Biomarkers, 2010; 15(1): 31–38

informa
healthcare

ORIGINAL ARTICLE

An investigation into the potential use of serum Hsp70 as a novel tumour biomarker for Hsp90 inhibitors

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Abstract

Hsp90 inhibitors are under investigation in multiple human clinical trials for the treatment of cancers, including myeloma, breast cancer, prostate, lung, melanoma, gastrointestinal stromal tumour and acute myeloid leukaemia. The pharmacodynamic activity of Hsp90 inhibitors in the clinic is currently assessed by Hsp70 induction in peripheral blood mononuclear cells using Western blot analysis, a method that is laborious, semiquantitative and difficult to implement in the clinic. Since Hsp70 was reported to be secreted by tumour cells and elevated in sera of cancer patients, serum Hsp70 has been evaluated as a potentially more robust, easily and reproducibly measured biomarker of Hsp90 inhibition as an alternative to cytosolic Hsp70. A highly sensitive and specific electrochemiluminescent ELISA was developed to measure serum Hsp70 and employed to evaluate Hsp70 levels in both *ex vivo* and xenograft samples. In *ex vivo* studies, maximal secretion of Hsp70 by tumour cells was observed between 48 and 72 h after exposure to Hsp90 inhibitors. In *in vivo* studies a 3–4-fold increase in serum Hsp70 was observed following treatment with BIIB021 in tumour-bearing mice. Strikingly, secreted Hsp70 was detectable in mice transplanted with human tumours but not in naive mice indicating a direct origination from the transplanted tumours. Analysis of clinical samples revealed low baseline levels (2–15 ng ml^{-1}) of Hsp70 in the serum of cancer patients and normal donors. Together these findings in laboratory studies and archived cancer patient sera suggest that serum Hsp70 could be a novel biomarker to assess reliably the pharmacological effects of Hsp90 inhibitors in clinical trials, especially under conditions where collection of tumour biopsies is not feasible.

Keywords: *Hsp70; serum biomarker; Hsp90 inhibitor; SCLC; cancer*

Introduction

Biomarkers are widely used in current clinical trials in cancer therapy. Biomarker data reveals not only if a drug is on target, but also helps to define responses at an early stage and stratify patients. In Hsp90 inhibitor clinical trials, the most commonly used biomarkers are Hsp70, Raf-1 and cdk4 from isolated human peripheral blood mononuclear cells (PBMC) (Workman 2003, Solit et al. 2007, Ramanathan et al. 2005, Workman et al. 2007). Hsp70 is a co-chaperone of the Hsp90 complex and is upregulated by Hsp90 inhibitors via activation of the transcription factor HSF-1. Raf-1 and cdk4 are Hsp90 clients and are degraded by the proteasome upon Hsp90 inhibition. Practical difficulties with clinical implementation of PBMC isolation protocols and extraction of intracellular biomarkers in the laboratory have prompted the search for novel serum biomarkers (Zhang et al. 2006). These efforts have led to the identification of two serum biomarkers, Her-2 ECD (extracellular domain) and IGFBP-2 (insulin-like growth factor-binding protein 2) by our group and are currently under clinical investigation (Zhang et al. 2006).

Hsp70 is a typical intracellular protein and mainly involved in intracellular transport processes and supporting the folding of nascent polypeptides. It was also

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(Received 16 April 2009; revised 12 August 2009; accepted 14 August 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK Ltd
DOI: 10.3109/13547500903261347

found to play key roles in tumour immunity, which employs the carrier function of the protein. Secreted Hsp70 was initially discovered and explored in the context of immunotherapy. Several studies have shown that secreted heat shock proteins, including gp96, gp170 and Hsp70, bound with antigenic peptides are targeted to dendritic cells and elicit tumour-specific T cell responses and antitumour effects (Tamura et al. 1997, Srivastava et al. 1994, Singh-Jasuja et al. 2000, Chang et al. 2007). Heat shock proteins are able to activate dendritic cells and promote immune responses. For these reasons, tumour cell-based antigens loaded on to soluble Hsp70 have been widely used as vaccines for controlling tumour growth (Massa et al. 2004, Wang et al. 2006). As Hsp70 does not have a leader sequence, the secretion of this protein by tumour cells has been a puzzle until a recent observation that Hsp70 secretion occurred via a non-classical pathway, involving lysosomal endosomes (Mambula & Calderwood 2006). Secreted Hsp70 binds to cell surfaces via scavenger receptors and participates in the recognition of the tumour cells by the immune system (Theriault et al. 2006). In addition, these studies also showed that the circulating Hsp70 secreted from tumour cells correlated with the stage of cancers.

Recently, Hsp70 was found to be elevated in the serum of small cell lung cancer (SCLC) patients (Seiwert et al. 2005), indicating that secreted Hsp70 not only binds to the tumour cell surface, but also circulates in soluble form in serum. As a transcriptional target of HSF-1, intracellular Hsp70 is activated by Hsp90 inhibition through activation of HSF-1 and has been used as a standard pharmacodynamic biomarker for Hsp90 inhibitors in both preclinical and clinical studies. We hypothesized that the increase of Hsp70 in tumour cells induced by Hsp90 inhibitors could also lead to elevated secretion of Hsp70, providing a convenient soluble correlate of intracellular Hsp70. To investigate whether or not Hsp90 inhibitors can induce the secretion of Hsp70, we examined the level of secreted Hsp70 from SCLC cell lines and xenografts upon treatment with two clinical stage Hsp90 inhibitors, 17-AAG and BIIB021. Herein, we report the development of a highly sensitive enzyme-linked immunosorbent assay (ELISA) to quantify accurately secreted and intracellular Hsp70 induced by both 17-AAG and BIIB021 in tissue culture and mice bearing H82 (SCLC) tumour. We observed both a dose- and time-dependent secretion of Hsp70 that correlates closely with the induction of intracellular Hsp70. Most importantly, utilizing animal models we demonstrated that the secreted Hsp70 originated exclusively from tumours, but not normal tissues, indicating that, unlike intracellular Hsp70 from PBMC, which is utilized as a surrogate biomarker in the clinic, secreted Hsp70 is a direct biomarker from tumour cells. In addition, the basal levels of serum Hsp70 were relatively low in many cancer patients, providing a baseline for experimental evaluation of secreted Hsp70 in clinical settings.

Materials and methods

Cell culture and cancer patient serum

Human SCLC lines, H82 and H146 were purchased from ATCC (Manassas, VA, USA) and maintained in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% FBS (ATCC) and 10 mM HEPES (Invitrogen, Carlsbad, CA, USA). Serum from five melanoma, breast, colorectal, SCLC and lung cancer patients were collected under an IRB-approved protocol no. BRI 0722 by Bioreclamation Inc. (Long Island, NY, USA).

Western blotting

One million human SCLC (H146) cells were lysed in 500 µl of Tris–lysis buffer (150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma, St Louis, MO, USA). Lysis was achieved by gentle rotation at 4°C for 30 min. Cell lysates were centrifuged (21 000*g*, 10 min) to remove cell debris and boiled for 5 min in Western loading sample buffer. Protein lysates were resolved on 4–12% Tris-glycine gradient gels (Invitrogen), transferred to PVDF membrane (Millipore, Billerica, MA, USA), and probed with mouse anti-HSP70 (Assay Design, Ann Arbor, MI, USA) and mouse anti-actin (Sigma). Protein transfer was monitored with biotinylated protein markers (Cell Signaling, Danvers, MA, USA). Immunoreactive bands were detected using goat antimouse polyclonal HRP conjugate (Jackson ImmunoResearch, Westgrove, PA, USA) and SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Protein levels on the PVDF membranes were quantified using the Fluor-S Max2 imaging system (Bio-Rad, Hercules, CA, USA).

ELISA

The assay was performed using the Total HSP70 Whole Cell Lysate Kits on either electrochemiluminescence platform (Cat. no. K111EVD-3, Meso Scale Discovery, Gaithersburg, MD, USA) or a colorimetric platform (Catalog no. DYC1663, R&D Systems, Minneapolis, MN, USA) as per manufacturers' instructions. Briefly, protein concentrations in tumour lysates were normalized using BCA assay (Pierce). The normalized cell lysates or the total serum samples (1:4 dilution) were added to 96-well plates coated with anti-total-Hsp70 antibody. Identified amounts of recombinant human Hsp70 (R&D) are used to make a standard curve. The captured Hsp70 was detected with anti-Hsp70 antibody labelled with a sulfo-tag (for electrochemiluminescence detection)

or biotin–streptavidin–HRP system (for colorimetric detection). Protein levels were quantified in terms of electrochemiluminescence units on Sector Imager 2400 (Meso Scale Discovery) or optical density units on a microplate reader set at 450/540 nm.

Analysis of Hsp70 in cellular samples

Cells were plated in 100 cm UptiCell chambers plates at 40–50% confluence in FBS growth medium for 24 h before treatment. BIIB021 was added in at 1:3 dilutions starting from 1 μM. Both supernatant and cell pellets were collected at 24, 48 and 72 h after treatment. Untreated cells and the culture medium were collected at the same time and used as control. Supernatants were centrifuged to get rid of cell debris and stored at –20°C for Hsp70 analysis. Cell pellets were frozen and thawed. Cell lysates were prepared in lysis buffer (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM DTT, 1 mM PMSF, 1 μg ml^{-1} Pepstatin A, 1 μg ml^{-1} leupeptin, 5 μg ml^{-1} Aprotinin) supplemented with additional protease inhibitors from Sigma. Protein concentrations of cell lysates were normalized by BCA method (Pierce) and stored at -80°C until analysis. Both supernatant and cell lysate samples were measured for Hsp70 level by Hsp70 ELISA kits as described above.

Analysis of serum Hsp70 in animal model

Five million H82 cells were inoculated subcutaneously into female nu/nu mice from Charles River. After tumour size reached an average of 500–600 mm^3, mice were randomized into four groups for three dose treatments and a control group. BIIB021 was formulated in acidified water and administered orally at 31 and 62.5 mg kg^{-1}. The vehicle was administered to the control group. Mice were sacrificed and sera were taken at 24 and 48 h after dosing and plasma was obtained with lithium heparin tubes to ensure proper anticoagulation and kept on ice before centrifuged. The samples were kept at –80°C until analysis.

Results

Development of a sensitive ELISA for the quantitation of Hsp70

As mentioned previously, the most widely used surrogate biomarker to monitor Hsp90 inhibitor activity in clinical trials is intracellular Hsp70 that is primarily quantified by Western blot (Ramanathan et al. 2005, Solit et al. 2007, Workman, 2003, Bagatell et al. 2007, Banerji et al. 2005, Nowakowski et al. 2006, Workman et al. 2007, Weigel et al. 2007). This assay by its nature is semiquantitative, relatively insensitive and not amenable to high throughput testing. To facilitate transition to a more quantitative and robust assay platform to test clinical samples, we developed a highly sensitive electrochemiluminescence-based ELISA and quantified intracellular Hsp70 induced in tumour cells treated with the Hsp90 inhibitor BIIB021. As illustrated in Figure 1, the ELISA (Figure 1A) had superior dynamic range when compared with the Western blot (Figure 1B); this was represented by its capacity to capture accurately both extremely low levels of Hsp70 protein in vehicle (DMSO)-treated samples and robust levels in BIIB021-treated samples. As a consequence, ELISA detected over a 20-fold increase in Hsp70 levels after treatment with BIIB021, whereas the Western blot revealed only a 3-fold difference in the same samples (Figure 1C). The specificity of the assay over other heat shock family proteins, including Hsp27 and Hsp90, as well as an unrelated protein, human β-actin was also assessed. The antibody specifically recognized Hsp70, but not the other control proteins (Figure 1D). Overall, the ELISA was more sensitive, specific, quantitative and amenable to high-throughput analysis.

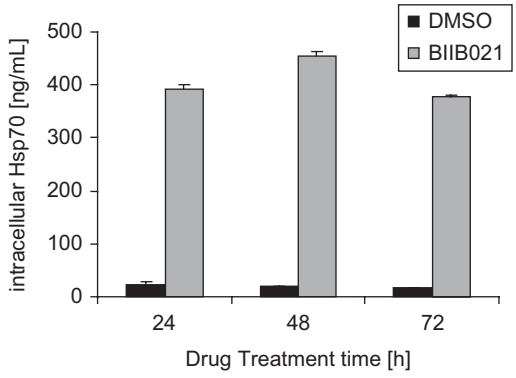
Correlative induction of intracellular and secreted Hsp70 by Hsp90 inhibitors

To determine if Hsp90 inhibitors affect Hsp70 secretion and if the secreted level correlates with the increase of intracellular Hsp70 level in tumour cells, we treated a panel of SCLC cell lines, including H69, H82 and H146 with increasing concentration of 17-AAG and BIIB021. Supernatant and cell pellets were collected at 48 and 72 h after treatment and analysed for Hsp70 level by ELISA. Both 17-AAG and BIIB021 increased intracellular and secreted forms of Hsp70 in a dose-dependent manner in all three cell lines tested (Figure 2). While the secreted Hsp70 in culture medium continued to increase up to 72 h in all three cell lines, intracellular Hsp70 reached its highest level at 48 h in H82 and H146 (Figure 2, parts I and II, A and B), suggesting a delay in the secretion event in these two cell lines that might retard the maximum secretion of Hsp70. This was not the case for H69 whose intracellular Hsp70 continued to increase up to 72 h (Figure 2, part III, A and B). Nevertheless, the induced Hsp70 in the two compartments correlated tightly at each time point for both 17-AAG and BIIB021 (Figure 2, part IC, IIC, IIIC), supporting the idea that secreted Hsp70 reflected the intracellular alteration of Hsp70 and thus could be a potential biomarker for Hsp90 inhibitors.

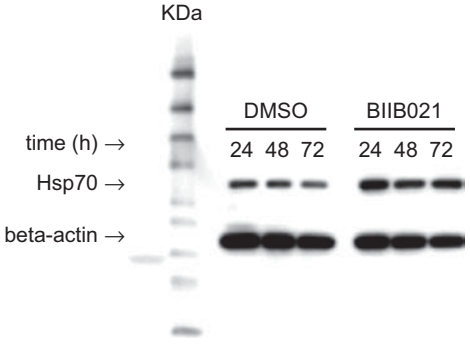
Hsp90 inhibitors induced secretion of Hsp70 in tumour-bearing mice but not naive mice

To determine if an Hsp90 inhibitor could induce Hsp70 secretion *in vivo*, a single dose of BIIB021 was

A. ELISA



B. Western blob assay



C. Fold change observed in ELISA versus Western blot

Drug Treatment Time (h) →	ELISA			Western blot		
	24	48	72	24	48	72
DMSO	1.0	1.0	1.0	1.0	1.0	1.0
BIIB021 [1uM]	16.6	21.8	22.9	2.7	1.9	3.2

D. Specificity

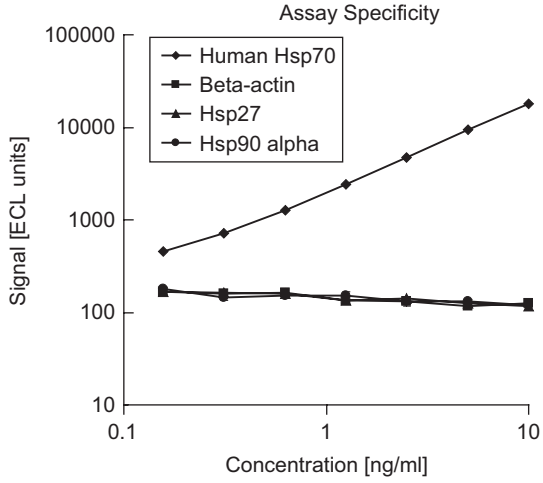


Figure 1. Meso Scale ELISA is a more sensitive and specific method for the quantitation of Hsp70. Small cell lung cancer line, H146 was treated with the Hsp90 inhibitor, BIIB021 at a concentration of 1 μM or an equivalent amount of DMSO (drug solvent) as a negative control. Intracellular Hsp70 levels were measured by Meso Scale ELISA (A) and Western blot (B) methods at 24, 48 and 72 h after drug exposure. Data points in panel A represent mean (error bars, SD) of triplicate samples. (C) A comparison of fold change in Hsp70 levels revealed by Meso Scale ELISA and Western blot methods. Before determining fold change (drug/DMSO), total protein concentrations in tumour cell lysates were normalized with β-actin (house keeping protein) levels. (D) Assay specificity was determined by comparing antibody reactivity to increasing amounts of heat shock family proteins reported to be part of Hsp90 chaperone complex.

administered to H82 tumour-bearing mice at two dose levels, 31 and 62.5 mg kg^{-1}. Twenty-four and 48 h later, mice were sacrificed and plasma was collected and measured for Hsp70 level by ELISA. In accordance with the *in vitro* data, we observed a dose-dependent increase in serum Hsp70 levels (see Figure 3A). To confirm the tumour origin of the secreted Hsp70, BIIB021 was administered to naive mice without transplanted tumours. As shown in Figure 3B, the basal level of Hsp70 in naive mice was significantly lower than that in tumour mice, (<0.1 ng ml^{-1} vs 30–40 ng ml^{-1}), suggesting that the high Hsp70 in the serum of tumour-bearing mice originated from tumour cells and not normal tissues. In addition, no appreciable increases in the serum Hsp70 levels were observed in BIIB021-treated mice compared with control mice treated with vehicle (Figure 3B), confirming that the BIIB021-induced increase in Hsp70 levels is attributable to tumour cells and not normal tissues.

To eliminate the possibility that the apparent lack of induction of Hsp70 secretion in naive mice was due to the lack of recognition of murine Hsp70 by the antibody we used, we examined the specificity of the Hsp70 antibody by the same ELISA assay, using purified murine and human recombinant Hsp70. Here we showed that the antibody recognized the murine Hsp70 equally well, if not better than human Hsp70 (Figure 3C). This confirmed that the basal serum Hsp70 level in naive mice was low and proved that the source of high serum Hsp70 in tumour-bearing mice was a secreted product from tumour cells. This is an important observation, which identifies and supports the use of serum Hsp70 in the clinic to monitor directly the activity of tumour-derived Hsp70 as opposed to intracellular Hsp70 from normal PBMC, which can serve only as a surrogate biomarker of Hsp90 inhibition in tumour cells.

Hsp70 is quantifiable in cancer patient serum

To qualify serum Hsp70 as a response biomarker for 17-AAG and BIIB021 (Hsp90 inhibitors) in the clinic, we evaluated if we could detect and quantify pretreatment levels of Hsp70 in the sera of normal and diseased donors. A group of serum samples from healthy people and patients representing different cancer

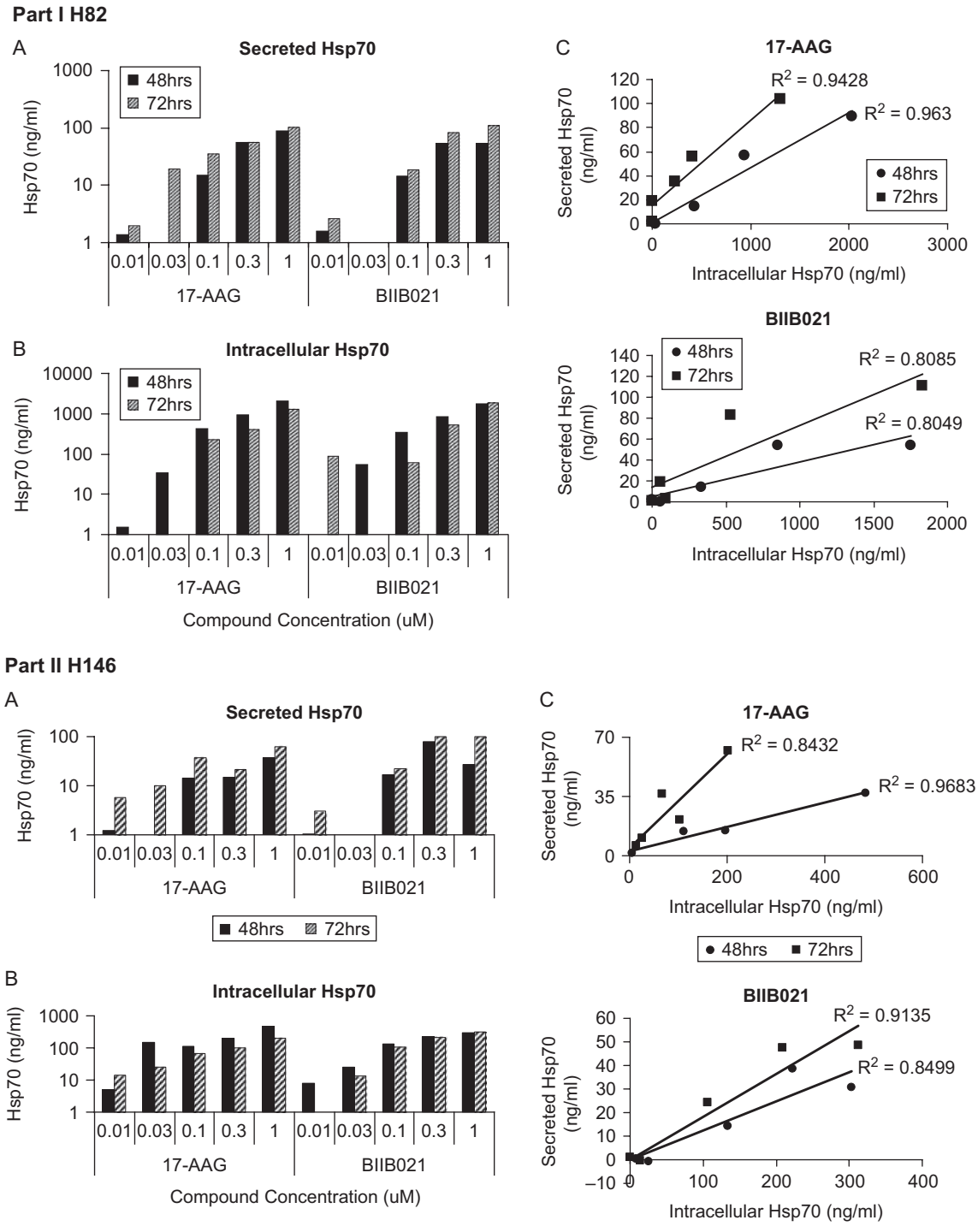


Figure 2. Correlative induction of intracellular and secreted Hsp70 by Hsp90 inhibitors. H82 (Part I), H146 (Part II) and H69 (Part III) cells were treated with increasing amount of either 17-AAG or BIIB021. DMSO was used as the control. Both cells and culturing medium were collected at 48 and 72 h after treatment and were measured for Hsp70 levels in both compartments by colorimetric ELISA. (A) Hsp70 in culture medium was induced by 17-AAG and BIIB021 in a dose-dependent manner. (B) Intracellular Hsp70 was induced by 17-AAG and BIIB021 in a dose-dependent manner. (C) Correlation of the induction of Hsp70 by 17-AAG and BIIB021 in culture medium and within cells.

Figure 2. continues on next page.

types was obtained and examined for basal Hsp70 levels. The result in Figure 4 illustrated that the Hsp70 level in normal donor serum remained in the low range, less than 15 ng ml^{-1}. For cancer patient samples, based on the statistical analysis, at least four out of the eight tumour types we analysed have significantly greater amounts of serum Hsp70 than normal samples indicating a potential tumour origin. Prostate cancer

Figure 2. Continued.

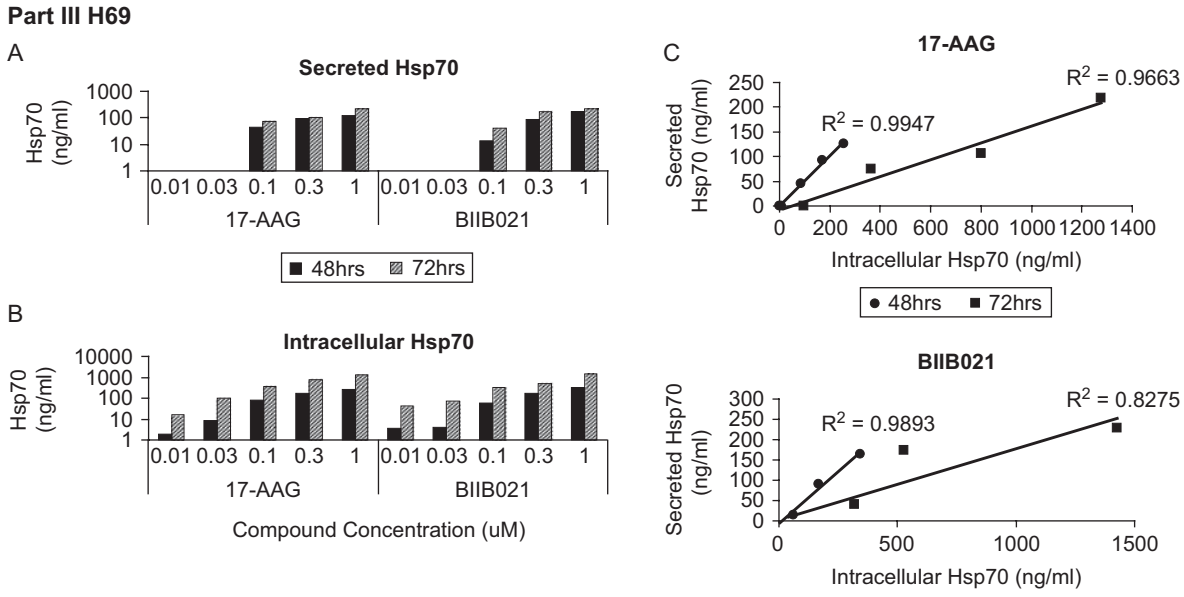


Figure 2. Correlative induction of intracellular and secreted Hsp70 by Hsp90 inhibitors. H82 (Part I), H146 (Part II) and H69 (Part III) cells were treated with increasing amount of either 17-AAG or BIIB021. DMSO was used as the control. Both cells and culturing medium were collected at 48 and 72 h after treatment and were measured for Hsp70 levels in both compartments by colorimetric ELISA. (A) Hsp70 in culture medium was induced by 17-AAG and BIIB021 in a dose-dependent manner. (B) Intracellular Hsp70 was induced by 17-AAG and BIIB021 in a dose-dependent manner. (C) Correlation of the induction of Hsp70 by 17-AAG and BIIB021 in culture medium and within cells.

barely missed the cut ($p = 0.07$) due to the availability of very few donors.

Discussion

The Hsp90 complex is a molecular chaperone that governs the stability and functionality of an increasing group of oncogenic proteins. Many proteins in this group are activated, mutated or overexpressed during tumour development and progression, and thus tumours rely intimately on the optimal function of Hsp90 complex for their survival. Degradation of certain client proteins in tumour cells by Hsp90 inhibitors has been used as a pharmacodynamic biomarker to monitor Hsp90 inhibitor activity in clinical trials. A review of the literature reveals that each cancer indication appears to have a different dominant Hsp90 client protein. For example, in breast cancer, degradation of HER-2 was utilized as a sensitive biomarker of Hsp90 inhibitor activity (Basso et al. 2002, Chandarlapaty et al. 2008, Smith-Jones et al. 2006), while in BRaf mutant melanoma, disappearance of BRaf was used as a measurement for the on-target effect of these compounds (Banerji et al. 2008, da Rocha Dias et al. 2005, Grbovic et al. 2006). A commonly used biomarker in different types of indications in the clinical trials of Hsp90 inhibitors is the co-chaperone, Hsp70, which is upregulated as a result of activation of HSF-1 following Hsp90 inhibition. However, all of these biomarkers have to be examined by a Western blot assay in PBMC cells, which is a surrogate tissue and may not reflect the direct activity of clinical agents on tumour cells. By contrast, secreted Hsp70 we report here has the potential to help monitor tumour cell activity directly, as it was shown to be secreted from tumour cells and was not detected in tumour-free naive mice treated with the same Hsp90 inhibitor. The serum Hsp70 appeared to be induced in a dose-dependent manner by Hsp90 inhibitors and exhibited a tight correlation with the change of intracellular Hsp70 (Figure 2), indicating that the serum Hsp70 reflected the alteration of intracellular Hsp70 in tumour cells, and therefore could be used potentially as a tumour biomarker and be useful for the determination of optimal dose and schedules of Hsp90 inhibitors in human clinical trials.

The basal serum Hsp70 level in various human cancer patients has been widely examined and is found to be elevated in several types of cancer patients. Our studies provide evidence that Hsp70 is both detectable and quantifiable in a variety of cancer patients and healthy volunteers. These findings correlate well with elevated serum Hsp70 levels observed in SCLC patients (Seiwert et al. 2005) and strongly support the utility of serum Hsp70 as a universal biomarker for measuring Hsp90 inhibitory activity in many types of cancer. We noted that in the SCLC samples pool we studied, Hsp70 levels were relatively low compared with other cancer types. This may be due to the different stages of the samples we

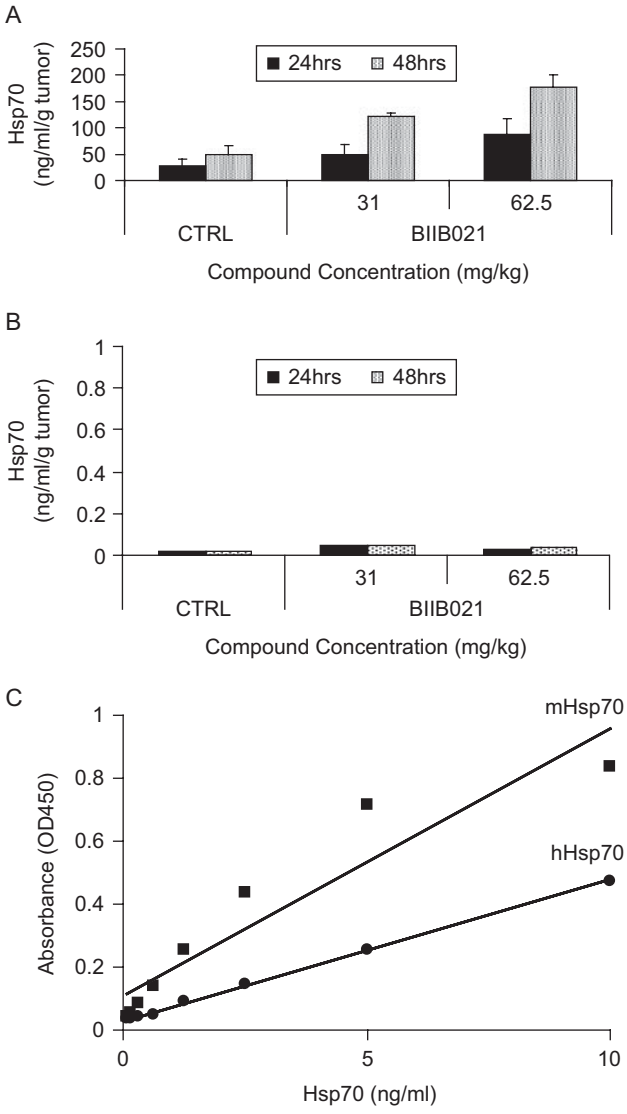


Figure 3. BIIB021 induces secretion of Hsp70 in tumour-transplanted mice, but not naive mice. H82-tumour bearing mice (A) or naive mice (B) (five in each group) were dosed with either vehicle or BIIB021 at indicated dose levels. Mice sera were collected at either 24 or 48 h after dosing and quantified for Hsp70 level by colorimetric ELISA. Error bars represent SE. (C) The specificity of the antibody for mouse and human Hsp70 were examined. The antibody recognized the protein from both species.

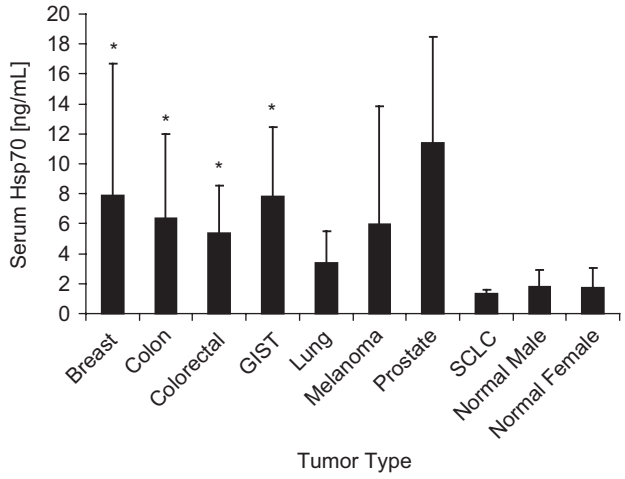


Figure 4. Baseline Hsp70 levels are quantifiable in cancer patient serum. Serum from donors (n = 4–25) representing various cancer types was diluted 1:4 and Hsp70 levels were determined independently by a validated Meso Scale ELISA. All three recombinant Hsp70 quality controls recovered within 20% of their expected values (8, 2 and 0.6 ng ml^{-1}). Each bar represents mean (error bars, SD) of Hsp70 levels in a tumour type. *Statistically significant (p <0.05) value when compared with Hsp70 levels in normal human serum by two-tailed unpaired Student's t-test.

have, as the circulating Hsp70 level does vary with the stages of cancer (Seiwert et al. 2005, Wang et al. 2006, Mambula & Calderwood 2006), or simply because of the small sample size we examined. However, we did observe an elevation of serum Hsp70 in breast, colon, colorectal cancer and GIST, which is increased 2–4-fold compared with the normal samples, indicating that elevated secretion of Hsp70 may be a generalized phenomenon in many types of cancer. A systematic analysis of more serum samples from different types of cancer patients at various stages would provide additional information on the actual status of serum Hsp70 levels in cancer patients. Nevertheless, the low background level of circulating Hsp70 in cancer patients may be beneficial and provide a sensitive means for the measurement of Hsp90 inhibitor activity in clinical settings. Furthermore, we are monitoring serum Hsp70 levels in the ongoing BIIB021 trials in breast cancer and solid-tumour patients; the outcome of these studies may validate the utility of this biomarker for predicting pathway interdiction, dose or schedule.

During the measurement of Hsp70 in cultured tumour cell lines, we noted differences in both the quantity and kinetics of Hsp70 induction in different cell lines in response to different Hsp90 inhibitors. For example, both H82 and H146 secreted comparable levels of Hsp70 in response to 17-AAG and BIIB021, even though they had dramatic differences between the intracellular Hsp70 levels induced by the compounds (Figure 2). Moreover, intracellular Hsp70 reached its highest level around 48 h after treatment in both cell lines, whereas Hsp70 secretion continued to increase up to 72 h. In H69, another SCLC cell line, we found that both intracellular and secreted Hsp70 reached their highest level at 72 h (Figure 2). These findings imply that there are differences between intracellular and secreted Hsp70 in terms of quantity, kinetics and response to different Hsp90 inhibitors and variability from cell line to cell line. Whether these issues influence their utility as biomarkers of Hsp90 inhibitors in the clinic remains to be revealed. Nevertheless, quantifiable and correlative induction of secreted Hsp70 in all three cell lines and xenograft-bearing mice combined with observations

in cancer patient sera provide strong evidence for its potential use as a universal biomarker to monitor the activity of a promising group of Hsp90 inhibitors currently undergoing clinical investigation. Furthermore, examination of secreted Hsp70 in an expanded range of cancer types and additional clinical experience would provide more information on the utility of serum Hsp70 to guide both dose-scheduling and patient stratification decisions.

Acknowledgements

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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