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Letters

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Affinity Purification Probes of Potential Use To Investigate the Endogenous Hsp70 Interactome in Cancer

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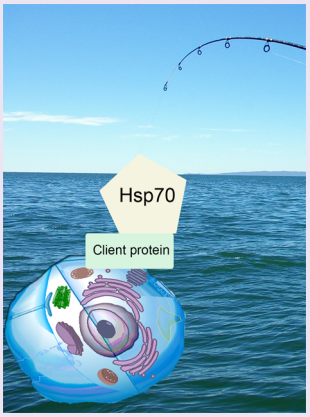
Supporting Information

ABSTRACT: Heat shock protein 70 (Hsp70) is a family of proteins with key roles in regulating malignancy. Cancer cells rely on Hsp70 to inhibit apoptosis, regulate senescence and autophagy, and maintain the stability of numerous onco-proteins. Despite these important biological functions in cancer, robust chemical tools that enable the analysis of the Hsp70-regulated proteome in a tumor-by-tumor manner are yet unavailable. Here we take advantage of a recently reported Hsp70 ligand to design and develop an affinity purification chemical toolset for potential use in the investigation of the endogenous Hsp70-interacting proteome in cancer. We demonstrate that these tools lock Hsp70 in complex with onco-client proteins and effectively isolate Hsp70 complexes for identification through biochemical techniques. Using these tools we provide proof-of-concept analyses that glimpse into the complex roles played by Hsp70 in maintaining a multitude of cell-specific malignancy-driving proteins.



The heat shock protein 70 family members (Hsp70s) are important cancer chaperones. They are abundantly expressed in malignant tumors of various origins, and their expression correlates with increased cell proliferation, poor differentiation, metastases, resistance to therapies, and poor therapeutic outcome in human cancers.[1−9] A variety of mechanisms have been assigned to account for the observed reliance of cancer cells on Hsp70s, including inhibition of apoptosis, induction of autophagy, control of senescence, and regulation of the stability of onco-proteins.[1−9]

Much of our knowledge on Hsp70 function in cancer is derived from genetic knockdown approaches.[6] Performing knock-down studies can however be challenging as Hsp70 is a family of at least 8 isoforms, some of interchangeable functions, a significant number with long half-lives and high constitutive expression.[6,7] Genetic studies also treat Hsp70 as a monolithic entity and are unable to tackle the acknowledged contribution of epigenetics to the activity of these proteins. Further, due to feed-back synthesis of one Hsp70 member after the knock-down of another, such studies often lead to no observable phenotypes.[6−9] Cellular manipulations that are often conducted to investigate the function of a protein and its potential interactors, *i.e.*, by transfection of mutants, tagged proteins, or overexpression systems, need also caution as they may lead to "false positives" for Hsp70. This is of no surprise because Hsp70 and other heat shock proteins are "buffers" of cellular stress, and thus such manipulations that lead to proteome stress may impose artificial interactions of Hsp70s with the transfected protein.[1−5] Such interactions are not native to a cancer cell. Together, these facts help explain why information ensuing from such studies is often conflicting.[6−9]

An accurate understanding of the role of Hsp70 in cancer is further confounded by the large number of binding partners that function to allosterically regulate its activity in a dynamic manner.[1−9] Furthermore, these Hsp70 complexes are likely to be cancer cell- and type-specific and in addition subject to the profound implications induced by post-translational modifications.[10−12] Depending on the particular cellular context, Hsp70 may display distinct functions such that the phenotype observed following perturbation by genetic knockdown versus small-molecule probe can be significantly different. This realization cannot be ignored as it has a tremendous impact on the development of novel therapeutics for human disease.

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Thus, the field is sorely missing Hsp70-directed chemical tools that could be used both as therapeutics and as reagents to dissect in a spatiotemporal manner the cell-specific endogenous effects and mechanisms associated with Hsp70 in cancers and other diseases.[4,5] The complex regulatory nature of Hsp70 lends itself to the possibility for a variety of strategies aimed at modulating its activity, including inhibition of ATPase activity, inhibition of substrate binding, and inhibition of co-chaperone binding. Indeed, a number of small molecules from a range of structural classes have been reported to modulate Hsp70 activity.[1−5] Hampering the use of numerous such ligands as chemical tools are, however, their pleiotropic effect in cells, little structure−activity tractability, and limited potential for chemical modifications such as is required for probe optimization.

We have recently reported the use of a homology model that enabled the identification of a novel allosteric site located in the nucleotide binding domain of Hsp70.[13] Combining structure-based design and phenotypic testing, we then designed a novel inhibitor of this site, YK5. In cancer cells, this compound acted as a potent and selective binder of the cytosolic but not the organellar human Hsp70s and had biological activity partly by interfering with the formation of active oncogenic Hsp70/Hsp90/onco-client protein complexes.[13] Further medicinal chemistry around the YK5 scaffold led to extensive SAR knowledge that confirmed the proposed binding mode of the ligand and provided insights on the attachment of specific linkers for the construction of Hsp70-directed chemical tools.[14,15] On the basis of this information, we here design and validate an affinity purification chemical toolset that we propose will be of use for the investigation of the endogenous Hsp70-interacting proteome in cancer.

Cytosolic Hsp70s are ATPases and require the binding and hydrolysis of ATP for activity (see Figure 1A). They are composed of two major domains, a ∼40 kDa, N-terminal nucleotide-binding domain (NBD) and a ∼25 kDa, C-terminal substrate-binding domain (SBD), connected to each other by a short linker.[16,17] Together, the two domains function in a complex, highly coordinated fashion regulated by the binding and release of ATP/ADP, substrate, and co-chaperones (*i.e.*, Hsp40, BAG1, Hsp110). Although Hsp70 has very weak ATPase activity alone, the binding of J-domain-containing proteins such as Hsp40 increase its ATPase rate by ∼7-fold. The function of Hsp70 is further regulated by the binding of nucleotide exchange factors (NEFs), such as BAG1 and Hsp110, which bind to the NBD and promote ADP release. The conformation of Hsp70 is highly dependent upon whether ATP or ADP is bound. When ATP is bound, Hsp70 adopts a conformation with weak affinity for substrate, and when ADP is bound, it adopts a conformation with high affinity for substrate. These conformational changes are further regulated by the J-domain-containing proteins and the NEFs.

On the basis of the biochemical regulation of Hsp70, we hypothesized that ligands interacting with Hsp70 in the ADP-conformation may trap Hsp70 in complex with its substrates and therefore could function as affinity purification tools (Figure 1A). As appreciated in the field, heat shock proteins chaperone large proteome subsets; the nature and identity of these are determined by the specific alterations characteristic of a particular tumor or tumor subset.[1−9] Several protein-chaperoning functions of Hsp70 are as part of the Hsp90 machinery (Figure 1A, Function **1**), where Hsp70 is a key co-chaperone of Hsp90. It is thought to load client proteins onto the Hsp90 machinery through the action of another co-chaperone, heat shock organizing protein (HOP). The Hsp90 machinery is an important mechanism by which cancer cells regulate the function of several cancer-driving proteins, such as those involved in altered signaling, cell cycle, and transcriptional regulation. In addition to "loading" substrates for increased activation by the Hsp90 machinery, Hsp70 has also "stand-alone" functions (Figure 1A, Function **2**) in regulating anti-apoptotic molecules and other yet not fully characterized functions.[5,18] Having affinity purification chemical tools that capture Hsp70 bound to its client proteins would enable an inquiry on the spectrum of proteins regulated by Hsp70 in any given tumor.

The basis for our Hsp70 toolset design is YK5 (**1**, Figure 1B). This compound inserts into the allosteric pocket on Hsp70 located outside the ATP/ADP pocket and forms a covalent link with an active cysteine, Cys 267, deep inside the protein cavity (Figure 1B).[13] The YK5-binding pocket is available to the ligand specifically in the ADP conformation. In the ATP-bound state, the N-terminal domain cleft closes, reducing by approximately half the volume of the allosteric cavity.[16] On the other hand, in the ADP-bound conformation considerable rearrangements in this domain open the cleft and render the allosteric pocket available for ligand binding. Therefore, we posit that an affinity probe based on YK5, because of its ADP-conformation preference and also its covalent modification of Hsp70, may indeed be able to affinity purify Hsp70 bound to its protein clientele.

Biotinylated analogues of YK5 could be of use for such purpose. Biotinylated compounds have the potential to be cell-permeable and may be used to investigate Hsp70 complexes in live cells. These complexes could then be captured on a solid support containing avidin or streptavidin (SBs, streptavidin agarose beads). Alternatively, YK5 could be directly immobilized on a solid support (*i.e.*, YK5-beads) resulting in a potentially higher localized concentration of ligand; this may enable more efficient affinity purifications of Hsp70 complexes. However, YK5-beads are limited to cell homogenates.

There are several requirements for a probe to be useful in dissecting the tumor Hsp70 proteome. First, the probe should retain selective and tight binding to tumor Hsp70. Second, for analysis of complexes in live cells, the probe (*i.e.*, biotinylated probe) should permeate cells and while inside bind to the tumor Hsp70 complexes. Upon cell permeabilization, the biotinylated probe should retain Hsp70 binding and concomitantly bind to streptavidin, allowing subsequent isolation of Hsp70 protein complexes. Third, because isolation of Hsp70 in complex with its tumor-specific client proteins is the desired outcome, the probe should also trap and lock the Hsp70/protein complex, to maintain these complexes throughout the subsequent permeabilization and purification steps.

When preparing such probes it is crucial that attachment of the linker to the molecule be in a position that does not adversely affect binding to the target. The two most synthetically tractable sites for biotinylation were on piperazine ring **A** (site 1) and on pyrimidine ring **B** (site 2) (Figure 1B). Therefore, we first synthesized biotinylated analogues of YK5 attached through a tetra(ethylene glycol) (TEG) linker by either replacing the *N*-methyl group from piperazine ring **A** (**2**, Figure 1B) or one of the *O*-methyl groups from pyrimidine ring **B** (**3**, Figure 1B). Testing of these two compounds in a battery of phenotypic assays designed to read fingerprints of Hsp70 inhibition in cancer cells[13−15] indicated that attachment of the linker to site

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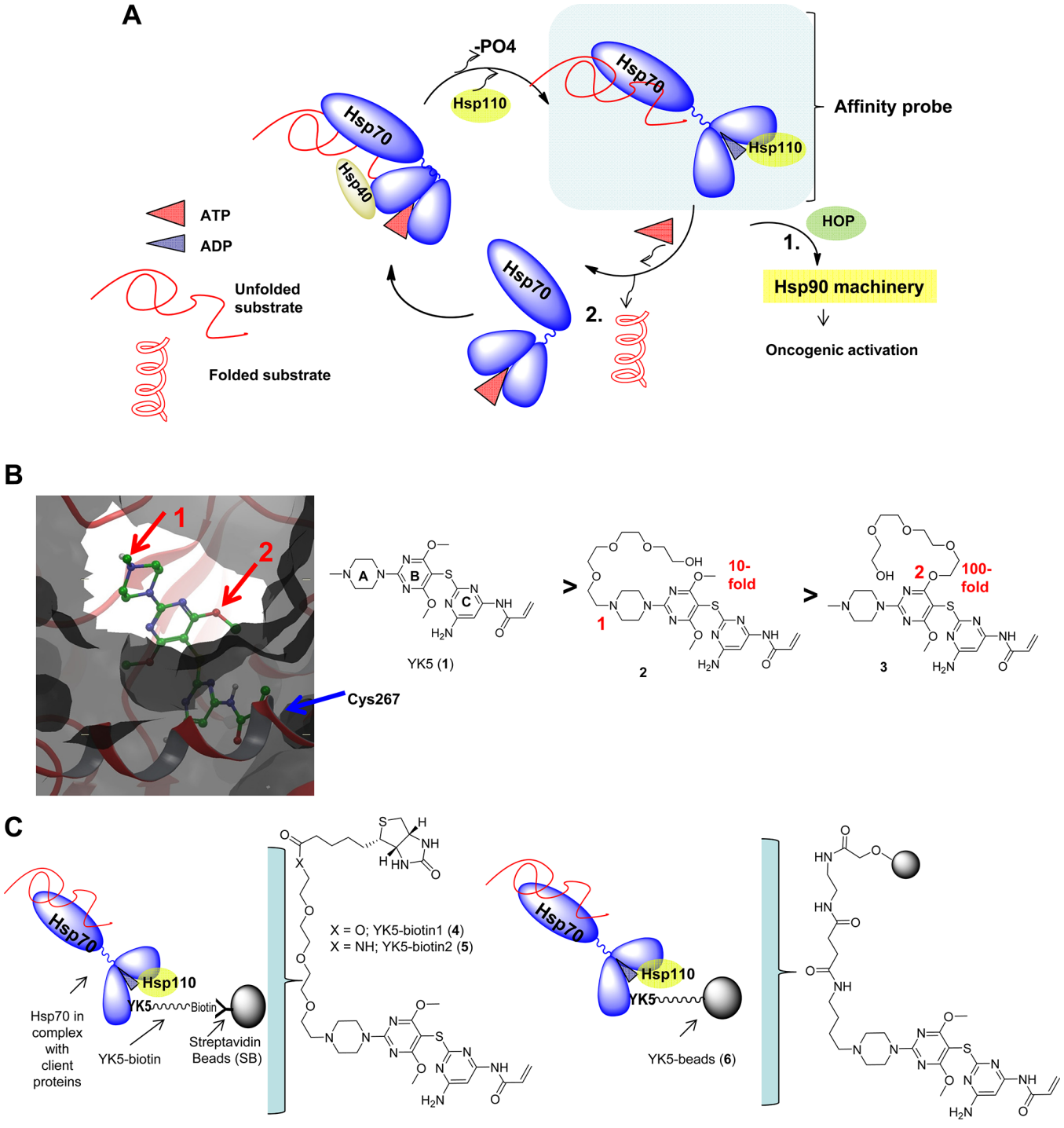

Figure 1. Design of the Hsp70 affinity-probe for use in Hsp70 proteome investigations. (A) Schematic representation of the Hsp70 chaperoning cycle. The function of Hsp70 is regulated by ATP/ADP and by the binding of nucleotide exchange factors such as Hsp110. The conformation of Hsp70 is highly dependent upon whether ATP or ADP is bound. When ATP is bound, Hsp70 adopts a conformation with weak affinity for substrate, and when ADP is bound, it adopts a conformation with high affinity for substrate. Through this cycle the substrate is folded and rendered active (function **2**). For certain substrates a more specialized activation is required, and the protein is transferred onto the Hsp90 machinery (function **1**). A small molecule-based affinity probe that interacts with Hsp70 in the substrate-bound conformation and, further, traps Hsp70 in a substrate (*i.e.*, client protein)-bound conformation may isolate the endogenous Hsp70/client protein complexes in a tumor-by-tumor manner. (B) Computer-generated representation of the best Glide-predicted binding pose derived for compound YK5 within the allosteric site of Hsp70. Two sites for potential attachment of a linker are indicated by the red arrows. The effect of linker attachment on Hsp70-mediated activity is also presented. (C) Structure of the three designed Hsp70 probes.

2 was detrimental to activity (100-fold loss of activity when compared to YK5). Attachment to site 1 led to an only 10-fold activity drop (Figure 1B).[13,14] Based on these observations, three YK5-based tools were designed incorporating linkers that enabled either biotin attachment *via* an ester or an amide linkage (**4**, YK5-biotin1 and **5**, YK5-biotin2, respectively) or

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direct attachment to Affigel 10 beads (**6**, YK5-beads) (Figure 1C). The synthesis of these probes is described in the Supporting Information (Figures S1−S2). Although ester-linked probes are generally considered less ideal than their amide analogues because of their susceptibility to hydrolysis, the synthesis of the ester derivatives was more synthetically feasible and hence more practical in identifying the optimal site of attachment of the biotin moiety.

We first evaluated the cell permeability profile of the two biotinylated analogues (Figure 2A). YK5-biotin1 retained the activity of the precursor hydroxy compound **2**, *i.e.*, it was approximately 10-fold less active than YK5, whereas YK5-biotin2 showed little activity in cells. For YK5-biotin2, we attributed the loss of Hsp70-mediated activity in cells majorly to its inability to permeate live cells, because YK5-biotin2 and YK5-biotin1 isolated a comparable Hsp70 amount when incubated with cell extracts (Figure 2B). While the ester linker in YK5-biotin1 may be a liability in cells and become hydrolyzed, we found that under the conditions of the assay (*i.e.*, 4 h of incubation in cells prior to cell lysing and affinity purification) it remained stable as confirmed by the immunoreactivity of the YK5-biotin1 isolated band with antibodies against both Hsp70 and biotin (Figure 2C). Tandem liquid chromatography−mass spectrum analysis of peptide digests obtained from the YK5-biotin1 isolated band confirmed the presence of two inducible Hsp70 isoforms (Hsp70-1 and Hsp70-6) and of Hsc70, the constitutive Hsp70 member (Hsp70-8), all three cytosolic Hsp70 members.[13] The BB70 antibody recognizes all the major Hsp70 isoforms, namely, Hsp70, Hsc70, Grp75, and Grp78.[13]

Next we modulated the stringency of the buffer used to wash the protein complexes isolated on the YK5-biotin1 beads (Figure 2D). With a buffer typically used to capture tertiary and quaternary protein complexes, we observed that, in addition to the major 70 kDa band, numerous others appeared upon staining of the gel with either Coomassie blue (left) or silver (right). The majority of these bands seemed to be specific as they were not present in the control pulldowns (Figure 2D, D-biotin) or if the affinity purified complexes were washed with a buffer containing 1 M NaCl (Figure 2D, high-salt buffer), a salt concentration that typically displaces protein−protein interactions but also washes off a majority of problematic proteins that tend to stick to resins (*i.e.*, actin, tubulin). Preincubation of cells with YK5 prior to addition of the YK5-biotin1 and to the affinity purification step (Figure 2E), and also affinity purifications with YK5-biotin1 in physiological buffer conditions (*i.e.*, with 150 mM NaCl or KCl) (Figure 2F) further substantiate that the majority of the bands are indeed bound to YK5 *via* Hsp70 and thus are potential Hsp70 interactors.

We next tested affinity purifications performed in the cell extract rather than in live cells. When using the preformed YK5-biotin1/streptavidin beads or the YK5-beads to isolate the Hsp70 complexes, we noted a lower signal-to-noise ratio (*i.e.*, lower ratio of specific versus sticky proteins such as tubulin and actin; see red arrows Figure 2G−I). Binding of "sticky" proteins such as actin and tubulin was majorly resin- and not Hsp70-mediated (Figure 2H,I). This observation was further confirmed by attaching to the Affigel 10 resin a derivative of YK5 with over 10-fold lower affinity for Hsp70 (Figure 2I, control beads). Therefore, to improve the signal-to-noise ratio in this case, preclearing of the cell lysates by incubation with control beads is recommended.

Hsp70s (Hsp70 and Hsc70) represent 1% (or more) of the total cellular proteins in mammalian cells. In HeLa cancer cells, Hsp90 and Hsp70 were determined to be 2.8 and 2.7% of the total protein mass, respectively.[19] If one considers that there are ∼7,000−8,000 proteins per cell, such content of Hsp70 proteins would be 1 to 3 log higher than most cellular proteins that are usually chaperoned by the Hsp70/Hsp90 system, *i.e.* signaling proteins, transcription factors, anti-apoptotic proteins. In the affinity purification, the tool isolates Hsp70 in complex with over 1,000 proteins (as we have determined by mass spectrum analyses of the entire gel, unpublished data). A similar interactome number was reported by us for Hsp90 using Hsp90 inhibitor-based affinity purification tools.[10,20] Several may be direct interactors of Hsp70 while others may be part of mega-Hsp70 containing protein complexes. Also of importance, potential client proteins of Hsp70 may be in distinct activation states and/or cellular locations, thus only a fraction of such proteins may be Hsp70 bound, at any time. With these in mind, we caution that any individual band observed on the Hsp70-interactome gel is a mixture of tens of proteins that associate with Hsp70. These "real" interactors are intermixed with "nonspecific" bands. Therefore, it is important that for mass sprectrum proteomic analyses, one includes control experiments as indicated above (*i.e.*, preclear the lysate with resin and/or perform an analysis of YK5-biotin1 beads in lysates pretreated with YK5 and/or use beads with an inactive control attached).

To validate the utility of YK5-biotin1 to affinity purify Hsp70 in complex with protein clients, we first went on to show that the Hsp70 pool isolated by YK5-biotin1, but not biotin, contained an established Hsp70 client such as the serine/threonine kinase Raf-1 (Figure 2F and Figure 3A, left).[21] Because the anti-Hsp70 Ab BB70 can deplete cell extracts of Hsp70 but is weak at capturing Hsp70 in complex with the activating co-chaperone Hsp110 and thus with onco-protein clients (Figure 3B−D), we were able to probe the specific interaction of YK5-biotin1/streptavidin beads (SB) with Raf-1 through Hsp70. Namely, when incubated with extracts depleted of Hsp70 by BB70, YK5-biotin1/SB failed to significantly interact with the oncogenic Raf-1 kinase, confirming that interaction of the probe with Raf-1 occurred through an Hsp70-mediated complex (Figure 3D). The Hsp70/Hsp110 complex constitutes a small fraction of the total Hsp70; however, it is an "active" complex in the sense that it is involved in active chaperoning of client proteins.[22,23] In HeLa cancer cells about 15% of the cytosolic Hsp70 pool may be involved in the formation of the Hsp70−Hsp110 complex.[19] The observation that the Hsp70 species depleted by BB70 contains little if any Hsp110 supports the conclusion that this antibody fails to remove most of the active complexes and therefore the client proteins bound to Hsp70. The human cytosol contains three distinct Hsp110s: HSPH2 (Apg-2), HSPH1 (Hsp105), and HSPH3.[23] The antibody used to blot for Hsp110 recognizes Hsp105.

Next, we enquired on known Hsp70/Hsp90 machinery interactors specific to a genetic background (Figure 3E).[24] We validated several onco-proteins involved either in increased signaling through a pathogenic pathway or in aberrant cell cycling to be in complex with Hsp70, as isolated by the affinity probe. These include Cyclin D1 and the HER2 kinase in the HER2 overexpressing SKBr3 breast cancer cells, cyclin dependent kinase 1 (CDK1), and phosphoinositide-dependent kinase-1 (PDK1) in the MDA-MB-468 breast cancer cells and mutant androgen receptor (mAR) in LNCaP prostate cancer cells (Figure 3E). Preincubation of cell lysates with YK5 diminished in a dose-dependent manner the ability of the probe

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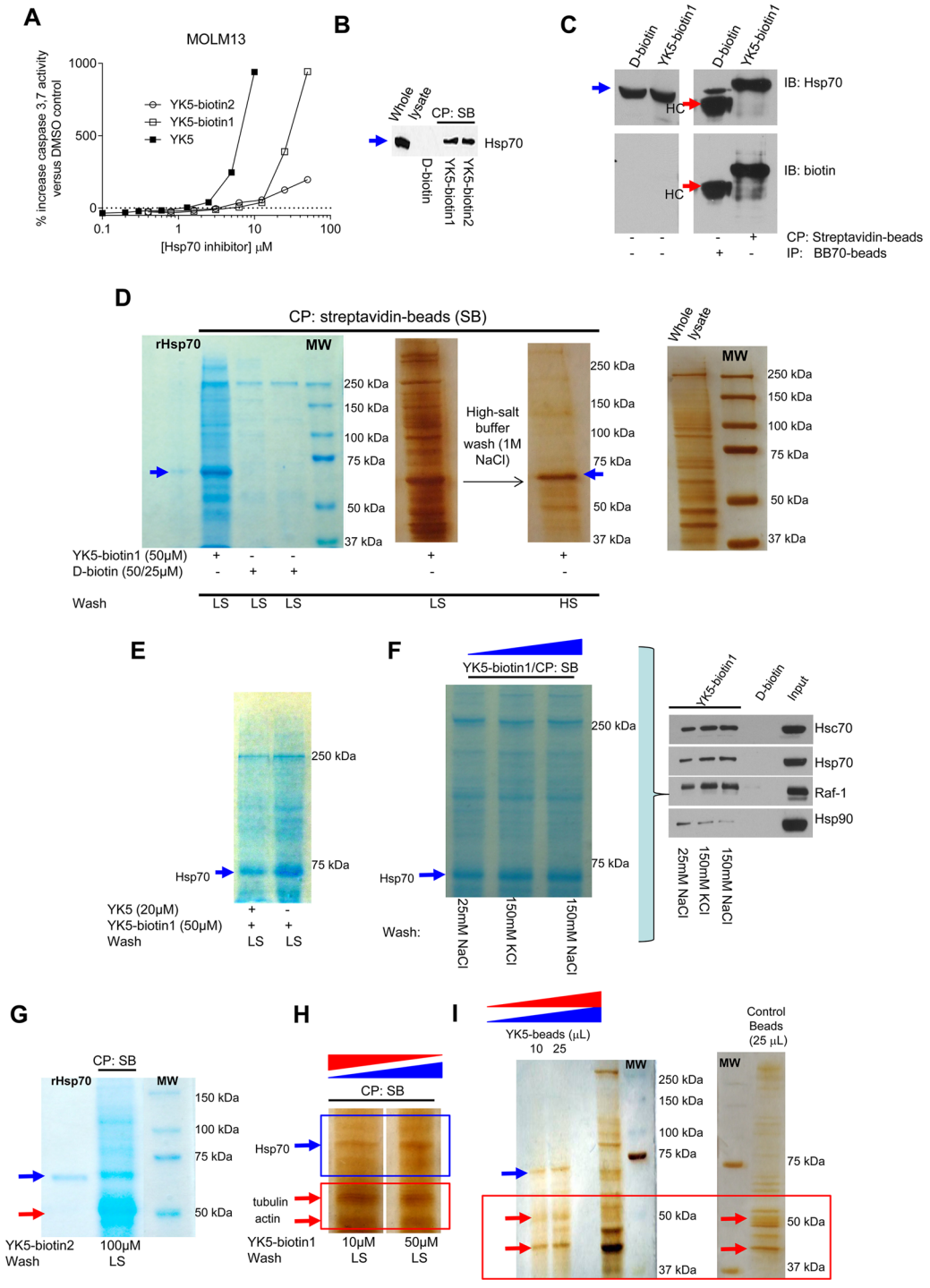

Figure 2. Evaluation of the probe for use in affinity purification. (A) YK5-biotin1 but not YK5-biotin2 favorably permeates live cells and maintains an Hsp70-mediated effect.[14] (B) Cell extracts were incubated overnight with the indicated biotinylated probes and protein complexes isolated on streptavidin beads (SBs). (C) Cells were incubated for 4 h with YK5-biotin1 or D-biotin, and then protein complexes were isolated on either SBs (for YK5-biotin1) or the BB70 antibody-beads (for D-biotin). Following separation on a denaturing gel, proteins were probed with the indicated antibodies. HC, heavy chain; CP, chemical precipitation; IP, immunoprecipitation. The BB70 antibody recognizes Hsp70, Hsc70, Grp75, and Grp78.[13] (D) Cancer cells were treated with the indicated concentrations of YK5-biotin1 or D-biotin for 4 h prior to lysing and precipitation of protein complexes on SBs. Beads were washed with either low-salt (LS) (25 mM NaCl) or high-salt (HS) (1 M NaCl) buffer, as indicated, and proteins were eluted by boiling in 2% SDS and then separated on a denaturing gel. Gels were stained with Coomassie blue or silver, as indicated. rHsp70, recombinant Hsp70; MW, molecular weight ladder; whole lysate, total protein content. (E) Cells were pretreated for 1 h with YK5 prior to addition of YK5-biotin1 and affinity purification as in panel D. (F–I) Experiment setup as in panel B. In panel F the LS buffer was modified to increase its content from 25 mM NaCl to 150 mM NaCl or KCl. Blue arrow indicates the location of the Hsp70 protein. Red arrows indicate the location of unspecific proteins, such as tubulin and actin. These experiments were repeated twice with comparable results.

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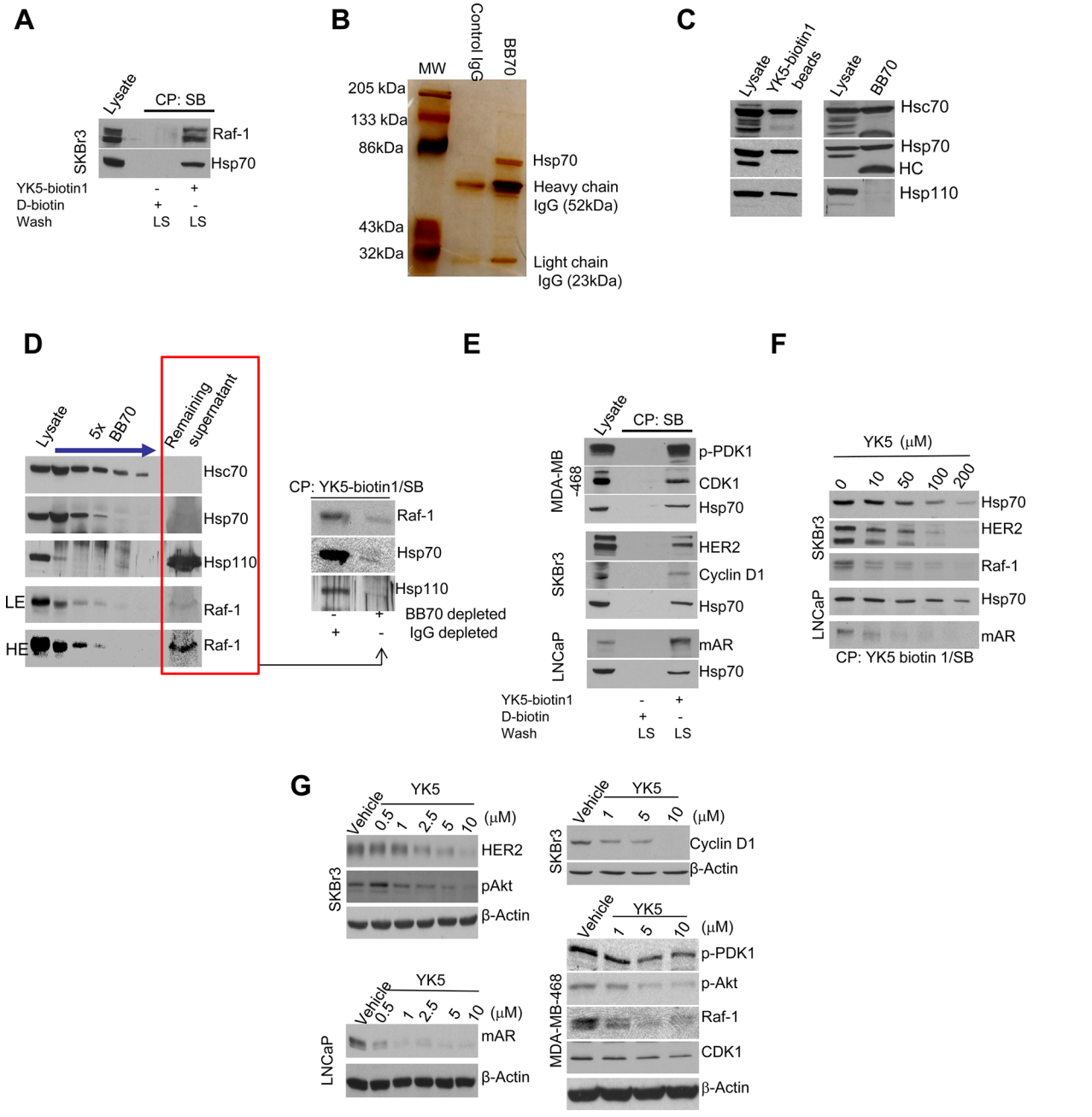

Figure 3. Validation of the Hsp70 proteome isolated by the chemical probes. (A–C) SKBr3 cancer cell extracts were incubated overnight with the indicated biotinylated probes (A,C) or the indicated antibodies (B,C). Protein complexes isolated on streptavidin agarose beads (A,C) or protein G agarose beads (B,C) were probed with the indicated antibodies (A,C) or silver stained (B). (D) Cell lysates immunodepleted with either BB70 or a control IgG were subjected to affinity purification with YK5-biotin1/SBs. Isolated proteins were probed with the indicated antibodies. LE and HE, low- and high-exposure, respectively (E) Experiment setup as in panel A for the indicated cancer cell extracts. (F) As in panel E for cell extracts preincubated for 3 h with the indicated concentrations of YK5. (G) Cancer cells were treated for 24 h with the indicated concentrations of vehicle (DMSO) or YK5, and proteins were analyzed by Western blot. These experiments were repeated twice with comparable results.

to interact with Hsp70 and resulted in displacement of bound onco-proteins (Figure 3F). Further confirming that these affinity purified proteins are indeed clients of Hsp70 and depend on the chaperone for their function and stability, we next investigated whether addition of YK5 to these cancer cells led to a reduction in the steady-state levels and activity of these proteins (Figure 3G). As known, inhibition of chaperone function leads to client protein clearance.[1−9,13] For YK5, we have recently shown that when Hsp70 is inhibited by YK5, Hsp90 machinery onco-proteins cannot be transferred onto Hsp90 and become destabilized and targeted for clearance, at least in part by the proteasome.[13] In accord with this mechanism and proof that the proteins are true Hsp70-clients in these cancer cells, addition of YK5 led to a dose-dependent reduction in their steady-state levels. The effects occurred in a concentration range that YK5 alters the Hsp70/Hsp90 complexes in cancer cells.[13,14]

In conclusion, we here report on the design and validation of affinity probes that enable the unique isolation of Hsp70 in complex with onco-client proteins endogenous to the specific cellular context. We show that these tools are superior in their

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capture ability to widely used immuno-purification tools, such as the BB70 antibody. They lock and preserve the endogenous Hsp70/protein complexes throughout the subsequent experimental steps (*i.e.*, permeabilization, washes). In addition to classical investigation of complexes in cellular extracts and because of its ability to permeate live cells, one such probe, YK5-biotin1, enables the investigation of Hsp70 complexes in live cells. While it is true that, following the addition of the biotinylated tool, cells will be permeabilized and thus no longer alive, the capture of the Hsp70 complexes takes place in the live cell prior to the homogenization step. This "in live cell" method may provide certain advantages over affinity purifications performed in cell extracts. When adding the biotinylated probe to cell homogenates, one may encounter two potential limitations. First, due to the dynamic nature of the Hsp70−client protein interactions, the endogenous complexes may be lost during the protein extraction process and thus pull-downs from homogenates may miss important interactors. Second, during homogenization, certain proteins may lose their well-regulated conformation and potentially aggregate. Such misfolded proteins are prone to be captured by chaperones resulting in "false positives" (*i.e.*, nonendogenous Hsp70 client proteins). False positives increase the "background" on the affinity resin, and the higher the background, the poorer the identification of relevant endogenous Hsp70 complexes will be.

To our knowledge, these are the only Hsp70 probes to have such combined characteristics and thus represent unique useful tools to investigate the Hsp70 proteome. Information of such Hsp70-chaperoned proteome will be extremely valuable not only to understand tumor-specific roles of Hsp70 and associated mechanisms but also to develop rational strategies for the clinical implementation of these agents to cancer treatment. Last but not least, they may provide clues on the altered functional proteome in individual tumors, a quest yet elusive by today's proteomics methods.

METHODS

Synthesis. The detailed synthetic procedures and compound characterizations are described in the Supporting Information.

Cell Lines. We purchased the human cancer cells MDA-MB-468, LNCaP, MOLM13, and K562 from the American Type Culture Collection (Manassas, VA). SKBr3 cells were a gift from Dr. Neal Rosen, MSKCC. Cells were cultured routinely in DME/F12 (MDA-MB-468 and SKBr3) or in RPMI (LNCaP, MOLM13, and K562) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin, and streptomycin.

Buffers. To wash protein complexes isolated by the YK5-derived tools or BB70-Ab, we used either high-salt buffer (20 mM Tris pH 7.4, 1 M NaCl, 0.1% NP-40) or low-salt buffer (20 mM Tris pH 7.4, 25 mM NaCl, 0.1% NP-40), as indicated. To elute protein complexes from solid support we used elution buffer (62.5 mM TrisHCl pH 6.8, 2% SDS, 10% glycerol, 15.5 mg mL^{-1} DTT, bromophenol blue 0.02 mg mL^{-1}) and boiled the sample at 100 °C for 3 min.

Chemical Precipitation. Protein lysates were prepared using 20 mM Tris pH 7.4, 25 mM NaCl, 0.1% NP-40 lysis buffer. High capacity streptavidin agarose beads (50 μL) (Thermo Scientific) were washed three times with the lysis buffer. The indicated concentration of YK5-biotin was added to the streptavidin beads, and the mixture was incubated at 4 °C for 1 h. Upon a three-time wash with the buffer, YK5-biotin/SBs were added to 500 μg aliquots of total cellular protein in the lysis buffer. Samples were incubated at 4 °C overnight, washed five times with the lysis buffer, and applied to SDS-PAGE. Gels were either subjected to Western blotting procedure or stained by Coomassie blue (Bio-Rad) or silver (Invitrogen) as indicated.

Competition Assay. Protein lysates were prepared using 20 mM Tris pH 7.4, 25 mM NaCl, 0.1% NP-40 lysis buffer, and 300 μg aliquots of total cellular protein were incubated for 3 h at 4 °C with the indicated concentrations of soluble competitor in 20 mM Tris pH 7.4, 25 mM NaCl, 0.1% NP-40 buffer. Meanwhile, YK5-biotin1/streptavidin beads were prepared by incubating streptavidin agarose beads (50 μL) (Thermo Scientific) with 50 μM YK5-biotin1 at 4 °C for 1 h. Upon a three-time wash of the YK5-biotin1/SBs with the buffer, the above soluble competitor containing lysates were added to the beads. Samples were incubated at 4 °C overnight, washed five times with the lysis buffer, and applied to SDS-PAGE. The gels were subjected to Western blotting procedure.

Hsp70 Depletion. Four microliters of BB70 anti-Hsp70 antibody or normal mouse IgG and 30 μL of protein G agarose beads (Upstate) suspension were added to 200 μg of MDA-MB-468 cancer cell lysate in 20 mM Tris pH 7.4, 25 mM NaCl, 0.1% NP-40 buffer. Following incubation at 4 °C for 3 h, samples were centrifuged, the supernatant was collected, and the bead pellet was discarded. The procedure was repeated twice. YK5-biotin1/SBs were prepared as described above, added to the supernatants, and incubated at 4 °C overnight. Beads were washed five times with 20 mM Tris pH 7.4, 25 mM NaCl, 0.1% NP-40 buffer and applied to SDS-PAGE.

Immunoprecipitation. Cells were collected and lysed in 20 mM Tris pH 7.4, 25 mM NaCl, 0.1% NP-40 buffer. Appropriate antibody (BB70 for Hsp70) (5 μL) or normal IgG (5 μL) (as a negative control) was added to each sample (500 μg of total protein) together with protein G agarose beads (30 μL) (Upstate) and incubated at 4 °C overnight. Purified complexes were washed five times with the lysis buffer and applied on SDS-PAGE followed by silver staining according to the manufacturer's instructions (Invitrogen) or Western blotting procedure.

Western Blotting. Cells were grown to 60−70% confluence and treated with inhibitor or DMSO vehicle for 24 h. Protein lysates were prepared in 50 mM Tris pH 7.4, 150 mM NaCl, and 1% NP-40 lysis buffer. Protein concentrations were measured using a BCA kit (Pierce) according to the manufacturer's instructions. Protein lysates (10−50 μg) were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with the indicated primary antibodies: anti-erbB2 (HER2) from rabbit (1:250, 28-0004, Zymed), anti-Hsc70 from rabbit (1:500, SPA-816, Enzo), anti-biotin from mouse (1:250, B7653, Sigma-Aldrich), anti-Hsp70 from mouse (1:500, SPA-810, Enzo), anti-phospho-Akt (Ser 473) from rabbit (1:500, 9271, Cell Signaling), anti-Raf-1 from rabbit (1:500, sc-133, Santa Cruz), antiphospho-PDK1 (Ser 241) from rabbit (1:500, 3061, Cell Signaling), anti-CDK1 from mouse (1:1000, 905-777-100, Assay Designs), anti-cyclin D1 from mouse (1:125, 2926, Cell Signaling), anti-androgen receptor from mouse (1:500, 554225, Biosciences), anti-Hsp110 from rabbit (1:500, SPA-1101, Enzo) and anti-β-actin from mouse (1:2500, A1978, Sigma-Aldrich). Membranes were then incubated with a corresponding peroxidase-conjugated secondary antibody (1:3,000 dilution). The anti-Hsp70 (BB70) antibody was a gift of Dr. D. Toft or was purchased from Stressmarq. This antibody recognizes Hsp70, Hsc70, Grp75, and Grp78.

Caspase3,7 Activity Assay. MOLM-13 cells (30,000 cells/well) were plated in black 96-well plates (Corning no. 3603) in 40 μL of RPMI media, and left in an incubator (37 °C, 5% CO_2) for up to 24 h. Cells were treated for 16 h with compounds or DMSO (control) at desired concentrations in 50 μL of the media. Drugs were added in triplicate wells. Following exposure of cells to Hsp70 inhibitors, 50 μL of buffer containing 10 mM HEPES (pH 7.5), 2 mM EDTA, 0.1% CHAPS and the caspase substrate Z-DEVD-R110 (Invitrogen) at 25 μM was added to each well. Plates were incubated until the signal stabilized (about 45 min), and then the fluorescence signal of each well was measured in an Analyst GT microplate reader. The percentage increase in apoptotic cells was calculated by comparison of the fluorescence reading obtained from treated versus control cells.

ASSOCIATED CONTENT

Supporting Information

Synthesis procedures and characterization of YK5-based chemical tools. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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