviation of the signal obtained from positive control (*i.e.*, Hsp90 inhibitor)-reacted wells and StdBkg, the standard deviation of the signal obtained from control DMSO-reacted cells.³² AvgSample and AvgBkg are the av-

erage of the corresponding signals. AvgPositive and AvgNegative are the average of the corresponding signals. Signal-to-background ratio (S/B) is defined as the mathematical ratio of signal obtained in positive control wells (*i.e.*, Hsp90 inhibitor treated) and background control wells (DMSO-only treated).

Western Blotting

For Western blot analysis, primary neurons were treated 7 days after plating with vehicle (DMSO) or PU-H71 at concentrations of 0.5, 1, and 2.5 μ M. SK-S-NH or SKBr3 cells were grown to 60%–70% confluence and treated with inhibitor or DMSO vehicle. Cells were collected at 24 h and lysed in 50 mM Tris pH 7.4, 150 mM NaCl, and 1% NP-40 lysis buffer. Protein concentrations were measured using the BCA kit (Pierce) according to the manufacturer's instructions. Protein lysates (10–100 μ g) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and incubated with the indicated primary antibodies: anti-Hsp90 from mouse (1:500, SPA-830; Stressgen), anti-Hsp70 from mouse (1:250, SPA-810; Stressgen), and anti- β -actin from mouse (1:2,500, A1978; Sigma-Aldrich).

Hsp90 Binding Assay

We performed measurements in black 96-well microtiter plates (Corning #3650) as previously described.³³ In brief, each 96-well plate contained 3 nM of a Cy3B-labeled Hsp90 inhibitor, Cy3B-GM, 2.5 μ g of SK-N-SH cell homogenate, and tested inhibitor (initial stock in DMSO) in a final volume of 100 μ L. The plate was left on a shaker at 4°C for 24 h, and the fluorescence polarization values in mP were recorded. EC₅₀ values were determined as the competitor concentrations at which 50% of the Cy3B-GM was displaced. Fluorescence polarization measurements were performed on an Analyst GT instrument (Molecular Devices).

Statistics

We performed statistical analysis and graph plotting with Prism 4.0 (GraphPad Software). We presented all data as the mean $\pm$ standard deviation. A *P* value of <0.05 was considered significant.

RESULTS

The Hsp70 Assay Design

Evaluation and identification of compounds that alter protein levels in an un-engineered cellular model requires cumbersome *in vitro* analyses. This may involve treating cultured cells with individual drugs, followed by detergent lysis, polyacrylamide gel electrophoresis of total cellular proteins, and Western blotting to determine protein levels. This methodology is decidedly unsuitable for rapid small molecule testing.

Our goal is to overcome these hurdles, and to develop a method that is characterized by higher speeds and is less labor intensive. For this purpose, we investigated whether a hybrid Western blotting and enzyme-linked immunosorbent assay^{32,34} could be modified for quantifying an increase or decrease in Hsp70 expression in neuronal and cancer cells. In principle, cells could be plated in

microtiter dishes and treated for 12 to 24 h with small molecules at equal concentrations to identify agents that alter cellular levels of Hsp70. The concentration of one compound could also be varied to determine its potency (Table 1). Regardless of the purpose, cells in the microtiter dish must be fixed and permeabilized to expose the antigen and preserve it for antibody detection by one of many widely used techniques, such as immunodetection coupled with enhanced chemiluminescence or fluorescence (Table 1).

Development and Optimization of the 96-Well Hsp70 Assay

To be successful, the assay requires identification of a suitable cell line and of best possible primary and secondary antibodies. The fixative conditions and required cell numbers must also be optimized. For the Hsp70 assay, we chose SK-N-SH, a neuroblastoma cell line with robust growth in culture. In addition, the cell line is an accepted *in vitro* human neuronal cell model for studies on apoptosis, neuroprotection, and cancer. Several small molecule Hsp90 inhibitors, such as PU24FCL and 17-AAG, were chosen as positive controls for the assay.^{29,35} This is possible because Hsp90 represses the activity of the primary heat shock transcription factor, HSF-1. Inhibition of Hsp90 releases HSF-1 from the Hsp90 complex, resulting in subsequent production of HSPs, including Hsp70.³⁶ Induction of Hsp70 by Hsp90 inhibitors has been reported in both cancer and neurodegenerative disease models.^{8,25,26}

Optimization of Cell Number and Reagents

We next screened a panel of antibodies in search for an optimal signal-to-noise ratio, and we tested two widely used cell fixation and permeabilization methods, the Bouin's fixative, which uses a mixture of acetic acid, formaldehyde, and picric acid, and MeOH fixation, which uses 100% of the organic solvent (Fig. 1). Among the primary anti-Hsp70 antibodies, the anti-Hsp70 from Stressgen (AssayDesign) (clone C92F3A-5, cat. no. SPA-810), which is a mouse monoclonal antibody that binds to amino acid residues 436–503 of human Hsp70 and has no reactivity with the constitutive Hsc70 (Hsp73), was most effective (Entry 1 and 2, Figs. 1A and 2B). The mouse anti-Hsp70 from Calbiochem (also clone C92F3A-5, cat. no. 386032) performed similarly in the assay (Entry 5 and 6, Fig. 1A, B); however, this product was twice as expensive, so the Stressgen product was employed. In addition, MeOH outperformed the Bouin's fixative in this assay, with higher signal/background ratios being obtained, regardless of the antibody combinations used (Fig. 1C, D). The two Hsp90 inhibitors, PU24FCL and 17-AAG, performed similarly with respect to Hsp70 induction (Fig. 1B), and were used interchangeably throughout the study.

Because of the excellent performance of the antibody pair identified in Figure 1, the assay was very sensitive: Measurable changes in Hsp70 levels in as low as 1,000 plated cells per well were detected (Fig. 2A). The assay signal rose with increasing cell numbers, and reached a plateau at $\sim$ 20,000 plated cells per well (Fig. 2A).

Table 1. Heat Shock Protein 70 Assay Protocol Table for Compound EC₅₀ Value Determination

Step	Parameter	Value	Description
1	Plate cells	100 μ L	8,000 SK-N-SH cells
2	Incubation time	Overnight	37°C, 5% CO ₂
3	Controls	100 μ L	$\pm$ DMSO, PU-H71 (1 μ M), media
4	Compounds	100 μ L	As desired
5	Incubation time	12–24 h	37°C, 5% CO ₂
6	Washing step	1 $\times$ 200 μ L, 5 min	TBST
7	Fixation/permeabilization	100 μ L, 10 min	Methanol, 4°C
8	Washing step	1 $\times$ 200 μ L, 5 min	TBST
9	Blocking	200 μ L, 90 min	SuperBlock (Pierce #37535)
10	Primary antibody	100 μ L, Overnight	1:1,000 dilution in SuperBlock; 4°C
11	Secondary antibody	200 μ L, 2 h	1:2,000 dilution in SuperBlock, RT
12	Detection reagents	100 μ L, 10 min	ECL (Amersham #95038-566)
13	Assay readout	530 nm	Analyst GT (Molecular Devices)

Step Notes

1. Solid black 96-well plates (Corning #3603), 8-tip dispense cells to all wells
 2. Cells are allowed to attach to the plate.
 3. Column 1, media only; column 2, the Hsp90 inhibitor PU-H71 in media as a positive control; column 11–12, DMSO (vehicle) in media; row A to row H by 1/2 serial dilution
 4. Columns 3–10, four compounds in duplicate; row A to row H by 1/2 serial dilution
 5. 12–24 h drug treatments are considered for observation of maximal effects.
 6. Wash with of ice-cold TBST for 5 min.
 7. Methanol should be cooled at -20°C before use.
 8. Remove methanol by vacuum suction with an 8-tip aspirator followed by washing with of ice-cold TBST.
 9. 8-tip dispense reagent to all wells
 10. Column 1, nonspecific primary antibody IgG; columns 2–12, anti-Hsp70 antibody in SuperBlock
 11. Wash with ice-cold 2 $\times$ 200 μ L TBST before adding the secondary antibody.
 12. Wash with ice-cold 1 $\times$ 200 μ L TBST before adding the ECL reagent; plates lidded while developing.
 13. Scan each well for 0.1 s. Luminescence height = 0.6 mm; maximum integration = 100,000 μ s
- DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; Hsp, heat shock protein; TBST, Tris buffer saline +0.1% Tween 20.

The specificity of the antibody for the correct antigen is an intrinsic component of the Western blot data that are lost in dot blot or cytoblot format. For this reason, we tested the specificity of the Hsp70 antibody in Western blot. Seven cancer cells that encompass breast, prostate, and lung cancer were used to demonstrate detection of a single band of 70 kDa, corresponding to Hsp70 (Fig. 2B).

Assessment of Assay Signal

A good signal window is essential for a robust and reproducible assay. The S:N and Z' parameters take into account the dynamic range of the assay signal and the data variation associated with the

reference control measurement.³³ An excellent assay is described by Z' values higher than 0.5 and S:N of over 8. These assay parameters are also accepted as defining an assay that will perform well in screens. For cell-based assays these values have been more difficult to achieve due to unavoidable biological variation. Notwithstanding these possible limitations, the Hsp70 assay performed exceedingly well, with Z' values over 0.7 and an S:N over 8 when 1,000 to 30,000 cells were plated per well in a 96-well microtiter plate (Fig. 2C).

DMSO is a common solvent used to dissolve many compounds for biological testing. In our assay, interference cannot be envisioned in the immunoblot steps because DMSO is removed before Hsp70-detection. However, cultured cells are sensitive to higher concentrations of this organic solvent when added into their growth media. For the Hsp70 assay, only a 15% reduction in the assay signal was detected in the presence of 5% (v/v) DMSO added to SK-N-SH cells (Fig. 2D). DMSO tolerance, nonetheless, should be evaluated for each cell line that will be used in the assay.

Hsp70 Assay Validation: Inducers of Hsp70 Expression in Neurodegenerative Cellular Models

As mentioned above, pharmacologic induction of Hsp70 in neuronal cells has a potential therapeutic value. We and others have reported that Hsp70 levels may be augmented in neurons upon inhibition of Hsp90.⁸ As described above, Hsp70 induction is a downstream effect of Hsp90 inhibition through activation of HSF-1.³⁶ This mechanism of Hsp70 induction allowed us to probe the specificity of the assay. To demonstrate target-specific induction of Hsp70 in the Hsp70 assay, we should observe a correlation between the potency of Hsp90 inhibitors to bind to Hsp90 and their ability to induce Hsp70 in SK-N-SH cells. As seen

in Figure 3, the amount of Hsp90 binding reflected the amount of Hsp70 induction (compare Fig. 3A to Fig. 3B and see correlation plot in Fig. 3C), validating that the Hsp70 assay results in a biologically relevant signal and substantiating its specificity.

The Western blot is the classical method to obtain information of the type generated by the Hsp70 assay. In this method, cells are plated, treated with small molecules, and lysed for separation using gel electrophoresis. The resolved proteins are then transferred to a membrane and probed using specific antibodies. Testing of the nine compounds from Figure 3 in a comparable concentration range and in three experimental replicates would require at least 27 gels, undoubtedly a lengthy and labor intensive option. On the other hand,

Fig. 2. Cell number optimization, assay performance, and assay validation. **(A)** SK-N-SH neuroblastoma cells were plated at the indicated number of cells per well and treated in triplicate with an Hsp70 inducer (17-AAG at 200 nM) or Vehicle (DMSO) for 24 h. The Hsp70 assay was then performed. Raw data expressed in relative luminescence numbers are presented as obtained for each cell count. **(B)** Specificity of the Hsp70 antibody was determined by Western blot. Partially trypsinized recombinant Hsp70 protein was used to detect and differentiate Hsp70 cleavage fragment from potential unspecific bands. **(C)** The assay performance parameters S:N and Z' for the experiment in **(A)** were calculated as described in the Materials and Methods section. Error bars represent the SD of the mean ($n = 3$). **(D)** SK-N-SH neuroblastoma cells were plated at 8,000 cells per well and treated in triplicate with the indicated amount of Vehicle (DMSO, v/v) for 24 h. The Hsp70 assay was then performed. Readings in each experimental condition (Y_{DMSO}) were compared to those obtained in the presence of no added DMSO (Y_0), and graphed as % Control = $Y_{\text{DMSO}}/Y_0 \times 100$.

the Hsp70 assay delivered this information in a rapid and effortless fashion with the use of only three 96-well plates (accounting for running the assay in triplicate), and resulted in data comparable to the Western blot (Fig. 4).

Specifically, when PU24FCl was tested concomitantly in the Hsp70 blot (Fig. 4A) and the Western blot (Fig. 4B), both methods demonstrated a dose-dependent induction of Hsp70. We noted similar effects when $>1 \mu\text{M}$ of the agent was added to the SK-N-SH cells. The Hsp70 assay, however, allowed for a better quantitation of the effect over the range of drug concentrations, because in this optimized setting the signal does not saturate, as observed in the Western blot. Further, the Hsp70 assay could be implemented in primary neuronal cultures. When PU-H71, a more potent derivative of PU24FCl, was added to rat

cortical neuronal cultures, a robust Hsp70 induction resulted (Fig. 4C) that was also detectable by the assay (Fig. 4D).

Hsp70 Assay Validation: Inhibitors of Expression of Endogenous and Chemically Induced Hsp70 in Cancer Cellular Models

High levels of Hsp70 augment the aggressiveness of tumors and allow cells to survive lethal conditions, including chemotherapeutic killing.^{17–19} In addition to conferring resistance to treatment, elevated Hsp70 expression promotes cancer by inhibiting programmed cell death and by promoting autonomous growth. This suggests that strategies that reduce Hsp70 expression or inhibit its induction may serve as possible therapeutic interventions in cancer.^{17–19} Inhibition

Fig. 3. Hsp70 assay validation—specificity. **(A)** SK-N-SH neuroblastoma cells were plated at 8,000 cells per well and treated in triplicate with a panel of Hsp90 inhibitors for 24 h. The Hsp70 assay was then performed. Raw data expressed in relative luminescence were normalized to β -actin levels³² and the adjusted values were presented. **(B)** The ability of the indicated inhibitors to compete with GM-Cy3B, a fluorescently labeled Hsp90 inhibitor,⁴⁵ for Hsp90 binding in SK-N-SH cell extracts was examined by fluorescence polarization. Values recorded in wells with added inhibitor were normalized to values read in control wells and plotted against the concentration of tested inhibitor. Drugs were assayed in triplicate. All compounds were used as DMSO stocks. Points, mean; bars, SD. **(C)** EC₅₀ values from the Hsp90 bind assay were plotted against those from the Hsp70 assay and a linear regression analysis, as implemented in Prism 4.0 was performed. Inset: correlative analysis for the most potent compounds.

of Hsp70 expression has been documented after pharmacological intervention with the flavanoid Quercetin.³⁷ In accord with reduction of Hsp70 expression, Quercetin induces apoptosis in several tumor cells and renders cells more susceptible to apoptotic inducers.³⁸ Although Quercetin has been reported to inhibit the induction of Hsp70 synthesis at the mRNA level, the inhibitory mechanism has not been clearly established.^{39,40} Overall, in spite of its evident utility in cancer treatment, Quercetin is not potent enough for clinical use (i.e., potency is often observed at 100–500 μ M). Nonetheless, the agent is an appropriate chemical tool to validate the Hsp70 assay.

We next tested whether the assay can be applied to identify inhibitors of Hsp70 induction. As above, 17-AAG was used as a chemical inducer of Hsp70, whereas Quercetin served as a model inhibitor of stimulated Hsp70 induction (Fig. 5A, B). The effects of the vehicle (DMSO) and of the two agents alone (17-AAG and Quercetin) are presented in Figure 5A. As seen in Figure 5B, preincubation of SK-N-SH and SKBr3 cells with Quercetin led to a dose-dependent reduction in Hsp70 induction by 17-AAG. The Hsp70 assay also allowed for quantification of the inhibitor's effect. For Quercetin, half maximal

inhibitory concentrations (IC₅₀) of 47 ± 5 and 140 ± 3 μ M in SK-N-SH and SKBr3 cells, respectively, were obtained.

When compared to SK-N-SH, SKBr3 cells express seven times higher endogenous levels of Hsp70 (Fig. 5A, DMSO). We therefore used these cells in the Hsp70 assay to evaluate and identify small molecules that inhibit endogenous expression of Hsp70. In this context, Quercetin again reduced Hsp70 expression in a dose-dependent manner, with a determined IC₅₀ of 125 ± 6 μ M (Fig. 5C).

Hsp70 Assay Cost and Implementation

An important feature to consider for an assay to be of common laboratory use is cost and availability. The Hsp70 assay uses cells, media, buffers, and antibodies that are commercially available, and bulk amounts of these reagents can be purchased at reduced costs. The anti-Hsp70 antibody will ultimately be the decisive factor for the overall cost of the assay. Used at a 1:1,000 dilution in the 96-well format at 100 μ L assay solution/well, the assay required ~ 10 μ g antibody per plate. If purchased in bulk, 50 mg of antibody can be obtained from Stressgen (<http://assaydesigns.com>) for \$25,000,

Fig. 4. Hsp70 assay validation—comparison to Western blot. **(A, B)** SK-N-SH neuroblastoma cells were treated with the indicated concentrations of PU24FCl for 24 h. The Hsp70 assay **(A)** and the Western blot **(B)** were then performed to evaluate Hsp70 expression. **(C, D)** Rat primary cortical neurons were treated for 24 h with the indicated concentrations of the Hsp90 inhibitor PU-H71, and Hsp70 induction was analyzed by Western blot **(C)** and the Hsp70 assay **(D)**. β -actin was used to control for potential cytotoxicity (in the Hsp70 assay) and equal protein loading (in the Western blot). The data are consistent with those obtained from multiple repeat experiments ($n \geq 3$).

which is $\sim$ \\$5/plate. This cost, compared to the assay kit price charged by most commercial vendors ($\sim$ \\$500), suggests that the Hsp70 assay is 100-times more cost effective. If instead the assay is implemented for library screening rather than compound evaluation, 50 mg of antibody would be sufficient for duplicate testing of a 200,000 compound library in 96-well plates, and the antibody cost would be $\sim$ 13 cents/compound.

Chemiluminescence is the read-out for this assay. Because chemiluminescence has served as the read-out for a large number of commercial kits, which measure everything from cell viability to kinase activity, plate readers with luminescent capability have become commonly used in laboratories and research institutes. Thus, the assay can be implemented in most research settings.

DISCUSSION

We have developed and optimized for the 96-well microtiter format a facile assay that relies on whole-cell immunodetection of Hsp70. Moreover, the assay can speedily evaluate small molecules that induce or inhibit endogenous Hsp70, and can quantitate their effect. In addition to the method presented here, others have developed assays that evaluate Hsp70 expression in human cells. Recently,

an elegant duplex assay that immunologically measures Hsp70 induction concomitantly with histone acetylation was reported by Hardcastle *et al.*⁴¹ The assay uses an europium chelate for antibody labeling and requires time-resolved fluorescent detection, making it difficult to implement in most research laboratories. In addition, a signal to background of only twofold was obtained, which was defined by the values of induced and endogenous Hsp70 levels. Therefore, the assay exhibits a poor level of robustness to detect agents that inhibit Hsp70 induction, whether stimulated or endogenous. Further, while no Z or S:N values were provided, an analysis of the presented data and of available standard deviations indicated a less than optimal assay. Nonetheless, Hardcastle *et al.* used the assay to screen a library of small molecules in a 384-well format and identified potential Hsp70 inducers.⁴¹ In contrast, the Hsp70 assay described here is far more robust, as demonstrated by $Z' > 0.6$ and $S:N > 8$. Our assay is also optimized to perform at a difference between the induced and the endogenous Hsp70 levels of over 15-fold, thus allowing for clear-cut experimental evaluations. Further, because of a good S:N ratio, the assay allows one to determine a specific decrease in the amount of the Hsp70 protein. Testing for the presence of a gene product is far simpler than testing for the absence or

Fig. 5. Hsp70 assay testing—detection of inhibition of chemically induced and of endogenous Hsp70 expression. SK-N-SH neuroblastoma and SKBr3 breast cancer cells were treated with DMSO (vehicle), 17-AAG (20 nM), and Quercetin (250 μ M) (A) or pretreated for 4 h with the indicated concentrations of Quercetin before the addition of 17-AAG (20 nM) (B). The Hsp70 assay was performed after 24 h of incubation. Readings in each experimental condition ($Y_{\text{Quercetin}}$) were compared to those obtained in the presence of no added Quercetin ($Y_{0,\text{DMSO}}$), and graphed as % Control = $Y_{\text{Quercetin}}/Y_{0,\text{DMSO}} \times 100$. EC_{50} values were determined by fitting data to a 4-parameter dose-response model, as implemented in GraphPad Prism4. Points, mean; bars, SD. (C) SKBr3 breast cancer cells were treated for 24 h with the indicated concentrations of Quercetin, after which the Hsp70 assay was performed. Data were analyzed as indicated above. Points, mean; bars, SD.

near absence of a gene product because the amount of protein presumably increases with time in the first instance, and thus distinguishes itself from the background. In contrast, when the disappearance of a product is measured, the best resolution from background is at time zero. Notwithstanding this theoretical difficulty, we show here that meaningful measurements of a decrease in Hsp70 expression levels could be obtained.

An alternative and widely used method to measure Hsp70 expression is the Hsp70 reporter assay.⁴² A notable limitation of this assay is that it requires engineering of the cell by transfection of plasmids containing the promoter region of Hsp70 inserted into the pGL3-basic luciferase or the beta-lactamase reporter vector. This can be both time consuming and costly, and potentially changes the endogenous biological wiring of the cell. Recently, an HSF-responsive beta-lactamase reporter element was integrated into the HeLa human cervical cancer cell background, and a stable line was isolated.⁴² This

cell line was then used to investigate the ability of the assay to measure chemically stimulated HSF1-activation in 384- and 1,536-well format. While a 6-h incubation with 17-AAG resulted in a high response with a maximal response ratio of ~ 7.9 , comparable to our Hsp70 assay, overnight incubation with 17-AAG resulted in a substantially diminished assay window (maximal response ratio of ~ 2.0).⁴² A likely explanation for this phenomenon is that Hsp90 inhibition by 17-AAG leads to effects in the cell other than HSF-1 activation. In particular, Hsp90 is a chaperone protein that cells co-opt to buffer the introduction of a foreign protein, and to regulate the stability of structurally compromised proteins. As such, an effect of Hsp90 on the vector itself is possible. In fact, degradation of other vectors upon Hsp90 inhibition was previously observed (Chiosis, pers. comm.). Such effects of small molecules on the vector itself are likely to lead to false-positives and could complicate the interpretation of the data.

In contrast to these published methods, the Hsp70 assay presented here offers a cheap, straightforward, and less-interference-prone method to evaluate and identify compounds that modulate Hsp70 expression in un-engineered human cells. It also allows for robust evaluation of both an increase and a decrease in endogenous levels of Hsp70. Together, the assay offers a method that complements, and in several aspects provides advantages over currently available assays.

In addition to giving information on Hsp70 modulation in human cells, the use of the Hsp70 cell-based assay over a biochemical assay is advantageous because it reads not only compound activity but also cell permeability and solubility. Therefore, compounds with poor cell permeability and low water solubility are weeded-out early in the screening process. The Hsp70 assay can also detect compounds that kill cells. Such behavior would be noted for compounds exhibiting a bell-shaped curve: At lower drug concentrations there is an increase in the Hsp70 signal, which then drops with an increase in compound concentration. Collectively, the Hsp70 assay could identify and eliminate at early screening stages toxic, impermeable, and poorly soluble compounds, which is a significant benefit for a small molecule development program.

Cell-based assays, such as the Hsp70 assay, also tend to result in fewer false-positives than biochemical assays. Nevertheless, false-positives may be generated. Compounds could induce cell proliferation, and thus the signal may be falsely attributed to an increase in Hsp70 levels. As a result, cell proliferation data should be correlated with Hsp70 assay data over the experiment's time range to eliminate false-positives. To identify such compounds, the Hsp70 assay plate can be re-blotted for an abundant cytoskeletal protein, such as β -actin.³² Alternatively, a secondary assay that measures cell proliferation over the time period of the Hsp70 assay could be implemented. Several assays are available, such as the Sulforhodamine B assay that measures total biomass⁴³ or the Alamar Blue assay.⁴⁴ This reagent offers a rapid, objective measure of cell viability in cell culture, and it uses the indicator dye resazurin to measure the metabolic capacity of cells, which is an indicator of cell viability.⁴⁴

In addition to being used as an assay to evaluate potential Hsp70 expression modulators, the strategy developed here may be implemented and used to conduct medium-throughput screens for the identification of Hsp70 inducers in neuronal cells, or Hsp70 expression inhibitors in cancer cells. The compounds identified from these efforts could represent important leads that serve as the starting point to obtain drugs that modulate Hsp70 expression.

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